IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Hannon et al.	Confirmation No.:	8161
Application No:	11/894,676	Art Unit:	1635
Filed:	August 20, 2007	Examiner:	K. CHONG
Title:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Commissioner for Patents
P.O. Box 1450
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INTERVIEW SUMMARY AND SUPPLEMENTAL AMENDMENT AND RESPONSE TO OFFICE ACTION

This paper is further to the response filed on November 4, 2009 to the May 4, 2009 Office Action. The Director is authorized to charge any fees occasioned by this paper to Deposit Account No. 08-0219.

AMENDMENT

In the Claims

Please amend the claims as follows, without prejudice. This listing of the claims will replace all prior versions and listings of claims in the application:

1-49. (Cancelled)

50. (Previously presented) A method for attenuating expression of a target gene in a mammalian cell, the method comprising

introducing into a mammalian cell a library of RNA expression constructs, each expression construct comprising:

(i) an RNA polymerase promoter, and

(ii) a sequence encoding a short hairpin RNA molecule comprising a double-stranded region wherein the double-stranded region consists of at least 20 nucleotides but not more than 29 nucleotides, such that the short hairpin RNA does not trigger a protein kinase RNA-activated (PKR) response in the mammalian cells,

wherein the double-stranded region of the short hairpin RNA molecule comprises a sequence that is complementary to a portion of the target gene, and

wherein the short hairpin RNA molecule is stably expressed in the mammalian cell in an amount sufficient to attenuate expression of the target gene in a sequence specific manner, whereby expression of the target gene is inhibited.

51. (Cancelled)

52. (Previously presented) The method of claim 50, wherein the expression construct further comprises LTR sequences located 5' and 3' of the sequence encoding the short hairpin RNA molecule.

53. (Cancelled)

54. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of at least 21 nucleotides.

55. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of at least 22 nucleotides.

56. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of at least 25 nucleotides.

57. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of 29 nucleotides.

58. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule has a total length of about 70 nucleotides.

59. (Previously presented) The method of claim 50, wherein the RNA polymerase promoter comprises a pol II promoter or a pol III promoter.

60. (Previously presented) The method of claim 59, wherein the pol III promoter comprises a U6, an H1, or an SRP promoter.

61. (Withdrawn) The method of claim 59, wherein the pol II promoter comprises a U1 or a CMV promoter.

62. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule attenuates expression of the target gene in the mammalian cell by at least about 60%.

63. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule attenuates expression of the target gene in the mammalian cell by about 60% to about 90%.

64. (New) A method for attenuating expression of a target gene in a mammalian cell, the method comprising introducing into a mammalian cell a library of RNA expression constructs, each expression construct comprising:

(i) an RNA polymerase promoter, and

(ii) a sequence encoding a short hairpin RNA molecule comprising a double-stranded region wherein the double-stranded region consists of between 25 and 30 nucleotides, such that the short hairpin RNA does not trigger a protein kinase RNA-activated (PKR) response in the mammalian cells,

wherein the double-stranded region of the short hairpin RNA molecule comprises a sequence that is complementary to a portion of the target gene, and

wherein the short hairpin RNA molecule is stably expressed in the mammalian cell in an amount sufficient to attenuate expression of the target gene in a sequence specific manner, whereby expression of the target gene is inhibited.

REMARKS

I. INTERVIEW SUMMARY

A telephonic interview regarding the present application took place on December 17, 2009 among Examiner Chong, Acting SPE Vivlemore; Dr. Vladimir Drozdoff and Mr. John Maroney, both of Cold Spring Harbor Laboratory; and Dr. Anne-Marie Yvon and Dr. Jane Love, both of WilmerHale. Applicants explained that the intention of the interview was to advance what has been a very lengthy prosecution by providing any information that might assist the Examiners in view of the last response filed, and to reduce and clarify the issues in the case.

A. Claim Amendments filed on November 4, 2009

Applicants pointed out the amendments made to the claims and specifically explained the basis in the specification for support of those amendments in the pending application and in the parent '797 application. Applicants also discussed *In re Werthiem* as relevant case law in supporting the amendment reciting the length of the double-stranded region as "at least 20 nucleotides but not more than 29 nucleotides." Examiners Chong and Vivlemore stated that they did not see any written description issue with the claim amendment and agreed that the amendment is supported by adequate written description in the specification.

B. Length of Double-stranded Region As Claimed Not Disclosed in Fire et al. (U.S. Patent No. 6,506,599)

Applicants explained that Fire et al. does not anticipate the pending claims because Fire does not disclose all of the limitations of the claimed invention, as arranged in the claimed invention.

First, Applicants explained that Fire is silent with respect to the length of the double-stranded region. The Examiner pointed to the language in Fire at column 8, lines 5-6 which recites "The length of the identical nucleotide sequences may be at least 25, 50, …" and claim 15. Applicants pointed out that this disclosure in Fire reciting "identical nucleotides" refers back to the first sentence of that paragraph, at column 7, line 53, which recites "RNA containing a nucleotide sequences (sic) identical to a portion of the target gene are preferred…." Therefore, applicants made the point that the disclosure in Fire only refers to the length of the region that is complementary to the target gene, and does not refer to the length of the double-stranded region.

Applicants pointed out that the length of the double-stranded region in the context of the Fire disclosure could be much, much longer. Examiner Chong did not agree and maintained that Fire did disclose an embodiment having a 25-nucleotide double-stranded region.

C. Overlap in Ranges Is Not Sufficient for Anticipation

Applicants made the further point that even if, *arguendo*, Fire teaches a double-stranded region of 25 nucleotides (which Applicants dispute), the range recited in the pending claims, *i.e.*, "wherein the double-stranded region consists of at least 20 nucleotides but not more than 29 nucleotides…," only very slightly overlaps with Fire's recited range, *i.e.*, from "at least 25" nucleotides to an unspecified upper limit. Applicants stressed that according to the case law precedent in *Atofina*, a slight overlap in ranges is not anticipation.

Examiner Chong stated it was her position that the disclosure of "at least 25" not only discloses a range, but also discloses the 25 base length as a single species. The Examiner pointed to the recitation in the Fire document of "at least 25, 50, 100, 200, 300 or 400 bases as disclosing individual embodiments.

Applicants argued that the "at least 25, 50,…" recitation in Fire is a disclosure only of ranges, that the numbers refer only to the lower limit of those ranges, and that those ranges have no upper limit. In support, Applicants referred to the use of "at least…" language to describe ranges in *In re Werthiem*, 541 F.2d 257 (C.C.P.A. 1976). Additionally, Applicants referred to the *Atofina* case, which expressly notes that "[T]he disclosure of a range is no more a disclosure of the end points of the range than it is each of the intermediate points." *Atofina v. Great Lakes Chem. Corp.*, 441 F.3d 991, 1000, 78 U.S.P.Q. 2d 1417, 1424 (Fed. Cir. 2006), *as cited by* M.P.E.P. § 2131.03 (II).

D. Stable Expression Claim Recitation Is Not Disclosed in Fire

Applicants argued that the claim requirement of "stable expression" of the vector is not disclosed in Fire. Applicants pointed to language in Paragraph [0019] of the '797 publication regarding stable expression.

Examiner Chong inquired as to whether stable expression is a function of the construct. Applicants indicated that it is a function of its design in that it is designed for and can be used for stable expression. However, to avoid any misunderstanding, Applicants now clarify that as the

construct can also be used for transient expression, stable expression is not an inherent, that is, necessary function of the design.

Examiner Chong indicated that she would need to look further at the Fire disclosure and commented that if she could not find disclosure of "stable expression" in Fire, then this would be a good argument against anticipation.

E. Kreutzer (US 2004/0102408) Not a Proper Reference Under 35 U.S.C. § 102(e)

Applicants pointed out that the Kreutzer reference cited by the Examiner in the related '086 application is not a proper reference under 35 U.S.C. §102(e). Since the 102(e) rejection was made in the '086 application, Applicants have not previously submitted that argument in writing on the record in this case.

F. Declaration of Dr. Hernandez Evidence of Non-obviousness

Applicants explained that a 132 Declaration was filed to provide further factual evidence that one skilled in the art at the time of the invention would have understood the state of the art to teach away from the pending claims because longer dsRNA molecules elicit a PKR response and shorter dsRNA molecules were not thought to be effective.

G. Next steps

Applicants discussed the filing of this Interview Summary and Supplemental Response. Examiner Chong indicated that the '676 application next appears on her docket at about the middle of January 2010.

II. STATUS OF THE CLAIMS: ADDITION OF NEW CLAIM

Claims 50, 52, and 54-64 are pending in this application. Claim 64 is added. Claim 64 recites that "the double-stranded region consists of between 25 and 30 nucleotides." "Between" is the interval defined by two endpoints. Therefore, in claim 64, the interval between 25 and 30 defines a double-stranded region of 26, 27, 28, or 29 nucleotides. No new matter is added.

For the same reasons discussed during the interview, new claim 64 is fully supported by the specification and the parent application, U.S.S.N. 10,055,797 ("the '797 application") under 35 U.S.C. § 112. The facts of the present case are analogous to those in *In re Wertheim*, 541 F.2d 257 (C.C.P.A. 1976).

In *Wertheim*, the Applicant disclosed a range of 25-60% soluble solids in a freeze-dried coffee extract, and exemplified soluble solids of 36% and 50%. The court found that Wertheim had written description for 35-60% because "as a factual matter, persons skilled in the art would consider processes employing a 35-60% solids content range to be part of appellants' invention and would be led by the Swiss [priority] disclosure so to conclude." *Id.* at 265. In the present application, as in *Wertheim*, Applicants disclosed the setting of an upper limit based on an exemplified embodiment of the claimed invention. The upper limit is taught in the specification to be significant and necessary limit on the length of the double-stranded region, in order to avoid a PKR response in cells. As in *Wertheim*, one of skill in the art would therefore consider use of a short hairpin RNA having between 25 and 30 base pairs to all be part of the same invention the specification discloses for attenuating gene expression in mammalian cells without triggering a PKR response.

III. PENDING CLAIMS ARE NOT ANTICIPATED BY OR OBVIOUS OVER FIRE

A. Examiners Are Required to Provide Reasoned Rebuttal to Applicant's Arguments

According to M.P.E.P. § 707.07(f):

1. "Where the applicant traverses any rejection, the examiner should, if he or she repeats the rejection, take note of the applicant's argument and answer the substance of it."

2. "If it is the examiner's considered opinion that the asserted advantages are not sufficient to overcome the rejection(s) of record, he or she should state the reasons for his or her position in the record, preferably in the action following the assertion or argument relative to such advantages. By so doing the applicant will know that the asserted advantages have actually been considered by the examiner and, if appeal is taken, the Board of Patent Appeals and Interferences will also be advised."

If the amendments and arguments provided in the response and this supplemental response do not put the application in condition for allowance, Applicants request that the

Examiner, in her response, point out the specific reasons for continued rejection of the claims and the basis in the art and the precedential case law for those rejections.

B. Issue Clarified by Examiner Interview – Whether Fire Describes and, Therefore, Anticipates the Claimed Invention

1. Legal Standard of Anticipation

Anticipation requires that the prior art reference disclose each and every element recited in the pending claim. However, it is not enough that the reference disclose distinct teachings of each element of the claim within its four corners. The law requires that the elements in an anticipatory reference "be arranged as recited in the claim." *Net MoneyIN, Inc. v. Verisign, Inc.*, 545 F.3d 1362, 1371 (Fed. Cir. 2008).

The Federal Circuit in *Net MoneyIN* clarifies the law of anticipation:

> As we have stated numerous times (language on which VeriSign relies), in order to demonstrate anticipation, the proponent must show "that the four corners of a single, prior art document describe every element of the claimed invention." This statement embodies the requirement in section 102 that the anticipating invention be "described in a printed publication," and is, of course, unimpeachable. But it does not tell the whole story. Because the hallmark of anticipation is prior invention, the prior art reference—in order to anticipate under 35 U.S.C. § 102—must not only disclose all elements of the claim within the four corners of the document, but must also disclose those elements "arranged as in the claim.".

Id. at 1369 (citations omitted).

Ecolochem, Inc. v. Southern California Edison Co., 227 F.3d 1361 (Fed. Cir. 2000), is another case in which the Federal Circuit found that there was no anticipation due to the cited reference lacking disclosure that linked or arranged the elements as claimed. The court in *Net MoneyIN* commented on the *Ecolochem* case:

> After determining that the relevant figure and accompanying text described only the use of hydrogen to deoxygenate water, we concluded that the reference could not anticipate the claimed invention because there was no link between that figure and the general discussion of hydrazine as a deoxygenating agent. In other words, we concluded that although the reference taught all elements of the claim, it did not contain a discussion suggesting or linking hydrazine with the mixed bed in the figure, and thus did not show the invention arranged as in the claim.

Net MoneyIN, Inc., 545 F.3d at 1370 (citation omitted).

In Applicants' case, the prior art reference is Fire and the Examiner has identified locations in Fire that allegedly support anticipation of the claimed invention. In contrast, it is Applicants' position that for the sake of argument, even assuming that Fire does disclose every element of the claimed invention, by analogy to *Ecolochem*, there is no discussion in Fire suggesting or linking stable expression with use of a hairpin RNA having a double-stranded region of at least 20 nucleotides but no more than 29 nucleotides, and therefore Fire does not show the invention arranged as in the claim.

2. Fire does not anticipate the presently claimed range.

Fire does not disclose a short hairpin RNA molecule, where "the double-stranded region consists of at least 20 nucleotides but not more than 29 nucleotides."

M.P.E.P. § 2131.03, entitled "Anticipation of Ranges" makes it clear that disclosure of a large range does not anticipate a smaller, overlapping range. Relying upon the *Atofina* case, the M.P.E.P. also instructs that disclosure of a range is not disclosure of the endpoints of the range.

Section 2131.03 states that in order to anticipate the claims, the claimed subject matter must be disclosed in the reference with "sufficient specificity to constitute an anticipation under the statute." It goes on to state:

> What constitutes a "sufficient specificity" is fact dependent. If the claims are directed to a narrow range, and the reference teaches a broad range, depending on the other facts of the case, it may be reasonable to conclude that the narrow range is not disclosed with "sufficient specificity" to constitute an anticipation of the claims. *See, e.g., Atofina v. Great Lakes Chem. Corp*, 441 F.3d 991, 999, 78 U.S.P.Q. 2d 1417, 1423 (Fed. Cir. 2006) wherein the court held that a reference temperature range of 100-500 degrees C did not describe the claimed range of 330-450 degrees C with sufficient specificity to be anticipatory. Further, while there was a slight overlap between the reference's preferred range (150-350 degrees C) and the claimed range, that overlap was not sufficient for anticipation. "[T]he disclosure of a range is no more a disclosure of the end points of the range than it is each of the intermediate points." *Id.* at 1000, 78 U.S.P.Q. 2d at 1424.

M.P.E.P. § 2131.03 (II).

The pending claims of the present application are directed to a narrow range (at least 20 nucleotides but not more than 29 nucleotides; between 25 and 30). The Fire reference discloses

a very broad range (at least 25 nucleotides, with no upper limit). Although there is a slight overlap in our case, just as in *Atofina*, this overlap is not sufficient for anticipation.

Here, Applicants understand the Examiner's position to be that Fire's disclosure, "[t]he length of the identical nucleotide sequences may be at least 25, 50, 100, 200, 300 or 400 bases," not only discloses a range, but serves as a species disclosure, that is, a length of 25 nucleotides. The Examiner's position conflicts with binding precedent, as acknowledged by the M.P.E.P., which holds that simply because ranges must necessarily describe end points, describing an end point does not disclose that end point as a species: "[T]he disclosure of a range is no more a disclosure of the end points of the range than it is each of the intermediate points." *Atofina*, 441 F.3d at 1000; *see also*, M.P.E.P. § 2131.03 (II)(citing *Atofina*); M.P.E.P. § 2163.05 (III) ("at least…" describing a range).

The court in *Atofina* ruled that even though the broad range fully encompassed the narrower range, there was a "considerable difference between the claimed range and the range in the prior art" such that "no reasonable fact finder could conclude that the prior art describes the claimed range with sufficient specificity to anticipate this limitation of the claim." *Atofina*, 441 F.3d at 999.

The court's finding with respect to the preferred prior art range is also instructive. The court said that the slightly overlapping prior art range of 150-350° C did not anticipate the claimed range of 330-450° C. The court stated that the disclosure of a range "does not constitute a specific disclosure of the endpoints of that range." *Id.* at 1000. The court went on to say: "The disclosure is only that of a range, not a specific temperature in that range, and the disclosure of a range is no more a disclosure of the end points of the range than it is of each of the intermediate points." *Id.*

The supposed range disclosed by Fire is of a complementary region of at least 25 nucleotides, with no disclosed endpoint. The present claims recite a double-stranded region of at least 20 but not more than 29 nucleotides. The alleged overlap with Fire's range is even more slight here than in *Atofina*.

Furthermore, new claim 64 is not anticipated by Fire because, if, *arguendo*, one takes the Examiner's position as true (which Applicants do not) that Fire anticipates the species of a double-stranded region having a length of 25 nucleotides, then a claim requiring a length of double-stranded region of "between 25 and 30" is not anticipated by Fire. The alleged species

that the Examiner contends is disclosed by Fire of 25 nucleotides in length is excluded from the range recited in new claim 64. Accordingly, the alleged species disclosed by Fire falls outside the scope of the claim, and therefore does not anticipate new claim 64.

3. Fire's disclosure of length ranges refers to "a nucleotide sequence identical to a portion of a target gene" or the complementary region, and not to the claimed element of a "double-stranded region."

The Examiner relies upon col. 7-8 and claim 15 of Fire to support alleged anticipation. In particular, the Examiner relies on Fire's statement: "The length of the identical nucleotide sequences may be at least 25, 50, 100, 200, 300 or 400 bases." This statement in Fire uses the term "identical nucleotide sequences" which the Examiner believes to be the same as the "double-stranded region" in the pending claims. Applicants disagree.

The antecedent for "the identical nucleotide sequences" in Fire can be found at the beginning of that same paragraph, at col. 7, lines 53-55. Here, Fire states "RNA containing a nucleotide sequences [sic] identical to a portion of the target gene are referred for inhibition." Fire is merely describing the length of the complementary region of the RNA molecule, not the length of the double-stranded region. These two regions can be of very different lengths.

The Examiner's interpretation of the statement in Fire is inconsistent with the context of the surrounding disclosure. The only lengths mentioned by Fire are with respect to the region that is complementary to the target gene sequence. A complementary region of "at least 25" nucleotides could imply a much longer double-stranded region. In fact, Fire exemplifies dsRNAs several hundred base pairs in length. (See Table 1.) There is no explicit or inherent disclosure in Fire regarding the length of the double-stranded region of a hairpin RNA.

Claim 15 of Fire is expressly limited to a two-stranded RNA molecule. Therefore, the reference to "said double-stranded ribonucleic structure is at least 25 bases in length" refers only to the range of double-stranded region lengths in a two-stranded RNA molecule. This fails to disclose anything about a short hairpin RNA molecule. Under *Net MoneyIN* and *Ecolochem*, it would be improper to extrapolate the recitation in Fire claim 15, which refers to a two-stranded RNA molecule, to a different structure, *e.g.*, a single hairpin RNA, as Applicants are presently claiming.

4. Fire does not link the elements of the claimed invention together as they appear in the presently pending claims.

We understand that the Examiner's position is that she can take the disclosure of Fire into consideration as a whole. Nowhere, however, in Fire is there any description or discussion of using an expression construct as presently claimed for stable expression of a short hairpin RNA molecule. Moreover, Fire does not link or connect, in his disclosure, the use of a short hairpin RNA molecule with stable expression of that molecule in a mammalian cell. In addition, Fire does not link or connect the element of a hairpin RNA molecule with the element of the hairpin comprising a double-stranded region consisting of at least 20 nucleotides but not more than 29 nucleotides. Fire therefore does not show or describe the invention arranged as in the claims., These deficiencies in the Fire disclosure, under *Net MoneyIN* and *Ecolochem*, therefore demonstrate that Fire does not anticipate the claimed invention.

C. Kreutzer Is Not a Proper Section 102(e) Reference, And the Claims Are Not Anticipated by Kreutzer

Kreutzer et al. as cited in the parent application, U.S.S.N. § 10/997,086, is not proper prior art under 35 U.S.C. §102(e). Kreutzer et al. is based on an international application filed prior to November 29, 2000 and is therefore subject to the pre-"American Inventors Protection Act (AIPA) of 1999" version of 35 U.S.C. § 102(e). See M.P.E.P. 706.02(a) (II.B). The former version of Section 102(e) states:

> A person shall be entitled to a patent unless-
>
> . . .
>
> (e) the invention was described in a patent granted on an application for patent by another filed in the United States before the invention thereof by the applicant for patent, or on an international application by another who has fulfilled the requirements of paragraphs (1), (2), and (4) of section 371(c) of this title before the invention thereof by the applicant for patent.

The parent of the Kreutzer et al. publication cited in the Office Action was U.S. application Serial No. 09/889,802 ("the '802 application), which is now abandoned. The '802 application has no 102(e) date because it never issued as a patent. The 102(e) date of the cited Kreutzer et al. publication is its filing date, March 6, 2003, which is later than the January 22, 2002 priority date of the present application. See Example 6 of M.P.E.P. § 706.02(f)(1) and note

the section on Additional Benefit Claims. Therefore, Kreutzer et al. is not a proper reference under Section 102(e).

Regardless, Kreutzer does not teach target gene attenuation by expression of a short hairpin RNA in a mammalian cell. The only place in Kreutzer that expressly mentions a hairpin is Paragraph [0019], which discusses chemical modification of the nucleotides in the loop region to protect against degradation. This discussion does not describe a hairpin expressed in a mammalian cell, which cannot have such chemical modification. Instead, this paragraph is directed to a hairpin that is synthesized before being introduced into a mammalian cell, "in particular, when using a vector according to the invention," that is "by means of T7 and SP6 in-vitro transcription" (Kreutzer, Use Example 2 Paragraph [0068]).

Paragraph [0019] is in the midst of several paragraphs teaching how to chemically link the dsRNA to prevent dissociation of the strands. Taken in context, the hairpin mentioned in Paragraph [0019] of Kreutzer is not one that is or could ever be expressed from a vector in the cell containing the target gene.

Consequently, Kreutzer did not use "hairpin" in any part of the disclosure that may describe expression of a double-stranded RNA from a vector in a cell.

D. Non-Obviousness Over The Prior Art

The submitted 132 Declaration by Dr. Nouria Hernandez demonstrates that one of ordinary skill in the art would have had no expectation of successfully carrying out the claimed methods. The scientific literature taught away from the expression of short hairpins to attenuate target gene expression.

CONCLUSION

Consideration of this paper and allowance of this application are requested. If it would advance prosecution, the Examiner is invited to contact the undersigned to discuss the contents of this paper.

Dated: January 12, 2010

Respectfully submitted,

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Electronic Acknowledgement Receipt

EFS ID:	6794878
Application Number:	11894676
International Application Number:	
Confirmation Number:	8161
Title of Invention:	Methods and compositions for RNA interference
First Named Inventor/Applicant Name:	Gregory J. Hannon
Customer Number:	84834
Filer:	Anne-Marie Yvon/Patricia Ierardi
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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		287000_130US3_SuppAmend_011210.pdf	177414 82e93d0999580b8913826a7fe03286e35600492a	yes	14

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Supplemental Response or Supplemental Amendment	1	1
	Claims	2	3
	Applicant Arguments/Remarks Made in an Amendment	4	14
Warnings:			
Information:			
	Total Files Size (in bytes):	177414	

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New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/06 (07-06)
Approved for use through 1/31/2007. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number	Filing Date	☐ To be Mailed
11/894,676	08/20/2007	

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY ☒ RATE ($)	SMALL ENTITY ☒ FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT 01/12/2010	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 13	Minus	** 20	= 0	X $26 =	0	OR	X $ =	
Independent (37 CFR 1.16(h))	* 2	Minus	***3	= 0	X $110 =	0	OR	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))									
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE	0	OR	TOTAL ADD'L FEE	

AMENDMENT	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)		RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))									
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/LINDA W. BADIE/

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/894,676	08/20/2007	Gregory J. Hannon	287000.130US3	8161

84834 7590 01/27/2010

WilmerHale/Cold Spring Harbor Laboratory
399 Park Avenue
New York, NY 10022

EXAMINER	
CHONG, KIMBERLY	

ART UNIT	PAPER NUMBER
1635	

MAIL DATE	DELIVERY MODE
01/27/2010	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	11/894,676	HANNON ET AL.	
	Examiner	Art Unit	
	KIMBERLY CHONG	1635	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *12 January 2010*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *50,52 and 54-64* is/are pending in the application.

4a) Of the above claim(s) *61* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *50,52,54-60,62-64* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Status of Application/Amendment/Claims

Applicant's response filed 01/12/2010 has been considered. Rejections and/or objections not reiterated from the previous office action mailed 05/04/2009 are hereby withdrawn. The following rejections and/or objections are either newly applied or are reiterated and are the only rejections and/or objections presently applied to the instant application. The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

With entry of the amendment filed on 01/12/2010, claims 50, 52 and 54-64 are pending in the application. Claims 61 and non-elected subject matter is withdrawn ans being drawn to a non-elected invention.

Status of the Application

Claims 50-61 are pending. Claims 50-60 are currently under examination. Claim 61 and non-elected subject matter are withdrawn as being drawn to a non-elected invention.

Information Disclosure Statement

The submission of the Information Disclosure Statements on 01/10/2008, 08/08/2008, 08/14/2008 and 08/28/2008 is in compliance with 37 CFR 19.7. The information disclosure statements have been considered by the examiner and signed copies have been placed in the file.

Response to Declaration

The declaration filed on 11/04/2009 under 37 CFR 1.132 is insufficient overcome the rejection of record.

Professor Hernandez states the literature indicated that dsRNA less than 150 base pairs in length were less effective. This argument is not persuasive as it is clearly shown in Kreutzer et al. that a dsRNA 21 nucleotide base paired molecule was capable of efficiently reducing gene expression (see Examples). Applicant argues that one would not have expected short dsRNA, less than 30 base pairs to be efficiently processed into siRNA however the claims are drawn to dsRNAs at least 20 and claims specifically recite 21 or 22 nucleotides. Elbashir et al. 2001(a) as cited by applicant does in fact demonstrate that small dsRNAs, less than 30 nucleotides were capable of reducing gene expression and without a PKR response and found that specifically 21 to 22 dsRNAs were responsible for mediating RNAi. Thus a person of ordinary skill in the art would have expected smaller dsRNAs of less than 30 nucleotides to be capable of mediating RNAi.

Professor Hernandez argues that Elbashir et al. discourage the use of precursors, however the claims are not limited to the use of precursor RNA and as claimed, the dsRNA can be as small as 20 nucleotides in length which as stated above, the skilled artisan would have expected to work.

Thus, there is nothing in the prior art, before or at the time of filing of the instant application that would have discouraged the skilled artisan from using dsRNA of less than 30 to mediate RNAi.

New Claim Rejections - 35 USC § 112

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claim 64 is rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. This is a new matter rejection.

Claim 64 recites the shRNA molecule comprises a double stranded region wherein the region "consists of between 25 and 30 nucleotides."

In paragraph [0015] of the published application, reproduced below, the dsRNA is disclosed as being at least 20, 21, or 22 nucleotides in length or at lest 25, 50, 100...etc. bases. A 29 nucleotide shRNA is exemplified in Example 7.

> [0015]In certain preferred embodiments, the length of the dsRNA is at least 20, 21 or 22 nucleotides in length, e.g., corresponding in size to RNA products produced by Dicer-dependent cleavage. In certain embodiments, the dsRNA construct is at least 25, 50, 100, 200, 300 or 400 bases. In certain embodiments, the dsRNA construct is 400-800 bases in length.

The instant specification does not disclose the dsRNA wherein the double stranded region is between 25 and 30 nucleotides in length. Applicant explains that "between" means the interval defined by two endpoints and therefore the interval between 25 and 30 defines a double stranded region of 26, 27, 28 or 29 nucleotides. In other words, this new range in the claim limitation excludes a double stranded region of 25 and 30 nucleotides in length. There is no support in the instant specification for a definition of between as defining the interval between two endpoints and there is no support for a double stranded region that excludes 25 or 30 nucleotides as endpoints.

Applicant's cites Wertheim as support for the fact that "one of skill in the art would therefore consider use of a short hairpin RNA having between 25 and 30 base pairs to all be part of the same invention the specification discloses". This argument along with the facts of Wertheim are not relevant to the fact that the instant specification does not provide explicit support for a range of between 25 and 30 wherein the region excludes 20 or 30 nucleotides as endpoints or provide implicit support such that from the description of the length of the dsRNA in paragraph [0015], it is understood that the double stranded region is between 25-30 and excludes 25 and 30 as endpoints.

If Applicant believes that such support is present in the specification and claimed priority documents, Applicant should point, with particularity, to where such support is to be found.

For purposes of applying prior art, the limitation "wherein the double stranded region consists of between 25 and 30 nucleotides" is interpreted to mean the duplex region can have 25, 26, 27, 28, 29 or 30 nucleotides. Contrary to Applicant's

explanation of "between", in the context of the claim and based on the instant specification, the endpoints of 25 or 30 nucleotides are not excluded from this recitation.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claim 64 is rejected under 35 U.S.C. 102(b) as being anticipated by McSwiggen et al. (US 20050277133).

Claim 64 does not receive the benefit of the earlier filed applications as explained above in the new matter rejection. The later-filed application must be an application for a patent for an invention which is also disclosed in the prior application (the parent or original nonprovisional application or provisional application). The disclosure of the invention in the parent application and in the later-filed application must be sufficient to comply with the requirements of the first paragraph of 35 U.S.C. 112. See *Transco Products, Inc. v. Performance Contracting, Inc.*, 38 F.3d 551, 32 USPQ2d 1077 (Fed. Cir. 1994).

The claims are drawn to a method of attenuating expression of a target gene comprising introducing into mammalian cells a library shRNA consisting of between 25 and 30 nucleotides in length.

McSwiggen et al. teach methods of attenuation of target gene expression comprising administering a shRNA of up to 30 nucleotides in length and teach the use

of expression vectors and pol promoters for expression of a library of shRNA (see at least Examples 3-4, paragraphs 0488, 0017, 0054, 002 and Figure 17).

Thus, McSwiggen et al. anticipates the instant claim.

Response to Applicant's Arguments

Re: Double Patenting

The rejection of claims 50-60 under the judicially created doctrine of double patenting over claims 1, 2, 7-20, 24 and 59-63 of copending Application No. 10/350,798 is maintained. Applicant did not provide any arguments against this rejection.

The rejection of claims 50-60 under the judicially created doctrine of double patenting over claims 1, 2, 6-7, 9-10 and 23-28 of copending Application No. 09/858,862 is maintained. Applicant did not provide any arguments against this rejection.

Re- Claim Rejections - 35 USC § 112

The rejection of claim 52 under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement is withdrawn.

Re: Claim Rejections - 35 USC § 102

The rejection of claims 50-51 and 53-58 are rejected under 35 U.S.C. 102(e) as being anticipated by Fire et al. (US Patent Number 6,506,559) is withdrawn in response to claim amendments.

Re: Claim Rejections - 35 USC § 103

The rejection of claims 50, 52, 54-60 and 62-64 are rejected under 35 U.S.C. 103(a) as being unpatentable over Fire et al. (US Patent Number 6,506,559 cited on Applicant's IDS filed 01/10/2008), Good et al. (Gene Therapy 1997 cited on Applicant's IDS filed 01/10/2008) and Noonberg et al. (US Patent NO. 5,624,803) is maintained for the reasons of record.

The amendment to claim 50 reciting the hairpin RNA is stably expressed would have been rejected in the previous rejection of record (see Noonberg et al. at least column 17 beginning at line 20 which discusses stable expression of a RNA molecule using a construct comprising a U6 promoter) and therefore the response to arguments below applies.

New claims 62 and 63 would have been rejected in the previous rejection of record (see Fire et al. column 7 wherein Fire et al. teach inhibition of gene expression up to 95% would be routine) and and therefore the response to arguments below applies.

New claim 64 is drawn to subject matter that would have been rejected in the previous rejection of record and therefore the response to arguments below applies.

Applicant argues Fire et al. do not disclose the claimed range wherein the double stranded region consists of at least 20 nucleotides but not more than 29 nucleotides. Applicants argue Fire et al. disclose a very broad range of at least 25 nucleotides with no upper limit and although there is a slight overlap, this overlap is not sufficient for anticipation and relies on Atofina for support of this argument.

This argument is not persuasive because the disclosure in Fire et al. is not interpreted by the Examiner as a disclosure of a range. Fire et al. disclose RNA containing a nucleotide sequence identical to a portion of the target gene i.e. the antisense strand of the dsRNA (as well known in the prior art), to be at least 25 bases in length.

> 8) **RNA containing a nucleotide sequences identical to a portion of the target gene are preferred for inhibition**. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence have also been found to be effective for inhibition. Thus, sequence identity may optimized by sequence comparison and alignment algorithms known in the art (see Gribskov and Devereux, Sequence Analysis Primer, Stockton Press, 1991, and references cited therein) and calculating the percent difference between the nucleotide sequences by, for example, the Smith-Waterman algorithm as implemented in the BESTFIT software program using default parameters (e.g., University of Wisconsin Genetic Computing Group). Greater than 90% sequence identity, or even 100% sequence identity, between the inhibitory RNA and the portion of the target gene is preferred. Alternatively, the duplex region of the RNA may be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript (e.g., 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50.degree. C. or 70.degree. C. hybridization for 12-16 hours; followed by washing). **The length of the identical nucleotide sequences may be at least 25**, 50, 100, 200, 300 or 400 bases.

Applicant argues Fire is merely describing the length of the complementary region of the RNA molecule, not the length of the double-stranded region and these "two regions can be of very different lengths". Fire et al. clearly states in paragraph

(07) that the “double-stranded structure may be formed by a single self-complementary RNA strand or two complementary RNA strands” and it is clear that when Fire et al. recites a self-complementary RNA strand i.e. a hairpin RNA, this structure comprises a first strand that is complementary to the target RNA (the antisense strand) and a strand that is complementary to the first strand (the sense strand). There is no mention in the Fire et al. disclosure that the two strands of the hairpin RNA can be very different lengths as argued by Applicant.

Applicant argues claim 15 of Fire et al. is expressly limited to a tow-stranded RNA molecule and therefore the reference to a “double-stranded ribonucleic acid structure is at least 25 bases in length" refers only to the range of double-stranded regions in a two stranded RNA molecule. This argument is not convincing because nowhere in the claims does Fire et al. limit this structure to a two stranded RNA molecule. As stated above, Fire et al. clearly states in paragraph (07) that the “double-stranded structure may be formed by a single self-complementary RNA strand or two complementary RNA strands”. Thus, whenever a double stranded structure is recited in the specification or claims, this double stranded structure can be a hairpin RNA or comprised of two strands. As such, claim 15 recites a double stranded structure, a hairpin RNA, wherein the structure is at least 25 bases in length.

Applicant argues that Fire et al. does not discussion using an expression construct for stable expression of short hairpin RNA and Fire et al. do not link the elements of the claimed invention together as they appear in the presently pending claims. The arguments regarding the lack of Fire et al. discussing stable expression is

discussed above. With regard to Fire not linking the elements together as they appear in the pending claims, the Examiner assumes Applicant means Fire et al. do not describe the claimed invention in a single section or paragraph and this argument is not persuasive because it is unrealistic for the discussion of every element of the features of a dsRNA to be disclosed in any specification in one paragraph or sentence. As with most specification discussing RNAi and dsRNA, Fire et al. discloses a detailed description of dsRNA which involves describing the size of the dsRNA, the lengths of the strands, the cells to target, how to use dsRNA and this is not often disclosed in a single paragraph. Even Applicant's own specification does not link the elements of the claims invention together. The fact that Fire et al. do not "link" the elements of claimed invention does not negate the fact that Fire et al. teach a hairpin RNA having at least 25 bases and in combination with references above, obviates the claimed invention.

Applicant has not provided any other arguments regarding the remaining references, thus the rejection of record is maintained.

The rejection of claims 50, 52, 54-60 and 62-64 under 35 U.S.C. 103(a) as being unpatentable over Kreutzer et al. (US Application No. 20040102408), Lieber et al. (US Patent No. 6,130,092 cited on Applicant's IDS filed 01/10/2008), Good et al. (Gene Therapy 1997 cited on Applicant's IDS filed 01/10/2008) and Noonberg et al. (US Patent NO. 5,624,803) is maintained for the reasons of record.

The amendment to claim 50 reciting the hairpin RNA is stably expressed would have been rejected in the previous rejection of record (see Noonberg et al. at least column 17 beginning at line 20 which discusses stable expression of a RNA molecule using a construct comprising a U6 promoter) and therefore the response to arguments below applies.

New claims 62 and 63 would have been rejected in the previous rejection of record (it would have been obvious and routine to construct a dsRNA to reduce gene expression up to 90%) and therefore the response to arguments below applies.

New claim 64 is drawn to subject matter that would have been rejected in the previous rejection of record and therefore the response to arguments below applies.

Applicant argues Kreutzer et al. is not a proper 102(e) reference because the parent application US application 09/889,802 has no 102(e) date because it never issued and points to Example 6 of MPEP section 706.02(f)(1).

This argument is incorrect because regardless of whether or not the ‘802 application has a proper 102(e) date is not relevant to whether the applied reference has a proper 102(e) date. Kreutzer et al. (US Application No. 20040102408) has a 102(e) date which is the effective filing date of the ‘802 application, 09/17/2001, which is before the priority date of the instant application. Kreutzer et al. does not need the date of the international application from which the ‘802 arose to be used as a prior art reference in this instance. The '802 application was a national stage entry of the international application and therefore the national stage entry date is the filing date of

the application. Example 6 would be applicable if the Examiner is citing the '802 as prior art. Thus, Kreutzer et al. is available as a prior art reference.

Applicant argues Kreutzer et al. does not teach attenuation of gene expression by expression of a short hairpin RNA in a mammalian cell and claims the only mention of a hairpin is paragraph [0019] and this discussion of a the hairpin if taken in context is not one that could ever be expressed from a vector in the cell containing the target gene.

Applicant's argument is not convincing because Kreutzer et al. discusses dsRNA that is formed by a single auto complementary RNA comprising a loop and as interpreted is considered a hairpin RNA. Beginning at paragraph [0028] Kreutzer et al. disclose vectors comprising dsRNA for introduction into cells and therefore teach the expression in mammalian cells of dsRNA from a vector.

Applicant has not provided any other arguments regarding the remaining references, thus the rejection of record is maintained.

Conclusion

THIS ACTION IS MADE FINAL. Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the

shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Kimberly Chong whose telephone number is 571-272-3111. The examiner can normally be reached Monday thru Thursday between 6 and 3 pm.

If attempts to reach the examiner by telephone are unsuccessful please contact Tracy Vivlemore at 571-272-2914. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Patent applicants with problems or questions regarding electronic images that can be viewed in the Patent Application Information Retrieval system (PAIR) can now contact the USPTO's Patent Electronic Business Center (Patent EBC) for assistance. Representatives are available to answer your questions daily from 6 am to midnight (EST). The toll free number is (866) 217-9197. When calling please have your application serial or patent number, the type of document you are having an image problem with, the number of pages and the specific nature of the problem. The Patent Electronic Business Center will notify applicants of the resolution of the problem within 5-7 business days. Applicants can also check PAIR to confirm that the problem has been corrected. The USPTO's Patent Electronic Business Center is a complete service center supporting all patent business on the Internet. The USPTO's PAIR system provides Internet-based access to patent application status and history information. It also enables applicants to view the scanned images of their own application file folder(s) as well as general patent information available to the public. For more information about the PAIR system, see http://pair-direct.uspto.gov.

For all other customer support, please call the USPTO Call Center (UCC) at 800-786-9199.

/Kimberly Chong/
Primary Examiner
Art Unit 1635

Search Notes	Application/Control No.	Applicant(s)/Patent under Reexamination
	11/894,676	HANNON ET AL.
	Examiner	Art Unit
	KIMBERLY CHONG	1635

SEARCHED			
Class	Subclass	Date	Examiner

INTERFERENCE SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES (INCLUDING SEARCH STRATEGY)		
	DATE	EXMR
updated	1/20/2010	KC

Doc Code: M865 or FAI.REQ.INTV

PTOL-413A (10-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

Applicant Initiated Interview Request Form

Application No.: 10/997,086; 11/894,676 First Named Applicant: G. Hannon
Examiner: Kimberly Chong Art Unit: 1635 Status of Application: Pending

Tentative Participants:
(1) Jane Love (outside counsel) (2) Vladimir Drozdoff (in-house counsel)
(3) Anne-Marie Yvon (outside counsel) (4)

Proposed Date of Interview: March 18, 2010 **Proposed Time:** 2 PM (AM/PM)

Type of Interview Requested:
(1) [✓] Telephonic (2) [ ] Personal (3) [ ] Video Conference

Exhibit To Be Shown or Demonstrated: [ ] YES [✓] NO
If yes, provide brief description:

Issues To Be Discussed

Issues (Rej., Obj., etc)	Claims/ Fig. #s	Prior Art	Discussed	Agreed	Not Agreed
(1) 103	All		[ ]	[ ]	[ ]
(2) 102(e)	All	Kreutzer et al.	[ ]	[ ]	[ ]
(3)			[ ]	[ ]	[ ]
(4)			[ ]	[ ]	[ ]

[ ] Continuation Sheet Attached
[ ] Proposed Amendment or Arguments Attached
Brief Description of Arguments to be Presented:

(1) Weight and proper consideration of evidence presented under 37 CFR 1.132

(2) Application of 35 USC 102(e) to publication claiming priority to PCT application filed prior to November 29, 2000

An interview was conducted on the above-identified application on _____.
NOTE: This form should be completed by applicant and submitted to the examiner in advance of the interview (see MPEP § 713.01).
This application will not be delayed from issue because of applicant's failure to submit a written record of this interview. Therefore, applicant is advised to file a statement of the substance of this interview (37 CFR 1.133(b)) as soon as possible.

/Anne-Marie C. Yvon/
Applicant/Applicant's Representative Signature

Examiner/SPE Signature

Anne-Marie C. Yvon
Typed/Printed Name of Applicant or Representative

52,390
Registration Number, if applicable

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Electronic Acknowledgement Receipt

EFS ID:	7207698
Application Number:	11894676
International Application Number:	
Confirmation Number:	8161
Title of Invention:	Methods and compositions for RNA interference
First Named Inventor/Applicant Name:	Gregory J. Hannon
Customer Number:	84834
Filer:	Anne-Marie Yvon/Patricia Ierardi
Filer Authorized By:	Anne-Marie Yvon
Attorney Docket Number:	287000.130US3
Receipt Date:	15-MAR-2010
Filing Date:	20-AUG-2007
Time Stamp:	15:22:42
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Letter Requesting Interview with Examiner	287000_130US3_InterviewRequest_031510.pdf	61072 34a4f41d663e783f58c0514660cdc98605d4a0c2	no	2

Warnings:	
Information:	

Total Files Size (in bytes):	61072

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Doc Code: M865 or FAI.REQ.INTV

PTOL-413A (10-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

Applicant Initiated Interview Request Form

Application No.: 10/997,086; 11/894,676 First Named Applicant: G. Hannon
Examiner: Kimberly Chong Art Unit: 1635 Status of Application: Pending

Tentative Participants:
(1) Jane Love (outside counsel) (2) Vladimir Drozdoff (In-house counsel)
(3) Anne-Marie Yvon (outside counsel) (4)

Proposed Date of Interview: March 18, 2010 **Proposed Time:** 2 PM (AM/PM)

Type of Interview Requested:
(1) [✓] Telephonic (2) [ ] Personal (3) [ ] Video Conference

Exhibit To Be Shown or Demonstrated: [ ] YES [✓] NO
If yes, provide brief description:

Issues To Be Discussed

Issues (Rej., Obj., etc)	Claims/ Fig. #s	Prior Art	Discussed	Agreed	Not Agreed
(1) 103	All		[ ]	[ ]	[ ]
(2) 102(e)	All	Kreutzer et al.	[ ]	[ ]	[ ]
(3)			[ ]	[ ]	[ ]
(4)			[ ]	[ ]	[ ]

[ ] Continuation Sheet Attached
[ ] Proposed Amendment or Arguments Attached
Brief Description of Arguments to be Presented:

(1) Weight and proper consideration of evidence presented under 37 CFR 1.132

(2) Application of 35 USC 102(e) to publication claiming priority to PCT application filed prior to November 29, 2000

An interview was conducted on the above-identified application on _____.
NOTE: This form should be completed by applicant and submitted to the examiner in advance of the interview (see MPEP § 713.01).
This application will not be delayed from issue because of applicant's failure to submit a written record of this interview. Therefore, applicant is advised to file a statement of the substance of this interview (37 CFR 1.133(b)) as soon as possible.

/Anne-Marie C. Yvon/
Applicant/Applicant's Representative Signature

Examiner/SPE Signature

Anne-Marie C. Yvon
Typed/Printed Name of Applicant or Representative

52,390
Registration Number, if applicable

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

WILMERHALE

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anne-marie.yvon@wilmerhale.com

Date **March 15, 2010**

To **Examiner Kimberly CHONG**
Group Art Unit 1635
U.S. PATENT AND TRADEMARK OFFICE

Fax **(571) 273-8300**
Tel **(571) 272-1000**

cc

From **Anne-Marie C. Yvon, Ph.D.** Pages **3** (including cover)

Re **U.S. Appln. Nos. 10/997,086 + 11/894,676**
Our Ref. Nos.: 287000.130-US1 and 130-US3

ATTENTION: Examiner Kimberly Chong

Group Art Unit 1635

Dear Examiner Chong:

Attached is a courtesy copy of the Applicant Initiated Interview Request Form which was e-filed today in each of the above two pending applications.

Thank you for your attention to this matter.

Anne-Marie C. Yvon, Ph.D.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/894,676	08/20/2007	Gregory J. Hannon	287000.130US3	8161

84834 7590 03/25/2010

WilmerHale/Cold Spring Harbor Laboratory
399 Park Avenue
New York, NY 10022

EXAMINER	
CHONG, KIMBERLY	

ART UNIT	PAPER NUMBER
1635	

MAIL DATE	DELIVERY MODE
03/25/2010	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Interview Summary	Application No.	Applicant(s)	
	11/894,676	HANNON ET AL.	
	Examiner	Art Unit	
	KIMBERLY CHONG	1635	

All participants (applicant, applicant's representative, PTO personnel):

(1) *KIMBERLY CHONG*.

(2) *JANE LOVE*.

(3)*ANNE-MARIE YVON*.

(4)*VLADIMIR DROZDOFF*.

Date of Interview: *18 March 2010*.

Type: a)☒ Telephonic b)☐ Video Conference
c)☐ Personal [copy given to: 1)☐ applicant 2)☐ applicant's representative]

Exhibit shown or demonstration conducted: d)☐ Yes e)☐ No.
If Yes, brief description: _____.

Claim(s) discussed: _____.

Identification of prior art discussed: _____.

Agreement with respect to the claims f)☐ was reached. g)☐ was not reached. h)☒ N/A.

Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: *Discussed the outstanding Office action and the declaration submitted by Dr. Hernandez and whether the Examiner viewed the declaration as opinion or evidence. Discussed the Kreutzer et al. reference and whether it was properly cited as a 102(e) reference. The Examiner stated she will consider these issues in the next Office action.*.

(A fuller description, if necessary, and a copy of the amendments which the examiner agreed would render the claims allowable, if available, must be attached. Also, where no copy of the amendments that would render the claims allowable is available, a summary thereof must be attached.)

THE FORMAL WRITTEN REPLY TO THE LAST OFFICE ACTION MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP Section 713.04). If a reply to the last Office action has already been filed, APPLICANT IS GIVEN A NON-EXTENDABLE PERIOD OF THE LONGER OF ONE MONTH OR THIRTY DAYS FROM THIS INTERVIEW DATE, OR THE MAILING DATE OF THIS INTERVIEW SUMMARY FORM, WHICHEVER IS LATER, TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW. See Summary of Record of Interview requirements on reverse side or on attached sheet.

	/Kimberly Chong/ Primary Examiner AU1635

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

———

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:

1) A brief description of the nature of any exhibit shown or any demonstration conducted,
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
   (The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiner's version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, "Interview Record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Hannon et al.	Confirmation Nos:	9352 and 8161
Application Nos:	10/997,086 and 11/894,676	Art Unit:	1635
Filed:	November 23, 2004 and August 20, 2007	Examiner:	K. CHONG
Title:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INTERVIEW SUMMARY

This paper is filed in response to the March 25, 2010 Interview Summary, setting a one-month period for filing a statement of the substance of the interview. No fee is believed to be due for entry of this paper; however, the Director is authorized to charge any fee occasioned by this paper to Deposit Account No. 08-0219.

I. Background and Status

A telephonic interview was held on March 18, 2010 between Examiner Kimberly Chong, Jane Love and Anne-Marie Yvon of WilmerHale, and Vladimir Drozdoff of Cold Spring Harbor Laboratory. Applicants filed a Response to a non-final Office Action in U.S. application Serial No. 10/997,086 ("the '086 application") on February 26, 2010. A Response to a final Office Action in U.S. application Serial No. 11/894,676 ("the '676 application") is due on April 27, 2010.

The '676 application is a continuation of the '086 application. The claims in each of these applications are similar, but differ in that the '086 claims are directed to introducing an expression vector, and the claims in the '676 application are directed to introducing a library of RNA expression constructs. In particular, the claims of both applications are directed to the

attenuation of target gene expression in mammalian cells by RNAi. The claims of both applications require introduction into mammalian cells and stable expression of RNA expression constructs comprising a sequence encoding a short hairpin RNA molecule comprising a double-stranded region consisting of at least 20 nucleotides but not more than 29 nucleotides. The double-stranded region of the short hairpin RNA molecule expressed in the cell comprises a sequence that is complementary to a portion of the target gene. The claims further specify that the expressed short hairpin RNA does not trigger a protein kinase RNA-activated (PKR) response in the mammalian cells.

Applicant explained to the Examiner that the physical structure of shRNA is different from the structure of short interfering RNA (siRNA). Once expressed in a cell, shRNA must necessarily be processed by Dicer to mediate RNAi. The essence of the invention is that a sequence encoding an shRNA can be introduced into and expressed by a mammalian cell, and then the expressed shRNA can be processed into the 21 and 22-nt siRNA structures that inhibit target gene expression in a sequence-specific manner, and can do so without inducing a PKR response. See Hernandez Declaration, ¶¶ 2, 6-8.

II. Declaration of Dr. Nouria Hernandez

Applicants submitted a Declaration under 37 C.F.R. § 1.132 by Dr. Nouria Hernandez ("the Hernandez Declaration") in each of the '086 and '676 applications to demonstrate non-obviousness of the claimed inventions. The Hernandez Declaration, by one of ordinary skill at the time of the invention, demonstrates that one of ordinary skill in the art would have had no expectation of successfully carrying out the claimed methods. The scientific literature taught away from the expression of short hairpins to attenuate target gene expression.

During the interview, Applicants pointed out that a declaration by one of ordinary skill in the art is evidence, not argument, regarding how one of ordinary skill in the art would have viewed the state of the art based on the literature at the time of the invention. In this case, the Hernandez Declaration is evidence that one of ordinary skill in the art would have not expected to be able to use a short hairpin RNA comprising a double-stranded region consisting of 20-29 base pairs to attenuate target gene expression in mammalian cells, while avoiding a PKR response. As evidenced by the Hernandez Declaration, the fact that an expressed short hairpin could do so was unexpected in view of the state of the art at the time.

Applicants also pointed out that the Hernandez Declaration is not mere opinion about the prior art, it presents and factually states what the prior art said. In particular, Dr. Hernandez points to Elbashir et al. (24 May 2001) *Nature*, 411:494-98, which discloses target gene attenuation using dsRNAs of 38 base pairs or greater. Elbashir et al. explicitly teaches away from using shorter sequences, stating that dsRNAs of 29-36 base pairs are ineffective, and that "[s]hort 30-bp dsRNAs are inefficiently processed to 21- and 22-nt RNAs, which may explain why they are ineffective at mediating RNAi." Hernandez Declaration, ¶¶ 11, 12. These statements in the prior art are in direct contrast to the teachings of the present specification demonstrating that short hairpin RNAs could mediate RNAi. Thus, in view of the prior art, one of ordinary skill would not have reasonably expected the results demonstrated by the present inventors.

The Hernandez Declaration is factual evidence. To deem such evidence insufficient, the Examiner must do more than disagree with the declarant, she must provide factual evidence as to why the declarant is incorrect. There is no evidence in the record that rebuts the specific facts presented in the Hernandez Declaration. Therefore, these facts must be treated as evidence of non-obviousness. The Examiner agreed to reconsider the Declaration in view of these points made during the interview.

III. Rejection Over Kreutzer et al.

Applicant reiterated the fact that Kreutzer et al. is not a proper reference under 35 U.S.C. § 102(e). Applicant pointed Examiner Chong to M.P.E.P. § 706.02(f)(1), Example 6, particularly the section entitled "Additional Benefit Claims." The Kreutzer et al. application is an additional benefit claim of an international application filed prior to November 29, 2000 and has as its 102(e) date its actual filing date, not its effective filing date. Applicants noted that a full explanation of this argument was presented in the Response filed in the '086 application on February 26, 2010. The Examiner agreed to consider the argument.

Conclusion

Applicants thank Examiner Chong for the courtesy of the interview and her consideration of the issues raised. The Examiner is invited to contact the undersigned if it would advance prosecution.

Dated: April 7, 2010

Respectfully submitted,

/Anne-Marie C. Yvon/

Jane M. Love, Ph.D.
Registration No. 42,812

Anne-Marie C. Yvon, Ph.D.
Registration No. 52,390

Attorneys for Applicant(s)

Wilmer Cutler Pickering Hale and Dorr LLP
399 Park Avenue
New York, New York 10022
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Electronic Acknowledgement Receipt

EFS ID:	7368826
Application Number:	11894676
International Application Number:	
Confirmation Number:	8161
Title of Invention:	Methods and compositions for RNA interference
First Named Inventor/Applicant Name:	Gregory J. Hannon
Customer Number:	84834
Filer:	Anne-Marie Yvon/Patricia Ierardi
Filer Authorized By:	Anne-Marie Yvon
Attorney Docket Number:	287000.130US3
Receipt Date:	07-APR-2010
Filing Date:	20-AUG-2007
Time Stamp:	15:51:58
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Applicant summary of interview with examiner	287000_130US3_InterviewSummary_040710.pdf	85290 91d8e3771d93d5137fafe78a9e5bade1d8df65ae	no	4

Warnings:

Information:

Total Files Size (in bytes):	85290

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Hannon et al.	Confirmation No.:	8161
Application No:	11/894,676	Art Unit:	1635
Filed:	August 20, 2007	Examiner:	K. CHONG
Title:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT

This paper amends the reference in the specification to the applications to which the present application claims priority. A Supplemental Application Data Sheet accompanies this paper. The Commissioner is authorized to charge any fees due, or to credit any overpayment in fees, to Deposit Account No. 08-0219.

AMENDMENT

In the Specification

On page 1, please amend the paragraph immediately after the heading "Related Applications" as follows:

-- This application is a continuation application of ~~U.S. Serial No. 11/791,554, filed on May23, 2007, which is a national stage filing under 35 U.S.C. § 371 of International Application No. PCT/US2005/042488, filed on November 23,2005, which is a continuation application of~~ U.S. Serial No. 10/997,086, filed on November 23, 2004, ~~which is a continuation-in-part of U.S. Ser. No. 10/350,798, filed on January 24, 2003,~~ which is a continuation-in-part of U.S. Ser. No. 10/055,797, filed on January 22, 2002, which is ~~a continuation-in-part of International Application No. PCT/US01/08435, filed on March 16,2001, which claims the benefit of the filing date from U.S. Provisional Application Nos. 60/189,739, filed on March 16,2000, and 60/243,097, filed on October 24,2000. U.S. Ser. No. 10/350,798 is also a continuation-in-part of U.S. Ser. No. 09/866.557, filed on May 24, 2001, which is also a continuation-in-part of International Application No. PCT/US0l/08435, filed on March 16,2001. U.S. Ser. No. 10/350,798 is also a continuation-in-part of U.S. Ser. No. 09/858,862, filed on May 16,2001, which is also a continuation-in-part of International Application No. PCT/US0l/08435, filed on March 16,2001. The specifications of such applications are~~ incorporated by reference herein. ~~International Application PCT/US0l/08435 and International Application PCT/US2005/042488 were both published under PCT Article 21(2) in English.~~ --

REMARKS

The specification is amended to reference the applications to which the present application claims priority. No new matter is added. Consideration and entry of this paper are requested.

Dated: April 8, 2010

Respectfully submitted,

/Anne-Marie C. Yvon/

Jane M. Love, Ph.D.
Registration No. 42,812

Anne-Marie C. Yvon, Ph.D.
Registration No. 52,390

Attorneys for Applicant(s)

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399 Park Avenue
New York, New York 10022
(212) 230-8800 (telephone)
(212) 230-8888 (facsimile)

Supplemental Application Data Sheet

Application Information

Application number::	11/894,676
Filing Date::	08/20/2007
Application Type::	Regular
Subject Matter::	Utility
Suggested classification::	
Suggested Group Art Unit::	N/A
CD-ROM or CD-R?::	None
Number of CD disks::	
Number of copies of CDs::	
Sequence submission?::	Yes
Computer Readable Form (CRF)?::	Yes
Number of copies of CRF::	
Title::	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE
Attorney Docket Number::	0287000.00130US3
Request for Early Publication?::	No
Request for Non-Publication?::	No
Suggested Drawing Figure::	
Total Drawing Sheets::	67
Small Entity?::	No
Petition included?::	No
Petition Type::	
Licensed US Govt. Agency::	
Contract or Grant Numbers::	
Secrecy Order in Parent Appl.?::	No

Applicant Information

Applicant Authority Type::	Inventor
Primary Citizenship Country::	US
Status::	Full Capacity
Given Name::	Gregory
Middle Name::	J.
Family Name::	HANNON
Name Suffix::	
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State or Province of Residence::	NY
Country of Residence::	US
Street of mailing address::	34 Griffith Lane
City of mailing address::	Huntington
State or Province of mailing address::	NY
Country of mailing address::	
Postal or Zip Code of mailing address::	11743
Applicant Authority Type::	Inventor
Primary Citizenship Country::	
Status::	Full Capacity
Given Name::	Patrick
Middle Name::	
Family Name::	PADDISON
Name Suffix::	
City of Residence::	Northport
State or Province of Residence::	NY
Country of Residence::	US
Street of mailing address::	9 Moffett Street
City of mailing address::	Oyster Bay

State or Province of mailing address::	NY
Country of mailing address::	
Postal or Zip Code of mailing address::	11771

Applicant Authority Type::	~~Inventor~~
Primary Citizenship Country::	~~US~~
Status::	~~Full Capacity~~
Given Name::	~~Despina~~
Middle Name::	~~C.~~
Family Name::	~~SIOLAS~~
Name Suffix::	
City of Residence::	~~Mattituck~~
State or Province of Residence::	~~NY~~
Country of Residence::	~~US~~
Street of mailing address::	~~P.O. Box 412~~

City of mailing address::	~~Mattituck~~
State or Province of mailing address::	~~NY~~
Country of mailing address::	
Postal or Zip Code of mailing address::	~~11952~~

Applicant Authority Type::	Inventor
Primary Citizenship Country::	US
Status::	Full Capacity
Given Name::	Emily
Middle Name::	
Family Name::	BERNSTEIN
Name Suffix::	
City of Residence::	New York
State or Province of Residence::	NY

Country of Residence:: US
Street of mailing address:: 1161 York Avenue, Apt 11

City of mailing address:: New York
State or Province of mailing address:: NY
Country of mailing address::
Postal or Zip Code of mailing address:: 10021

Applicant Authority Type:: Inventor
Primary Citizenship Country:: US
Status:: Full Capacity
Given Name:: Amy
Middle Name::
Family Name:: CAUDY
Name Suffix::
City of Residence:: Lawrenceville
State or Province of Residence:: NJ
Country of Residence:: US
Street of mailing address:: 4221 Town Court N

City of mailing address:: Lawrenceville
State or Province of mailing address:: NJ
Country of mailing address::
Postal or Zip Code of mailing address:: 08648

Applicant Authority Type:: Inventor
Primary Citizenship Country:: US
Status:: Full Capacity
Given Name:: Douglas
Middle Name::

Family Name::	CONKLIN
Name Suffix::	
City of Residence::	Cold Spring Harbor
State or Province of Residence::	NY
Country of Residence::	US
Street of mailing address::	One Bungtown Road
City of mailing address::	Cold Spring Harbor
State or Province of mailing address::	NY
Country of mailing address::	
Postal or Zip Code of mailing address::	11724
Applicant Authority Type::	Inventor
Primary Citizenship Country::	US
Status::	Full Capacity
Given Name::	Scott
Middle Name::	
Family Name::	HAMMOND
Name Suffix::	
City of Residence::	Cold Spring Habor
State or Province of Residence::	NY
Country of Residence::	US
Street of mailing address::	One Bungtown Road, Nichols Bldg.
City of mailing address::	Cold Spring Harbor
State or Province of mailing address::	NY
Country of mailing address::	
Postal or Zip Code of mailing address::	11724

Correspondence Information

Correspondence Customer Number:: 84834

Representative Information

Representative Customer Number:: 84834

Domestic Priority Information

Application::	Continuity Type::	Parent Application::	Parent Filing Date::
This Application	Continuation of	~~11/791,554~~	~~05/23/07~~
~~11/791,554~~	~~National stage of~~	~~PCT/US2005/042488~~	~~11/23/05~~
~~PCT/US2005/042488~~	~~Continuation of~~	10/997,086	11/23/04
10/997,086	Continuation-in-part of	10/350,798	01/24/03
~~10/350,798~~	~~Continuation-in-part of~~	~~09/858,862~~	~~05/16/01~~
~~09/858,862~~	~~Continuation-in-part of~~	~~US01/08435~~	~~03/16/01~~
~~10/350,798~~	~~Continuation-in-part of~~	~~09/866,557~~	~~05/24/01~~
~~09/866,557~~	~~Continuation-in-part of~~	~~US01/08435~~	~~03/16/01~~
~~10/350,798~~	~~Continuation-in-part of~~	~~10/055,797~~	~~01/22/02~~
~~10/055,797~~	~~Continuation-in-part of~~	~~US01/08435~~	~~03/16/01~~
~~US01/08435~~	~~An application claiming the benefit under 35 USC 119(e)~~	~~60/189,739~~	~~03/16/00~~
~~US01/08435~~	~~An application claiming the benefit under 35 USC 119(e)~~	~~60/243,097~~	~~10/24/00~~

Foreign Priority Information

Assignee Information

Assignee name::	Cold Spring Harbor Laboratory
Street of mailing address::	One Bungtown Road
City of mailing address::	Cold Spring Harbor
State or Province of mailing address::	NY
State or Province of mailing address::	
Country of mailing address::	US
Postal or Zip Code of mailing address::	11724

A signature of the applicant or representative is required in accordance with 37 CFR 1.33 and 10.18. Please see 37 CFR 1.4(d) for the form of the signature.			
Signature	/Anne-Marie C. Yvon/	Date	April 8, 2010
Name (Print/Type)	Anne-Marie C. Yvon, Ph.D.	Registration No. (Attorney/Agent)	52,390

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Gregory J. Hannon et al.	Examiner:	Kimberly Chong
Application No.:	11/894,676	Art Unit:	1635
Filing Date:	August 20, 2007	Confirmation No.:	8161
For:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

REQUEST TO CORRECT INVENTORSHIP UNDER 37 C.F.R. §1.48(a)

Commissioner:

Applicants hereby request to correct the inventorship of the above-referenced patent application to add Emily Bernstein, Amy Caudy, Douglas Conklin, and Scott Hammond as inventors. In support of this Request, Applicants provide the following documents:

1. This paper setting forth the desired inventorship change (i.e., the addition of Emily Bernstein, Amy Caudy, Douglas Conklin, and Scott Hammond) under Rule 48(a)(1);
2. Copy of Statement by Emily Bernstein under §1.48(a)(2) submitted in parent application Serial No. 10/997,086, as permitted by 201.03.II.A;
3. Copy of Statement by Amy Caudy under §1.48(a)(2) submitted in parent application Serial No. 10/997,086, as permitted by 201.03.II.A;
4. Copy of Statement by Douglas Conklin under §1.48(a)(2) submitted in parent application Serial No. 10/997,086, as permitted by 201.03.II.A;
5. Copy of Statement by Scott Hammond under §1.48(a)(2) submitted in parent application Serial No. 10/997,086, as permitted by 201.03.II.A;
6. Declaration by the actual inventors under §1.48(a)(3), as required by § 1.63;
7. Payment of the processing fee under §1.48(a)(4), as set forth in § 1.17(i);
8. Written consent of the assignee under §1.48(a)(5);

9. Copy of the assignment executed by the originally named inventor under § 3.73(b)(1)(i) and §3.73 (c)(1) in support of the written consent of the assignee.

Applicants also provide:

10. Supplemental Application Data Sheet.

The processing fee of $130.00 accompanies this paper. The Commissioner is authorized to charge any other fee occasioned by this paper, and to credit any overpayment of fees, to Deposit Account No. 08-0219.

Dated: April 8, 2010

Respectfully submitted,

/Anne-Marie C. Yvon/

Jane M. Love, Ph.D.
Registration No. 42,812

Anne-Marie C. Yvon, Ph.D.
Registration No. 52,390

Attorneys for Applicant(s)

Wilmer Cutler Pickering Hale and Dorr LLP
399 Park Avenue
New York, New York 10022
(212) 230-8800 (telephone)
(212) 230-8888 (facsimile)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Gregory J. Hannon et al.	Examiner:	Kimberly Chong
Application No.:	11/894,676	Art Unit:	1635
Filing Date:	August 20, 2007	Confirmation No.:	8161
For:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Commissioner for Patents
P. O. Box 1450
Alexandria, Virginia 22313-1450

REQUEST TO AMEND INVENTORSHIP UNDER 37 C.F.R. § 1.48(b)(1)

Sir:

Applicants request correction of the inventorship of the above-identified application. Please delete Despina C. Siolas, whose invention is no longer being claimed.

The processing fee required by 37 C.F.R. § 1.48(b)(2) and set forth in 37 C.F.R. § 1.17(i) accompanies this paper. The Commissioner is authorized to charge any other fee occasioned by this paper, and to credit any overpayment of fees, to Deposit Account No. 08-0219.

Dated: April 8, 2010

Respectfully submitted,

/Anne-Marie C. Yvon/

Jane M. Love, Ph.D.
Registration No. 42,812

Anne-Marie C. Yvon, Ph.D.
Registration No. 52,390

Attorneys for Applicant(s)

Wilmer Cutler Pickering Hale and Dorr LLP
399 Park Avenue
New York, New York 10022
(212) 230-8800 (telephone)
(212) 230-8888 (facsimile)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Gregory J. Hannon et al.	Examiner:	Kimberly Chong
Application No.:	11/894,676	Art Unit:	1635
Filing Date:	August 20, 2007	Confirmation No.:	8161
For:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

CONSENT OF ASSIGNEE TO CORRECT INVENTORSHIP
IN A PATENT APPLICATION UNDER 37 C.F.R. §1.48(a)

Dear Commissioner:

I, John Maroney, certify that I am authorized to act on behalf of Cold Spring Harbor Laboratory, the assignee of the above-identified application, having a business address of 1 Bungtown Road, Cold Spring Harbor, NY 11724. I further certify that to the best of my knowledge and belief, Cold Spring Harbor Laboratory owns all title and interest in the above-identified application. I hereby consent to the addition of Emily Bernstein, Amy Caudy, Douglas Conklin, and Scott Hammond as inventors on the above-identified patent application. A copy of the Assignment for this application, signed by the originally-named inventors, accompanies this paper.

Respectfully submitted,
Cold Spring Harbor Laboratory

Date: 4/2/2010

John Maroney, Esq.
General Counsel

PTO/SB/01A (01-09)
Approved for use through 06/30/2010. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE

As the below named inventor(s), I/we declare that:

This declaration is directed to:

☐ The attached application, or

☒ Application No. 11/894676 filed on 08/20/2007

☒ As amended on April 9, 2009 (if applicable);

I/we believe that I/we am/are the original and first inventor(s) of the subject matter which is claimed and for which a patent is sought;

I/we have reviewed and understand the contents of the above-identified application, including the claims, as amended by any amendment specifically referred to above;

I/we acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me/us to be material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT International filing date of the continuation-in-part application.

WARNING:

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

All statements made herein of my/our own knowledge are true, all statements made herein on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and may jeopardize the validity of the application or any patent issuing thereon.

FULL NAME OF INVENTOR(S)

Inventor one: Gregory J. HANNON Date: 3/24/10

Signature: [signature] Citizen of: US

Inventor two: Patrick PADDISON Date:

Signature: Citizen of: US

☒ Additional inventors or a legal representative are being named on 1 additional form(s) attached hereto.

DECLARATION FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET	ADDITIONAL INVENTOR(S) Supplemental Sheet Page 1 of 1

Inventor three: Emily BERNSTEIN Date: _____

Signature: _____ Citizen of: US

Inventor four: Amy CAUDY Date: _____

Signature: _____ Citizen of: US

Inventor five: Douglas CONKLIN Date: _____

Signature: _____ Citizen of: US

Inventor six: Scott HAMMOND Date: _____

Signature: _____ Citizen of: US

Inventor seven: _____ Date: _____

Signature: _____ Citizen of: _____

Inventor eight: _____ Date: _____

Signature: _____ Citizen of: _____

Inventor nine: _____ Date: _____

Signature: _____ Citizen of: _____

Inventor ten: _____ Date: _____

Signature: _____ Citizen of: _____

Inventor eleven: _____ Date: _____

Signature: _____ Citizen of: _____

Inventor twelve: _____ Date: _____

Signature: _____ Citizen of: _____

Inventor thirteen: _____ Date: _____

Signature: _____ Citizen of: _____

PTO/SB/01A (01-09)
Approved for use through 06/30/2010. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE

As the below named inventor(s), I/we declare that:

This declaration is directed to:

☐ The attached application, or

☒ Application No. 11/894676 filed on 08/20/2007

☒ As amended on April 9, 2009 (if applicable);

I/we believe that I/we am/are the original and first inventor(s) of the subject matter which is claimed and for which a patent is sought;

I/we have reviewed and understand the contents of the above-identified application, including the claims, as amended by any amendment specifically referred to above;

I/we acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me/us to be material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT International filing date of the continuation-in-part application.

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All statements made herein of my/our own knowledge are true, all statements made herein on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and may jeopardize the validity of the application or any patent issuing thereon.

FULL NAME OF INVENTOR(S)

Inventor one: Gregory J. HANNON Date: _____

Signature: _____ Citizen of: US

Inventor two: Patrick PADDISON Date: 3/05/10

Signature: _____ Citizen of: US

☒ Additional inventors or a legal representative are being named on 1 additional form(s) attached hereto.

DECLARATION FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET	ADDITIONAL INVENTOR(S) Supplemental Sheet Page 1 of 1

Inventor three: Emily BERNSTEIN Date: _____

Signature: _____ Citizen of: US

Inventor four: Amy CAUDY Date: _____

Signature: _____ Citizen of: US

Inventor five: Douglas CONKLIN Date: _____

Signature: _____ Citizen of: US

Inventor six: Scott HAMMOND Date: _____

Signature: _____ Citizen of: US

Inventor seven: _____ Date: _____

Signature: _____ Citizen of: _____

Inventor eight: _____ Date: _____

Signature: _____ Citizen of: _____

Inventor nine: _____ Date: _____

Signature: _____ Citizen of: _____

Inventor ten: _____ Date: _____

Signature: _____ Citizen of: _____

Inventor eleven: _____ Date: _____

Signature: _____ Citizen of: _____

Inventor twelve: _____ Date: _____

Signature: _____ Citizen of: _____

Inventor thirteen: _____ Date: _____

Signature: _____ Citizen of: _____

PTO/SB/01A (01-09)
Approved for use through 06/30/2010. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE

As the below named inventor(s), I/we declare that:

This declaration is directed to:

☐ The attached application, or

☒ Application No. 11/894676 filed on 08/20/2007

☒ As amended on April 9, 2009 (if applicable);

I/we believe that I/we am/are the original and first inventor(s) of the subject matter which is claimed and for which a patent is sought;

I/we have reviewed and understand the contents of the above-identified application, including the claims, as amended by any amendment specifically referred to above;

I/we acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me/us to be material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT International filing date of the continuation-in-part application.

WARNING:

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All statements made herein of my/our own knowledge are true, all statements made herein on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and may jeopardize the validity of the application or any patent issuing thereon.

FULL NAME OF INVENTOR(S)

Inventor one: Gregory J. HANNON Date: _____

Signature: _____ Citizen of: US

Inventor two: Patrick PADDISON Date: _____

Signature: _____ Citizen of: US

☒ Additional inventors or a legal representative are being named on 1 additional form(s) attached hereto.

DECLARATION FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET	ADDITIONAL INVENTOR(S) Supplemental Sheet Page 1 of 1
Inventor three: Emily BERNSTEIN	Date: 3/10/10
Signature:	Citizen of: US
Inventor four: Amy CAUDY	Date:
Signature:	Citizen of: US
Inventor five: Douglas CONKLIN	Date:
Signature:	Citizen of: US
Inventor six: Scott HAMMOND	Date:
Signature:	Citizen of: US
Inventor seven:	Date:
Signature:	Citizen of:
Inventor eight:	Date:
Signature:	Citizen of:
Inventor nine:	Date:
Signature:	Citizen of:
Inventor ten:	Date:
Signature:	Citizen of:
Inventor eleven:	Date:
Signature:	Citizen of:
Inventor twelve:	Date:
Signature:	Citizen of:
Inventor thirteen:	Date:
Signature:	Citizen of:

PTO/SB/01A (01-09)
Approved for use through 06/30/2010. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE

As the below named inventor(s), I/we declare that:

This declaration is directed to:

☐ The attached application, or

☒ Application No. 11/894676 filed on 08/20/2007

☒ As amended on April 9, 2009 (if applicable);

I/we believe that I/we am/are the original and first inventor(s) of the subject matter which is claimed and for which a patent is sought;

I/we have reviewed and understand the contents of the above-identified application, including the claims, as amended by any amendment specifically referred to above;

I/we acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me/us to be material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT International filing date of the continuation-in-part application.

WARNING:

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All statements made herein of my/our own knowledge are true, all statements made herein on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and may jeopardize the validity of the application or any patent issuing thereon.

FULL NAME OF INVENTOR(S)

Inventor one: Gregory J. HANNON Date: _____

Signature: _____ Citizen of: US

Inventor two: Patrick PADDISON Date: _____

Signature: _____ Citizen of: US

☒ Additional inventors or a legal representative are being named on 1 additional form(s) attached hereto.

7346345
US1DOCS 7346345v1

DECLARATION FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET	ADDITIONAL INVENTOR(S) Supplemental Sheet Page 1 of 1

Inventor three: Emily BERNSTEIN Date: _____

Signature: _____ Citizen of: US

Inventor four: Amy CAUDY Date: 6 November 2009

Signature: _____ Citizen of: US

Inventor five: Douglas CONKLIN Date: _____

Signature: _____ Citizen of: US

Inventor six: Scott HAMMOND Date: _____

Signature: _____ Citizen of: US

Inventor seven: _____ Date: _____

Signature: _____ Citizen of: _____

Inventor eight: _____ Date: _____

Signature: _____ Citizen of: _____

Inventor nine: _____ Date: _____

Signature: _____ Citizen of: _____

Inventor ten: _____ Date: _____

Signature: _____ Citizen of: _____

Inventor eleven: _____ Date: _____

Signature: _____ Citizen of: _____

Inventor twelve: _____ Date: _____

Signature: _____ Citizen of: _____

Inventor thirteen: _____ Date: _____

Signature: _____ Citizen of: _____

PTO/SB/01A (01-09)
Approved for use through 06/30/2010. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE

As the below named inventor(s), I/we declare that:

This declaration is directed to:

☐ The attached application, or

☒ Application No. 11/894676 filed on 08/20/2007

☒ As amended on April 9, 2009 (if applicable);

I/we believe that I/we am/are the original and first inventor(s) of the subject matter which is claimed and for which a patent is sought;

I/we have reviewed and understand the contents of the above-identified application, including the claims, as amended by any amendment specifically referred to above;

I/we acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me/us to be material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT International filing date of the continuation-in-part application.

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FULL NAME OF INVENTOR(S)

Inventor one: Gregory J. HANNON Date: _____

Signature: _____ Citizen of: US

Inventor two: Patrick PADDISON Date: _____

Signature: _____ Citizen of: US

☒ Additional inventors or a legal representative are being named on 1 additional form(s) attached hereto.

7346345
US1DOCS 7346345v1

DECLARATION FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET	ADDITIONAL INVENTOR(S) Supplemental Sheet Page 1 of 1

Inventor three: Emily BERNSTEIN Date: _____

Signature: _____ Citizen of: US

Inventor four: Amy CAUDY Date: _____

Signature: _____ Citizen of: US

Inventor five: Douglas CONKLIN Date: 03-30-10

Signature: DBC Citizen of: US

Inventor six: Scott HAMMOND Date: _____

Signature: _____ Citizen of: US

Inventor seven: _____ Date: _____

Signature: _____ Citizen of: _____

Inventor eight: _____ Date: _____

Signature: _____ Citizen of: _____

Inventor nine: _____ Date: _____

Signature: _____ Citizen of: _____

Inventor ten: _____ Date: _____

Signature: _____ Citizen of: _____

Inventor eleven: _____ Date: _____

Signature: _____ Citizen of: _____

Inventor twelve: _____ Date: _____

Signature: _____ Citizen of: _____

Inventor thirteen: _____ Date: _____

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Title of Invention	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE

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☒ As amended on April 9, 2009 (if applicable);

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I/we acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me/us to be material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT International filing date of the continuation-in-part application.

WARNING:

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

All statements made herein of my/our own knowledge are true, all statements made herein on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and may jeopardize the validity of the application or any patent issuing thereon.

FULL NAME OF INVENTOR(S)

Inventor one: Gregory J. HANNON Date: _____

Signature: _____ Citizen of: US

Inventor two: Patrick PADDISON Date: _____

Signature: _____ Citizen of: US

☒ Additional inventors or a legal representative are being named on 1 additional form(s) attached hereto.

7346345
US1DOCS 7346345v1

DECLARATION FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET	ADDITIONAL INVENTOR(S) Supplemental Sheet Page 1 of 1

Inventor three: Emily BERNSTEIN Date:

Signature: Citizen of: US

Inventor four: Amy CAUDY Date:

Signature: Citizen of: US

Inventor five: Douglas CONKLIN Date:

Signature: Citizen of: US

Inventor six: Scott HAMMOND Date: 3/24/2010

Signature: Citizen of: US

Inventor seven: Date:

Signature: Citizen of:

Inventor eight: Date:

Signature: Citizen of:

Inventor nine: Date:

Signature: Citizen of:

Inventor ten: Date:

Signature: Citizen of:

Inventor eleven: Date:

Signature: Citizen of:

Inventor twelve: Date:

Signature: Citizen of:

Inventor thirteen: Date:

Signature: Citizen of:

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Gregory J. Hannon et al.	Examiner:	Kimberly Chong
Application No.:	10/997,086	Art Unit:	1635
Filing Date:	November 23, 2004	Confirmation No.:	9352
For:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

STATEMENT OF EMILY BERNSTEIN UNDER 37 CFR §1.48(a)(2)

Sir:

The fact that I was not named as an inventor in the above-identified application occurred without any deceptive intention on my part.

Respectfully submitted,

Dated: 3/10/10

Emily Bernstein

US1DOCS 7339909v1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Gregory J. Hannon et al.	Examiner:	Kimberly Chong
Application No.:	10/997,086	Art Unit:	1635
Filing Date:	November 23, 2004	Confirmation No.:	9352
For:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

STATEMENT OF AMY CAUDY UNDER 37 CFR §1.48(a)(2)

Sir:

The fact that I was not named as an inventor in the above-identified application occurred without any deceptive intention on my part.

Respectfully submitted,

Dated: 6 November 2009

Amy Caudy

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Gregory J. Hannon et al.	Examiner:	Kimberly Chong
Application No.:	10/997,086	Art Unit:	1635
Filing Date:	November 23, 2004	Confirmation No.:	9352
For:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

STATEMENT OF DOUGLAS CONKLIN UNDER 37 CFR §1.48(a)(2)

Sir:

The fact that I was not named as an inventor in the above-identified application occurred without any deceptive intention on my part.

Respectfully submitted,

Dated: 03.30.10

Douglas Conklin

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Gregory J. Hannon et al.	Examiner:	Kimberly Chong
Application No.:	10/997,086	Art Unit:	1635
Filing Date:	November 23, 2004	Confirmation No.:	9352
For:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

STATEMENT OF SCOTT HAMMOND UNDER 37 CFR §1.48(a)(2)

Sir:

The fact that I was not named as an inventor in the above-identified application occurred without any deceptive intention on my part.

Respectfully submitted,

Dated: 3/24/2010

Scott Hammond

ASSIGNMENT

WHEREAS, we, **Gregory J. Hannon, Patrick J. Paddison, and Despina C. Siolas,** have invented a certain improvement in **Methods and Compositions For RNA Interference** described in an application for Letters Patent of the United States, the specification of which:

[ ] is being executed on even date herewith; and is about to be filed in the United States Patent Office;

[x] was filed on **November 23, 2004** as Application No. **10/997,086**

[ ] was patented under U.S. Patent No. **[PATENT NUMBER]** on **[PUBLICATION DATE]**.

WHEREAS, **Cold Spring Harbor Laboratory,** (hereinafter "ASSIGNEE"), a corporation organized and existing under the laws of the **State of Delaware,** having principal offices at **One Bungtown Road, Cold Spring Harbor, New York 11724** desires to acquire an interest therein in accordance with agreements duly entered into with us;

NOW, THEREFORE, to all whom it may concern be it known that for and in consideration of said agreements and of other good and valuable consideration, the receipt of which is hereby acknowledged, we have sold, assigned and transferred and by these presents do hereby sell, assign and transfer unto said ASSIGNEE, its successors, assigns and legal representatives, the entire right, title and interest in and throughout the United States of America, its territories and all foreign countries, in and to said invention as described in said application, together with our entire right, title and interest in and to said application and such Letters Patent as may issue thereon, and any reissue, continuation, divisional and foreign counterparts thereof; said invention, application and Letters Patent to be held and enjoyed by said ASSIGNEE for its own use and behalf and for its successors, assigns and legal representatives, to the full end of the term for which said Letters Patent may be granted as fully and entirely as the same would have been held by us had this assignment not been made; we hereby convey all rights arising under or pursuant to any and all international agreements, treaties or laws relating to the protection of industrial property by filing any such applications for Letters Patent, all choses in action pertaining to the applications or Letters Patent including the right to sue for and collect damages and other recoveries for past infringement thereof, all rights to initiate proceedings before government and administrative bodies, and all files, records and other materials arising from the prosecution, exploitation, or defense of rights and registrations pertaining to the applications or Letters Patent. We hereby acknowledge that this assignment, being of our entire right, title and interest in and to said invention, carries with it the right in ASSIGNEE to apply for and obtain from competent authorities in all countries of the world any and all Letters Patent by attorneys and agents of ASSIGNEE's selection and the right to procure the grant of all such Letters Patent to ASSIGNEE for its own name as assignee of the entire right, title and interest therein;

AND, we hereby further agree for ourselves and our executors and administrators to execute upon request any other lawful documents and likewise to perform any other lawful acts which may be deemed necessary to secure fully the aforesaid invention to said ASSIGNEE, its successors, assigns and legal representatives, but at its or their expense and charges, including the execution of applications for patents in foreign countries, and the execution of substitution, reissue, divisional or continuation applications and preliminary or other statements and the giving of testimony in any interference or other proceeding in which said invention or any application or patent directed thereto may be involved;

AND, we do hereby authorize and request the Commissioner of Patents of the United States to issue such Letters Patent as shall be granted upon said application or applications based thereon to said ASSIGNEE, its successors, assigns, and legal representatives.

Inventor _____ Date: 4/29/05

Gregory J. Hannon

Address 34 Griffith Lane

Huntington, NY 11743

Witness Jo Leonardo Date: 4/29/05

Address 2 Robin Lane

Levittown, NY 11756

Inventor _____ Date: _____

Patrick J. Paddison

Address _____

_____

Witness _____ Date: _____

Address _____

_____

Inventor _____ Date: _____

Despina C. Siolas

Address _____

_____

Witness _____ Date: _____

Address _____

_____

ASSIGNMENT

WHEREAS, we, **Gregory J. Hannon, Patrick J. Paddison, and Despina C. Siolas,** have invented a certain improvement in **Methods and Compositions For RNA Interference** described in an application for Letters Patent of the United States, the specification of which:

[ ] is being executed on even date herewith; and is about to be filed in the United States Patent Office;

[x] was filed on **November 23, 2004** as Application No. **10/997,086**

[ ] was patented under U.S. Patent No. **[PATENT NUMBER]** on **[PUBLICATION DATE]**.

WHEREAS, **Cold Spring Harbor Laboratory,** (hereinafter "ASSIGNEE"), a corporation organized and existing under the laws of the **State of Delaware,** having principal offices at **One Bungtown Road, Cold Spring Harbor, New York 11724** desires to acquire an interest therein in accordance with agreements duly entered into with us;

NOW, THEREFORE, to all whom it may concern be it known that for and in consideration of said agreements and of other good and valuable consideration, the receipt of which is hereby acknowledged, we have sold, assigned and transferred and by these presents do hereby sell, assign and transfer unto said ASSIGNEE, its successors, assigns and legal representatives, the entire right, title and interest in and throughout the United States of America, its territories and all foreign countries, in and to said invention as described in said application, together with our entire right, title and interest in and to said application and such Letters Patent as may issue thereon, and any reissue, continuation, divisional and foreign counterparts thereof; said invention, application and Letters Patent to be held and enjoyed by said ASSIGNEE for its own use and behalf and for its successors, assigns and legal representatives, to the full end of the term for which said Letters Patent may be granted as fully and entirely as the same would have been held by us had this assignment not been made; we hereby convey all rights arising under or pursuant to any and all international agreements, treaties or laws relating to the protection of industrial property by filing any such applications for Letters Patent, all choses in action pertaining to the applications or Letters Patent including the right to sue for and collect damages and other recoveries for past infringement thereof, all rights to initiate proceedings before government and administrative bodies, and all files, records and other materials arising from the prosecution, exploitation, or defense of rights and registrations pertaining to the applications or Letters Patent. We hereby acknowledge that this assignment, being of our entire right, title and interest in and to said invention, carries with it the right in ASSIGNEE to apply for and obtain from competent authorities in all countries of the world any and all Letters Patent by attorneys and agents of ASSIGNEE's selection and the right to procure the grant of all such Letters Patent to ASSIGNEE for its own name as assignee of the entire right, title and interest therein;

AND, we hereby further agree for ourselves and our executors and administrators to execute upon request any other lawful documents and likewise to perform any other lawful acts which may be deemed necessary to secure fully the aforesaid invention to said ASSIGNEE, its successors, assigns and legal representatives, but at its or their expense and charges, including the execution of applications for patents in foreign countries, and the execution of substitution, reissue, divisional or continuation applications and preliminary or other statements and the giving of testimony in any interference or other proceeding in which said invention or any application or patent directed thereto may be involved;

AND, we do hereby authorize and request the Commissioner of Patents of the United States to issue such Letters Patent as shall be granted upon said application or applications based thereon to said ASSIGNEE, its successors, assigns, and legal representatives.

Inventor _____ Date: _____

Gregory J. Hannon

Address _____

_____

Witness _____ Date: _____

Address _____

_____

Inventor _____ Date: 4/24/05

Patrick J. Paddison

Address 39 Jefferson Ave
Northport, NY 11768

Witness _____ Date: 4/29/05

Address 1 BUNGTOWN RD.
COLD SPRING HARBOR, NY 11724

Inventor _____ Date: _____

Despina C. Siolas

Address _____

_____

Witness _____ Date: _____

Address _____

_____

ASSIGNMENT

WHEREAS, we, **Gregory J. Hannon, Patrick J. Paddison, and Despina C. Siolas,** have invented a certain improvement in **Methods and Compositions For RNA Interference** described in an application for Letters Patent of the United States, the specification of which:

[ ] is being executed on even date herewith; and is about to be filed in the United States Patent Office;

[x] was filed on **November 23, 2004** as Application No. **10/997,086**

[ ] was patented under U.S. Patent No. **[PATENT NUMBER]** on **[PUBLICATION DATE]**.

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NOW, THEREFORE, to all whom it may concern be it known that for and in consideration of said agreements and of other good and valuable consideration, the receipt of which is hereby acknowledged, we have sold, assigned and transferred and by these presents do hereby sell, assign and transfer unto said ASSIGNEE, its successors, assigns and legal representatives, the entire right, title and interest in and throughout the United States of America, its territories and all foreign countries, in and to said invention as described in said application, together with our entire right, title and interest in and to said application and such Letters Patent as may issue thereon, and any reissue, continuation, divisional and foreign counterparts thereof; said invention, application and Letters Patent to be held and enjoyed by said ASSIGNEE for its own use and behalf and for its successors, assigns and legal representatives, to the full end of the term for which said Letters Patent may be granted as fully and entirely as the same would have been held by us had this assignment not been made; we hereby convey all rights arising under or pursuant to any and all international agreements, treaties or laws relating to the protection of industrial property by filing any such applications for Letters Patent, all choses in action pertaining to the applications or Letters Patent including the right to sue for and collect damages and other recoveries for past infringement thereof, all rights to initiate proceedings before government and administrative bodies, and all files, records and other materials arising from the prosecution, exploitation, or defense of rights and registrations pertaining to the applications or Letters Patent. We hereby acknowledge that this assignment, being of our entire right, title and interest in and to said invention, carries with it the right in ASSIGNEE to apply for and obtain from competent authorities in all countries of the world any and all Letters Patent by attorneys and agents of ASSIGNEE's selection and the right to procure the grant of all such Letters Patent to ASSIGNEE for its own name as assignee of the entire right, title and interest therein;

AND, we hereby further agree for ourselves and our executors and administrators to execute upon request any other lawful documents and likewise to perform any other lawful acts which may be deemed necessary to secure fully the aforesaid invention to said ASSIGNEE, its successors, assigns and legal representatives, but at its or their expense and charges, including the execution of applications for patents in foreign countries, and the execution of substitution, reissue, divisional or continuation applications and preliminary or other statements and the giving of testimony in any interference or other proceeding in which said invention or any application or patent directed thereto may be involved;

AND, we do hereby authorize and request the Commissioner of Patents of the United States to issue such Letters Patent as shall be granted upon said application or applications based thereon to said ASSIGNEE, its successors, assigns, and legal representatives.

Inventor _____ Date: _____

Gregory J. Hannon

Address _____

_____

Witness _____ Date: _____

Address _____

_____

Inventor _____ Date: _____

Patrick J. Paddison

Address _____

_____

Witness _____ Date: _____

Address _____

_____

Inventor Despina C. Siolas Date: 5/2/05

Despina C. Siolas

Address PO Box 412 190 Central Drive

Mattituck NY 11952

Witness Jo Leonardo Date: 5/2/05

Address 2 Robin Ln.

Levittown, NY 11756

Electronic Patent Application Fee Transmittal

Application Number:	11894676
Filing Date:	20-Aug-2007
Title of Invention:	Methods and compositions for RNA interference
First Named Inventor/Applicant Name:	Gregory J. Hannon
Filer:	Anne-Marie Yvon
Attorney Docket Number:	287000.130US3

Filed as Small Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Petition fee- 37 CFR 1.17(h) (Group III)	1464	1	130	130
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
			Total in USD ($)	**130**

Electronic Acknowledgement Receipt

EFS ID:	7379316
Application Number:	11894676
International Application Number:	
Confirmation Number:	8161
Title of Invention:	Methods and compositions for RNA interference
First Named Inventor/Applicant Name:	Gregory J. Hannon
Customer Number:	84834
Filer:	Anne-Marie Yvon/sophie murray
Filer Authorized By:	Anne-Marie Yvon
Attorney Docket Number:	287000.130US3
Receipt Date:	08-APR-2010
Filing Date:	20-AUG-2007
Time Stamp:	18:06:48
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Credit Card
Payment was successfully received in RAM	$130
RAM confirmation Number	5826
Deposit Account	080219
Authorized User	LADD,CATHLEEN

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

Charge any Additional Fees required under 37 C.F.R. Section 1.16 (National application filing, search, and examination fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.17 (Patent application and reexamination processing fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.19 (Document supply fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		287000_130US3_Amendment_04082010.pdf	68465 b436cdb1d3dc3ad7bde7eb0c7836a926412f7026	yes	3

Multipart Description/PDF files in .zip description

Document Description	Start	End
Amendment After Final	1	1
Specification	2	2
Applicant Arguments/Remarks Made in an Amendment	3	3

Warnings:

Information:

2	Application Data Sheet	287000_130US3_Suppl_ADS.pdf	62309 97970fa9c6a91568245d9230277a3e5a6bb56e78	no	8

Warnings:

Information:

This is not an USPTO supplied ADS fillable form

3	Request under Rule 48 correcting inventorship	287000_130US3_Request_to_correct_inventorship.pdf	67751 a169f3f1273c2f63fbf21f055ad86bb719ddf5da	no	2

Warnings:

Information:

4	Request under Rule 48 correcting inventorship	287000_130US3_Request_to_Amend_inventorship.pdf	65558 a853123b703ee8a386fa44019bc5812db656eabb	no	1

Warnings:

Information:

5	Consent of Assignee accompanying the declaration.	287000_130US3_Consent_of_Assignee.pdf	37748 b0674d003c87c4c0bd77029d70d5f8880778ba32	no	1

Warnings:

Information:

6	Oath or Declaration filed	287000_130US3_Declaration.pdf	466930 72ce16edc18253173fbcf26b4d860c86f89a60ce	no	12

Warnings:

Information:					
7	Request under Rule 48 correcting inventorship	287000_130US_Statement_1_48.pdf	73990 ec2d63d786eed7795537dc1ba992a3bdc9fbb0d1	no	4
Warnings:					
Information:					
8	Assignee showing of ownership per 37 CFR 3.73(b).	287000_130US_assignment.PDF	420940 8ed4e97bfd1118ef817091117efd5d0c879feb05	no	9
Warnings:					
Information:					
9	Fee Worksheet (PTO-875)	fee-info.pdf	30070 3c6d39d78369fe0ffb6d7b8a5beee9590a225434	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	1293761	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/06 (07-06)
Approved for use through 1/31/2007. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number	Filing Date	☐ To be Mailed
11/894,676	08/20/2007	

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY ☒ RATE ($)	SMALL ENTITY ☒ FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT	04/08/2010	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 13	Minus	** 20	= 0	X $26 =	0	OR	X $ =	
	Independent (37 CFR 1.16(h))	* 2	Minus	***3	= 0	X $110 =	0	OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE	0	OR	TOTAL ADD'L FEE	

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)	OR	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/TINA J. BARDEN/

PTO/SB/06 (07-06)
Approved for use through 1/31/2007. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number	Filing Date	☐ To be Mailed
11/894,676	08/20/2007	

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY ☒ RATE ($)	SMALL ENTITY ☒ FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT 04/08/2010	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 13	Minus	** 20	= 0	X $26 =	0	OR	X $ =	
Independent (37 CFR 1.16(h))	* 2	Minus	***3	= 0	X $110 =	0	OR	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))									
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE	0	OR	TOTAL ADD'L FEE	

AMENDMENT	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)	OR	RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))									
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/TINA J. BARDEN/

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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Alexandria, Virginia 22313-1450
www.uspto.gov

Bib Data Sheet

CONFIRMATION NO. 8161

SERIAL NUMBER	FILING OR 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
11/894,676	08/20/2007 RULE	435	1635	287000.130US3

APPLICANTS

Gregory J. Hannon, Huntington, NY;
Patrick J. Paddison, Northport, NY;
Emily Bernstein, New York, NY;
Amy Caudy, Lawrenceville, NJ;
Douglas Conklin, Cold Spring Harbor, NY;
Scott Hammond, Cold Spring Harbor, NY;

**** CONTINUING DATA ***************************

This application is a CON of 10/997,086 11/23/2004
which is a CIP of 10/350,798 01/24/2003 ABN
which is a CIP of 10/055,797 01/22/2002 ABN
which is a CIP of PCT/US01/08435 03/16/2001
which claims benefit of 60/189,739 03/16/2000
and claims benefit of 60/243,097 10/24/2000
and said 10/350,798 01/24/2003
is a CIP of 09/866,557 05/24/2001 ABN
which is a CIP of PCT/US01/08435 03/16/2001
and said 10/350,798 01/24/2003
is a CIP of 09/858,862 05/16/2001
which is a CIP of PCT/US01/08435 03/16/2001

**** FOREIGN APPLICATIONS *********************

IF REQUIRED, FOREIGN FILING LICENSE GRANTED ** SMALL ENTITY **
** 11/02/2007

Foreign Priority claimed	STATE OR COUNTRY	SHEETS DRAWING	TOTAL CLAIMS	INDEPENDENT CLAIMS
☐ yes ☐ no	NY	67	14	2
35 USC 119 (a-d) conditions met ☐ yes ☐ no ☐ Met after Allowance				
Verified and Acknowledged Examiner's Signature Initials				

ADDRESS
84834

TITLE

Methods and compositions for RNA interference

FILING FEE RECEIVED	FEES: Authority has been given in Paper	☐ All Fees
970	No. _____ to charge/credit DEPOSIT ACCOUNT No. _____ for following:	☐ 1.16 Fees (Filing) ☐ 1.17 Fees (Processing Ext. of time)

		☐ 1.18 Fees (Issue) ☐ Other _____ ☐ Credit

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Hannon et al.	Confirmation No.:	8161
Application No:	11/894,676	Art Unit:	1635
Filed:	August 20, 2007	Examiner:	K. CHONG
Title:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

SUBMISSION OF A SUPPLEMENTAL APPLICATION DATA SHEET TO CORRECT APPLICATION DATA SHEET SUBMITTED ON 04/08/2010

Applicants submit a corrected Supplemental Application Data Sheet to update the address of inventor Patrick Paddison and to correct an inadvertent typographical error in the priority claimed on the Supplemental Application Data Sheet filed on April 8, 2010. The Commissioner is authorized to charge any fees due, or to credit any overpayment in fees, to Deposit Account No. 08-0219.

Dated: April 9, 2010

Respectfully submitted,

/Anne-Marie C. Yvon/

Jane M. Love, Ph.D.
Registration No. 42,812

Anne-Marie C. Yvon, Ph.D.
Registration No. 52,390

Attorneys for Applicant(s)

Wilmer Cutler Pickering Hale and Dorr LLP
399 Park Avenue
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(212) 230-8888 (facsimile)

Supplemental Application Data Sheet

Application Information

Application number::	11/894,676
Filing Date::	08/20/07
Application Type::	Regular
Subject Matter::	Utility
Suggested classification::	
Suggested Group Art Unit::	1635
CD-ROM or CD-R?::	None
Number of CD disks::	
Number of copies of CDs::	
Sequence submission?::	None
Computer Readable Form (CRF)?::	No
Number of copies of CRF::	
Title::	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE
Attorney Docket Number::	0287000.00130US3
Request for Early Publication?::	No
Request for Non-Publication?::	No
Suggested Drawing Figure::	
Total Drawing Sheets::	67
Small Entity?::	Yes
Petition included?::	No

Petition Type::	
Licensed US Govt. Agency::	
Contract or Grant Numbers::	
Secrecy Order in Parent Appl.?::	No

Applicant Information

Applicant Authority Type::	Inventor
Primary Citizenship Country::	US
Status::	Full Capacity
Given Name::	Gregory
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State or Province of mailing address::	NY
Country of mailing address::	
Postal or Zip Code of mailing address::	11743

Applicant Authority Type::	Inventor
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Name Suffix::	
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State or Province of Residence::	~~NY~~ WA
Country of Residence::	US
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City of mailing address::	~~Oyster Bay~~ Seattle
State or Province of mailing address::	~~NY~~ WA
Country of mailing address::	
Postal or Zip Code of mailing address::	~~11771~~ 98115
Applicant Authority Type::	Inventor
Primary Citizenship Country::	US
Status::	Full Capacity
Given Name::	Emily
Middle Name::	

Family Name::	BERNSTEIN
Name Suffix::	
City of Residence::	New York
State or Province of Residence::	NY
Country of Residence::	US
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State or Province of mailing address::	NY
Country of mailing address::	
Postal or Zip Code of mailing address::	10021
Applicant Authority Type::	Inventor
Primary Citizenship Country::	US
Status::	Full Capacity
Given Name::	Amy
Middle Name::	
Family Name::	CAUDY
Name Suffix::	
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State or Province of Residence::	NJ
Country of Residence::	US
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City of mailing address::	Lawrenceville
State or Province of mailing address::	NJ

Country of mailing address::

Postal or Zip Code of mailing address:: 08648

Applicant Authority Type:: Inventor

Primary Citizenship Country:: US

Status:: Full Capacity

Given Name:: Douglas

Middle Name::

Family Name:: CONKLIN

Name Suffix::

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State or Province of Residence:: NY

Country of Residence:: US

Street of mailing address:: One Bungtown Road

City of mailing address:: Cold Spring Harbor

State or Province of mailing address:: NY

Country of mailing address::

Postal or Zip Code of mailing address:: 11724

Applicant Authority Type:: Inventor

Primary Citizenship Country:: US

Status:: Full Capacity

Given Name::	Scott
Middle Name::	
Family Name::	HAMMOND
Name Suffix::	
City of Residence::	Cold Spring Habor
State or Province of Residence::	NY
Country of Residence::	US
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City of mailing address::	Cold Spring Harbor
State or Province of mailing address::	NY
Country of mailing address::	
Postal or Zip Code of mailing address::	11724

Correspondence Information

Correspondence Customer Number::	84834

Representative Information

Representative Customer Number::	84834

Domestic Priority Information

Application::	Continuity Type::	Parent Application::	Parent Filing Date::
This Application	Continuation of	10/997086	11/23/04
~~10/997086~~	~~Continuation-in-part of~~	~~10/350798~~	~~01/24/03~~
10/997086	Continuation-in-part of	10/055797	01/22/02

Foreign Priority Information

Assignee Information

Assignee name::	Cold Spring Harbor Laboratory
Street of mailing address::	One Bungtown Road
City of mailing address::	Cold Spring Harbor
State or Province of mailing address::	NY
State or Province of mailing address::	
Country of mailing address::	US
Postal or Zip Code of mailing address::	11724

Signature:

A signature of the applicant or representative is required in accordance with 37 CFR 1.33 and 10.18. Please see 37 CFR 1.4(d) for the form of the signature.			
Signature	/Anne-Marie C. Yvon/	Date	April 9, 2010
Name (Print/Type)	Anne-Marie C. Yvon	Registration No. (Attorney/Agent)	52,390

Electronic Acknowledgement Receipt

EFS ID:	7384486
Application Number:	11894676
International Application Number:	
Confirmation Number:	8161
Title of Invention:	Methods and compositions for RNA interference
First Named Inventor/Applicant Name:	Gregory J. Hannon
Customer Number:	84834
Filer:	Anne-Marie Yvon/sophie murray
Filer Authorized By:	Anne-Marie Yvon
Attorney Docket Number:	287000.130US3
Receipt Date:	09-APR-2010
Filing Date:	20-AUG-2007
Time Stamp:	14:22:48
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Miscellaneous Incoming Letter	287000_130US3_Transmittal_letter_re_Priority.pdf	59661 808ef8f09a9347004a0c4d8f47c00d83fa296202	no	1

Warnings:

Information:

2	Application Data Sheet	287000_130US3_Suppl_ADS_04092010.pdf	61449 ee45cdc886318827f1043799945cfa52956a714b	no	8

Warnings:	
Information:	
This is not an USPTO supplied ADS fillable form	
Total Files Size (in bytes):	121110

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

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United States Patent and Trademark Office
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Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/894,676	08/20/2007	Gregory J. Hannon	287000.130US3	8161

84834 7590 05/04/2010

WilmerHale/Cold Spring Harbor Laboratory
399 Park Avenue
New York, NY 10022

EXAMINER	
CHONG, KIMBERLY	
ART UNIT	PAPER NUMBER
1635	
MAIL DATE	DELIVERY MODE
05/04/2010	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Interview Summary	Application No.	Applicant(s)	
	11/894,676	HANNON ET AL.	
	Examiner	Art Unit	
	FEREYDOUN G. SAJJADI	1633	

All participants (applicant, applicant's representative, PTO personnel):

(1) *FEREYDOUN G. SAJJADI*. (3) *Vladimir Drozdoff*.

(2) *Jane Love*. (4)_____.

Date of Interview: *30 April 2010*.

Type: a)☒ Telephonic b)☐ Video Conference
c)☐ Personal [copy given to: 1)☐ applicant 2)☐ applicant's representative]

Exhibit shown or demonstration conducted: d)☐ Yes e)☒ No.
If Yes, brief description: _____.

Claim(s) discussed: *50*.

Identification of prior art discussed: *Fire et al. Kreutzer et al*.

Agreement with respect to the claims f)☐ was reached. g)☐ was not reached. h)☒ N/A.

Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: *Applicants' representative addressed issues regarding the separate rejections over the prior art of Fire et al. and Kreutzer et al, with particular reference to the Declaration under Rule 1.132 by Dr. Hernandez. The acting SPE indicated that Applicants' analysis pertaining to the priority date of Kreutzer did not fit the fact pattern at issue. With regard to the issue of teaching away by the prior art of Elbashir, the acting SPE indicated that Elbashir's teaching clearly indicated that short 30 bp dsRNAs are processed to 21 and 22-nt RNAs, albeit inefficiently; thereby not constituting a true teaching away*.

(A fuller description, if necessary, and a copy of the amendments which the examiner agreed would render the claims allowable, if available, must be attached. Also, where no copy of the amendments that would render the claims allowable is available, a summary thereof must be attached.)

THE FORMAL WRITTEN REPLY TO THE LAST OFFICE ACTION MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP Section 713.04). If a reply to the last Office action has already been filed, APPLICANT IS GIVEN A NON-EXTENDABLE PERIOD OF THE LONGER OF ONE MONTH OR THIRTY DAYS FROM THIS INTERVIEW DATE, OR THE MAILING DATE OF THIS INTERVIEW SUMMARY FORM, WHICHEVER IS LATER, TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW. See Summary of Record of Interview requirements on reverse side or on attached sheet.

/Fereydoun G Sajjadi/ Acting SPE, Art Unit 1635	

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

———

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:

1) A brief description of the nature of any exhibit shown or any demonstration conducted,
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
   (The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiner's version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, "Interview Record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/894,676	08/20/2007	Gregory J. Hannon	287000.130US3	8161

84834 7590 05/04/2010

WilmerHale/Cold Spring Harbor Laboratory
399 Park Avenue
New York, NY 10022

EXAMINER	
CHONG, KIMBERLY	
ART UNIT	PAPER NUMBER
1635	
MAIL DATE	DELIVERY MODE
05/04/2010	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

UNITED STATES DEPARTMENT OF COMMERCE
U.S. Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450

APPLICATION NO./ CONTROL NO.	FILING DATE	FIRST NAMED INVENTOR / PATENT IN REEXAMINATION	ATTORNEY DOCKET NO.
11894676	8/20/2007	HANNON ET AL.	287000.130US3

WilmerHale/Cold Spring Harbor Laboratory
399 Park Avenue
New York, NY 10022

EXAMINER	
KIMBERLY CHONG	
ART UNIT	**PAPER**
1635	20100503

DATE MAILED:

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner for Patents

Inventorship

In view of the papers filed 04/08/2010, it has been found that this nonprovisional application, as filed, through error and without deceptive intent, improperly set forth the inventorship, and accordingly, this application has been corrected in compliance with 37 CFR 1.48(a). The inventorship of this application has been changed by the deletion of Despina C. Siolas as an inventor and the addition of Emily Bernstein, Amy Caudy, Douglas Conklin and Scott Hammond as inventors.

The application will be forwarded to the Office of Initial Patent Examination (OIPE) for issuance of a corrected filing receipt, and correction of Office records to reflect the inventorship as corrected.

/Kimberly Chong/
Primary Examiner AU1635

PTO-90C (Rev.04-03)

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
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BIB DATA SHEET

CONFIRMATION NO. 8161

SERIAL NUMBER	FILING or 371(c) DATE RULE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
11/894,676	08/20/2007	514	1635	287000.130US3

APPLICANTS

Gregory J. Hannon, Huntington, NY;
Patrick J. Paddison, Northport, NY;
Emily Bernstein, New York, NY;
Amy Caudy, Lawrenceville, NJ;
Douglas Conklin, Cold Spring Harbor, NY;
Scott Hammond, Cold Spring Harbor, NY;

**** CONTINUING DATA ***************************

This application is a CON of 10/997,086 11/23/2004
which is a CIP of 10/350,798 01/24/2003 ABN
which is a CIP of 10/055,797 01/22/2002 ABN
which is a CIP of PCT/US01/08435 03/16/2001
which claims benefit of 60/189,739 03/16/2000
and claims benefit of 60/243,097 10/24/2000
and said 10/350,798 01/24/2003
is a CIP of 09/866,557 05/24/2001 ABN
which is a CIP of PCT/US01/08435 03/16/2001
and said 10/350,798 01/24/2003
is a CIP of 09/858,862 05/16/2001
which is a CIP of PCT/US01/08435 03/16/2001

**** FOREIGN APPLICATIONS ***************************

**** IF REQUIRED, FOREIGN FILING LICENSE GRANTED **** ** SMALL ENTITY **
11/02/2007

Foreign Priority claimed ☐ Yes ☑ No 35 USC 119(a-d) conditions met ☐ Yes ☑ No Verified and Acknowledged /KIMBERLY CHONG/ Examiner's Signature	☐ Met after Allowance Initials	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
		NY	67	14	2

ADDRESS

WilmerHale/Cold Spring Harbor Laboratory
399 Park Avenue
New York, NY 10022
UNITED STATES

TITLE

Methods and compositions for RNA interference

FILING FEE RECEIVED		
970	FEES: Authority has been given in Paper No._____ to charge/credit DEPOSIT ACCOUNT No._____ for following:	☐ All Fees ☐ 1.16 Fees (Filing) ☐ 1.17 Fees (Processing Ext. of time) ☐ 1.18 Fees (Issue)

BIB (Rev. 05/07).

		❑ Other _____
		❑ Credit

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Hannon et al.	Confirmation No.:	8161
Application No:	11/894,676	Art Unit:	1635
Filed:	August 20, 2007	Examiner:	K. CHONG
Title:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT IN RESPONSE TO JANUARY 27, 2010 FINAL OFFICE ACTION

This Amendment is filed in response to the January 27, 2010 Final Office Action for which a response was due April 27, 2010. Applicants hereby request a three-month extension of time to July 27, 2010. Accordingly, this paper is being timely filed. The Commissioner is authorized to charge any fees due, or to credit any overpayment in fees, to Deposit Account No. 08-0219.

Claim Listing

This listing of the claims will replace all prior versions and listings of claims in the application:

1-49. (Cancelled)

50. (Previously presented) A method for attenuating expression of a target gene in a mammalian cell, the method comprising

introducing into a mammalian cell a library of RNA expression constructs, each expression construct comprising:

(i) an RNA polymerase promoter, and

(ii) a sequence encoding a short hairpin RNA molecule comprising a double-stranded region wherein the double-stranded region consists of at least 20 nucleotides but not more than 29 nucleotides, such that the short hairpin RNA does not trigger a protein kinase RNA-activated (PKR) response in the mammalian cells,

wherein the double-stranded region of the short hairpin RNA molecule comprises a sequence that is complementary to a portion of the target gene, and

wherein the short hairpin RNA molecule is stably expressed in the mammalian cell in an amount sufficient to attenuate expression of the target gene in a sequence specific manner, whereby expression of the target gene is inhibited.

51. (Cancelled)

52. (Previously presented) The method of claim 50, wherein the expression construct further comprises LTR sequences located 5' and 3' of the sequence encoding the short hairpin RNA molecule.

53. (Cancelled)

54. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of at least 21 nucleotides.

55. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of at least 22 nucleotides.

56. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of at least 25 nucleotides.

57. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of 29 nucleotides.

58. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule has a total length of about 70 nucleotides.

59. (Previously presented) The method of claim 50, wherein the RNA polymerase promoter comprises a pol II promoter or a pol III promoter.

60. (Previously presented) The method of claim 59, wherein the pol III promoter comprises a U6, an H1, or an SRP promoter.

61. (Withdrawn) The method of claim 59, wherein the pol II promoter comprises a U1 or a CMV promoter.

62. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule attenuates expression of the target gene in the mammalian cell by at least about 60%.

63. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule attenuates expression of the target gene in the mammalian cell by about 60% to about 90%.

64. (Cancelled)

REMARKS

Claims 50, 52, 54-60 and 62-64 were pending and under examination. Herein, applicants have cancelled claim 64 without prejudice to pursue the subject matter of this claim in another application.

I. Interview

Applicants appreciate the Examiners agreement to participate in an interview to discuss the issues outstanding in this case.

II. Declaration of Professor Hernandez Under 37 C.F.R. §1.132

The Examiner has taken the position the Hernandez Declaration is "insufficient" to overcome the rejections of record.

In reply, applicants respectfully traverse the Examiner's position. The substance of the Hernandez Declaration will be discussed in detail below in the context of applicants' responses to the rejections under 35 U.S.C. § 103. However, applicants address here the Examiner's comments on pages 3-4 of the Final Office Action.

Rebuttal evidence can be submitted by way of a declaration. See 37 C.F.R. §1.132 and M.P.E.P. § 2141. Whenever an applicant submits additional evidence, the Examiner must reconsider patentability of the claimed invention, and any decision to maintain a rejection must show it was based on the totality of the evidence. *Id.* "Facts established by the rebuttal evidence must be evaluated along with the facts on which the conclusion of obviousness was reached, not against the conclusion itself." M.P.E.P. 2142 (citing *In re Eli Lilly & Co.*, 902 F.2d 943 (Fed. Cir. 1990))(emphasis added). "Consideration of rebuttal evidence and arguments requires Office personnel to weigh the proffered evidence and arguments. Office personnel should avoid giving evidence no weight, except in rare circumstances." See *In re Alton*, 76 F.3d 1168, 1174-75, 37 USPQ2d 1578, 1582-83 (Fed. Cir. 1996).

Here, applicants have met their burden by providing rebuttal evidence via the Declaration of Prof. Hernandez, a person of ordinary skill in the art at the time, including her testimony on how a person of ordinary skill in the art would have viewed the state of the art and the non-obviousness of the claimed invention. Instead of taking the factual evidence contained in the Hernandez Declaration into account, the Examiner improperly characterizes the statements made by Prof. Hernandez in her Declaration and data cited therein as "argument." (See page 3, line 3 and last paragraph.) This is clearly not attorney argument. Indeed, Prof. Hernandez is not "arguing" but is rather presenting evidence as a person of ordinary skill in the art at that time for the Examiner to consider. It is error to consider the content of the Hernandez Declaration as "argument." The statements therein are facts that must be taken into account.

Compounding that error, the Examiner, in dismissing the "argument," states that "the claims are not limited to the use of precursor RNA and as claimed, the dsRNA can be as small as 20 nucleotides in length which as stated above the skilled artisan would have expected to work" (Jan 27, 2010 Office Action at 3). The present claims are limited to the use of short hairpin RNA molecules. As evidenced by the present specification, as well as the Declaration of Prof. Hernandez, to mediate an RNAi response, it was understood that short hairpin RNA molecules would do so only if they were processed to siRNAs. In other words, in effecting gene silencing through RNAi, shRNAs are necessarily precursor RNAs. The Examiner's statement to the contrary belies a fundamental misunderstanding of the present specification and the Declaration, and misses a central point of the Declaration-- precursor RNA molecules with double-stranded regions of 36 bp or less were found to be ineffective in mediating RNAi and therefore such precursors, including short hairpin RNAs, were not expected to work. That Prof. Hannon found otherwise for short hairpin RNAs was surprising and unpredicted.

Finally, the Examiner does not provide any countervailing factual evidence that would rebut the statements of Prof. Hernandez. The Examiner improperly dismisses the factual evidence in the Declaration out of hand, and moreover, does not provide any further shred of evidence that would call into question the statements made by Prof. Hernandez. This wholesale dismissal of relevant evidence is contrary to the provisions of the M.P.E.P and to the patent law. *Id.* Applicants strongly request reconsideration of the facts set forth in the Hernandez Declaration.

III. Rejection Under 35 U.S.C. §112, first paragraph

The Examiner rejected claim 64 as allegedly failing to comply with the written description requirement.

In response, without conceding the correctness of the Examiner's position, and to accelerate prosecution of this application, applicants have canceled claim 64 without prejudice to pursue the subject matter of this claim in another application. Accordingly, applicants request the Examiner to reconsider and withdraw this ground of rejection.

IV. Rejection Under 35 U.S.C. § 102(b)

The Examiner rejected claim 64 as allegedly being anticipated by McSiggen et al. (US 20050277133).

In reply, without conceding the correctness of the Examiner's position, and to accelerate prosecution of this application, applicants have canceled claim 64 without prejudice to pursue the subject matter of this claim in another application. Accordingly, applicants request the Examiner to reconsider and withdraw this ground of rejection.

V. Obviousness-Type Double Patenting

The Examiner maintained rejections of claims 50-60 under the doctrine of obviousness-type double patenting over USSN 10/350,798.

The Examiner maintained rejections of claims 50-60 under the doctrine of obviousness-type double patenting over USSN 09/858,862.

In response, applicants traverse these rejections. As to the '798 application, applicants have previously stated that this rejection is improper. As applicants stated on page 7 of the response filed in connection with the present application on November 9, 2009, the '798 application is abandoned, as was indicated in a Notice of Abandonment dated May 21, 2008. Therefore, applicants request that the Examiner withdraw this ground of rejection.

The '862 application has now been allowed and issued as a USPN 7,732,714 on June 8, 2010. Claim 1 of the '714 patent reads as follows:

> 1. A method for attenuating expression of a target gene in a non-embryonic mammalian cell in culture, comprising introducing into the cell by transfection a double stranded RNA (dsRNA) in an amount sufficient to attenuate expression of the target gene, wherein the dsRNA is about 22 nucleotides in length and complementary across its length to a nucleotide sequences of the target gene and does not activate protein kinase RNA-activated (PKR) sequence-independent response, and wherein the cell is engineered with (i) a recombinant gene encoding a Dicer activity, (ii) a recombinant gene encoding an Argonaut activity, or (iii) both.

The claims of the '714 patent are patentably distinct from the claims of the present application. The grant of a second patent from the present application would not lead to an unjustified extension of the rights granted in the first patent, the '714 patent. Indeed, the claims of the present application are patentably distinct on a number of grounds, from the claims of the '714 patent. The claims of the present application are not an obvious variation of the invention defined in the claim of the '714 patent. The claims of the '714 patent require introducing double stranded RNA into a mammalian cell in culture. In contrast, the claims of the present application involve the introduction of an expression construct encoding a short hairpin RNA into a mammalian cell. In the claims of the '714 patent, RNA is introduced into the cell, whereas in the present claims, DNA is introduced into the cell. As is discussed below, and in the accompanying Declaration of Prof. Hernandez under 37 C.F.R. § 1.132, the introduction of an expression vector encoding short hairpin RNA is not obvious in view of a method whereby double-stranded RNA is introduced into a cell.

Moreover, the claims of the '714 patent require that the cell into which the double-stranded RNA is introduced, be engineered with a recombinant gene encoding a Dicer activity, an Argonaut activity, or both. Such a requirement is absent from the present claims, providing another basis of patentable distinctness.

Accordingly, applicants request that the Examiner reconsider and withdraw this ground of rejection.

VI. Obviousness Rejections Are The Only Outstanding Issues

Applicants request reconsideration of the Examiner's position alleging that the claims are obvious. For the following reasons, the Examiner (1) bases her rejections on clear errors of fact in the technical differences between the cited art and the claimed invention, (2) ignores critical evidence presented in a 132 Declaration of Prof. Hernandez, (3) lacks a proper basis for finding a reasonable expectation of success, based on the totality of the evidence in the record and (4) fails to provide a proper and sufficient articulation of a rationale for the finding of obviousness. Each of the two rejections under 35 U.S.C. §103 are discussed below.

VII. Rejection Under 35 U.S.C. § 103 Over Fire, Good and Noonberg

The Examiner rejected claims 50, 52, 54-60 and 62-64 as allegedly obvious in view of Fire et al. (USPN 6,506,599), Good et al., and Noonberg et al.

In reply, applicants respectfully traverse the rejection. The combination of Fire, Good and Noonberg would not make the claimed invention obvious to one of ordinary skill in the art at the time. These three references are not sufficient to make a *prima facie* case of obviousness for the reasons set out below.

According to the state of the art at the time of the invention, one would have understood the process of RNAi to be mediated by 21- and 22-nucleotide small interfering RNAs (siRNAs) generated from longer double-stranded RNAs (dsRNAs), as disclosed by Elbashir et al. (2001) *Nature* 411:494-98. However, in this regard, the skilled artisan would have had no reasonable expectation of success that sequence specific target gene attenuation could be achieved by using an expression vector encoding a short hairpin RNA molecule (shRNA) having a double-stranded region consisting of between 20 and 29 nucleotides.

In view of the state of the art at the time, the skilled artisan would have had no motivation to employ RNA molecules comprising short dsRNA structures that must be processed within the cell to activate RNAi. In particular, one of ordinary skill would have known the literature to indicate that the RNAi response was sharply length dependent. For example, both *in vitro* and *in vivo* analysis of the length requirements of dsRNA had revealed that dsRNAs of fewer than 150

bp in length appeared less effective than longer dsRNAs, and in some cases ineffective, in their ability to degrade target mRNA. *See* Elbashir et al. (2001) *Genes Dev.* 15:188-200; Bernstein et al. (2001) *Nature* 409:363-66. There was no realization that the ineffectiveness of such molecules in mediating RNAi could have been overcome by expressing RNA molecules within the cell in the form of a hairpin structure, as taught and claimed in the present application. In particular, the skilled artisan would not have expected that an RNA hairpin having a double-stranded region of 20 to 29 nucleotides in length would undergo processing to an siRNA or would be effective in triggering sequence specific gene attenuation through RNAi.

As evidence of the non-obviousness of the claimed invention, Applicants have submitted a Declaration under 37 C.F.R. § 1.132 from Professor Nouria Hernandez. As Prof. Hernandez states, it would not have been obvious to one of ordinary skill in the art at the time of the invention that one could attenuate target gene expression in a mammalian cell by introducing an expression construct encoding a short hairpin RNA molecule having a double-stranded region of 20-29 nucleotides. Indeed, according to Prof. Hernandez it was unexpected that the claimed method would result in effective target gene attenuation, and one of ordinary skill at the time of the invention would have had no reasonable expectation that it would do so.

Applicants will now address specifically the comments from the Examiner in the January 27, 2010 Final Office Action.

The claimed invention is directed to:

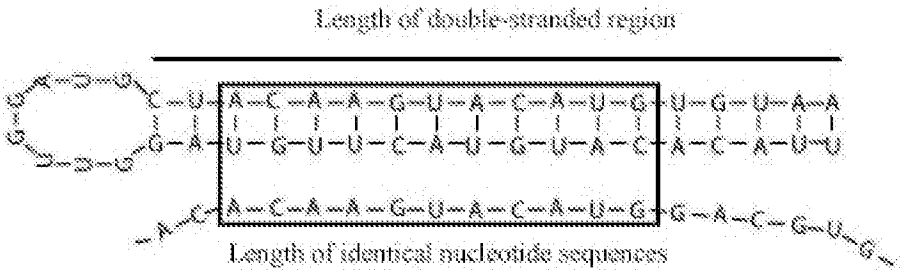
> Claim 50. A method for attenuating expression of a target gene in a mammalian cell, the method comprising
>
> introducing into a mammalian cell a library of RNA expression constructs, each expression construct comprising:
>
> (i) an RNA polymerase promoter, and
>
> (ii) a sequence encoding a short hairpin RNA molecule comprising a double-stranded region **wherein the double-stranded region consists of at least 20 nucleotides but not more than 29 nucleotides**, such that the short hairpin RNA does not trigger a protein kinase RNA-activated (PKR) response in the mammalian cells,

> wherein the double-stranded region of the short hairpin RNA molecule comprises a sequence that is complementary to a portion of the target gene, and
>
> wherein the short hairpin RNA molecule is **stably expressed** in the mammalian cell in an amount sufficient to attenuate expression of the target gene in a sequence specific manner, whereby expression of the target gene is inhibited.

Applicants claims require the double-stranded region to consist of at least 20 but not more than 29 nucleotides. The Examiner's § 103(a) rejection over Fire expressly relies on the erroneous factual finding that Fire discloses a dsRNA of 25 bases in length. Here, the Examiner improperly argues that the disclosure in Fire et al. of a range of lengths ("the length of the identical nucleotide sequences may be at least 25…") constitutes a disclosure of the endpoint of that range (25 bases) as a species. This constitutes clear legal error in view of *Atofina* as "may be at least 25…" discloses only a range and not any endpoint. See M.P.E.P. § 2163.03(II), *Atofina v. Great Lakes Chem. Corp*, 441 F.3d, 991, 1000 (Fed. Cir. 2006) ("[T]he disclosure of a range is no more a disclosure of the end points of the range than it is each of the intermediate points.") Therefore, the disclosure in Fire of "at least 25…" is a range and does not, as per the Federal Circuit in *Atofina*, disclose the end point 25 as a single species.

Moreover, Fire lacks any disclosure of a short hairpin RNA molecule as presently claimed, that is, a single-stranded RNA molecule comprising a double-stranded region having a length of at least 20 nucleotides but not more than 29 nucleotides. The Examiner erroneously alleges that Fire discloses the length of the dsRNA region "to be at least 25 bases in length." However, the language to which the Examiner expressly refers states only that "the length of *the identical nucleotide sequences* may be at least 25, 50, 100, 200, 300 or 400 bases." (Fire, 8:5-6, emphasis added.) The language makes no reference whatsoever to the length of the double-stranded region. The italicized phrase above refers back to sentence at 7:53-54 which recites "nucleotide sequences identical to a portion of the target gene…" This sentence does not refer to the length of the double-stranded region of a hairpin, but rather refers to the sequence that is identical to a portion of the target gene. These are two different things. Applicants illustrate this point with the diagram below. The length of the sequence identical to a portion of the target

gene does not disclose the length of the double-stranded region, as exemplified below:



In stating "there is no mention in the Fire et al. disclosure that the two strands of the hairpin RNA can be very different lengths as argued by Applicant," the Examiner entirely mischaracterizes Applicants' argument, which merely points out that the Fire specification never discloses the length of the duplex portion of a hairpin RNA molecule.

Moreover, Claim 15 of Fire cannot provide any basis for the Examiner's erroneous contention that Fire et al describes a short hairpin RNA molecule comprising a double-stranded region having a length of 25 base pairs. The Examiner asserts that Fire claim 15 discloses a hairpin RNA because "Fire clearly states in ¶ (07) that 'the double-stranded structure may be formed by a single self-complementary strand [can be a hairpin] or two complementary RNA strands [or comprised of two strands].'" The specific text the Examiner cites as describing a hairpin says that RNA is formed by a single strand. Fire claim 15 depends on claim 12, which expressly states that the claimed RNA is (and is limited to) a double-stranded molecule, and specifies it is a double-stranded molecule with a first strand…and a second strand. In other words it is made up of <u>two strands</u> and therefore cannot be a single strand, or a hairpin. The Examiner's contention that it can be is clear factual error.

The rejection omits another critical factual finding in failing to set forth any evidence establishing a reasonable expectation of success. On the contrary, the evidence in the record instead establishes that there was no reasonable expectation of success. The failure of the Examiner to provide any evidence otherwise precludes a finding of obviousness. Taking into account the record as a whole, including the Hernandez Declaration and Elbashir et al. (2001) Genes Dev. 15:188-200 (Elbashir 2001(a)) cited by Dr. Hernandez as additional evidence, there was no reasonable expectation that the presently claimed methods would be successful for attenuating expression of a target gene in a sequence specific manner. As one of ordinary skill,

who at the time of the invention was working in the field of RNA expression, the sworn testimony of Prof. Hernandez constitutes evidence of the state of the art, including the reasonable expectation of those of ordinary skill at the time.

In particular, Prof. Hernandez presents evidence:

> (1) that one of skill would have understood the process of RNAi to be mediated by 21- and 22- nucleotide small interfering RNAs (siRNAs) generated from longer double-stranded RNA (dsRNA) precursors;
>
> (2) given this fact, that expressed hairpin molecules, in order to mediate RNAi, similarly had to be processed in the cell to 21- and 22-nt siRNAs;
>
> (3) that Elbashir (2001a) reports, using an established *in vitro* system, dsRNA precursors of 29-36 bp failed to produce an RNAi response;
>
> (4) that the failure of 29-36 bp dsRNA precursors to do so could be explained by the fact that such dsRNA precursors were not effectively processed into the 21- and 22- nucleotide siRNAs mediating the RNAi response,
>
> (5) that, for this same reason, the data of Elbashir (2001a) would have caused one of skill to expect that a short hairpin RNA with a double-stranded region consisting of 20-29 bp region would also be ineffective in mediating RNAi, and
>
> (6) that in view of these data, there would have been no reasonable expectation that one could successfully use an RNA molecule comprising a double-stranded region consisting of 20-29 bp, such as the short hairpin RNA molecule recited in the present claims, to mediate RNAi.

Subsequent to a recent interview, the SPE has stated that "Elbashir's teaching clearly indicated that short 30 bp dsRNAs are processed to 21 and 22-nt RNA's, albeit inefficiently; thereby not constituting a teaching away." (See Interview Summary, May 4, 2010.) This view ignores the factual evidence in Elbashir itself, and the facts set out by Prof. Hernandez (*e.g.*, that

that dsRNAs of 29-36 bp failed to mediate any RNAi response. See Decl. ¶11, Elbashir Fig. 1. The authors of Elbashir state on page 189, first column, that "[s]pecific inhibition of target RNA expression was detected for dsRNAs as short as 38 bp, but dsRNAs of 29–36 bp were not effective in this process." In Fig. 2 of Elbashir, the authors observe that processing of a 29 bp dsRNA into 21-23nt siRNAs was dramatically delayed and decreased, as compared to the processing of dsRNA 39bp in length or longer. Interpreting Figure 2, the authors state: "This observation is consistent with a role of 21–23-nt fragments in guiding mRNA cleavage and provides an explanation ***for the lack of RNAi by 30-bp dsRNAs.*** The length dependence of 21–23 mer formation is likely to reflect ***a mechanism to prevent the undesired activation of RNAi by short intramolecular base-paired structures of cellular RNAs***." (See Elbashir et al. Genes & Development, 2001, p. 189, 2nd col. first partial ¶, emphasis added.) The authors themselves provide the clear conclusion that RNAi is not achieved with 30-bp dsRNAs. This conclusion is echoed by Prof. Hernandez's reading of Elbashir as set out in her sworn Declaration.

Here, Prof. Hernandez states that Elbashir "discourages" one from using short hairpin RNAs with a ds region of less than 38 bp (*see* ¶11). Prof. Hernandez declares that one would have been taught away from using short hairpins, as claimed, in view of Elbashir (see ¶ 16) and that Elbashir "expressly teaches away" from using shRNAs of less than 30 bp (see ¶ 14). See also Hernandez Declaration ¶¶ 7, 9-13, 16 and 19. Prof. Hernandez states that in view of Elbashir, there "would have been no expectation of success…" (see ¶ 19). The SPE's view that Elbashir does "not constitute[ing] a true teaching away" is not supported by any evidence which would rebut the evidence filed by Applicants. Again, the Patent Office is improperly choosing to ignore the factual record set out by applicants including the sworn testimony of Prof. Hernandez, one of ordinary skill in the art at the time. There is no evidence proffered by the Examiner or the SPE otherwise. The small, significantly delayed processing of dsRNAs of 29 bp in length does not provide any expectation of success (as per Prof. Hernandez, Declaration ¶ 19). The Examiner's unsubstantiated conclusion that data such as in Fig. 2 would have provided a reasonable expectation that RNA precursors having a double-stranded region of 29 base pairs or less would successfully mediate RNAi expressly contradicts the evidence set forth in the Declaration, including the contemporaneous interpretation by Elbashir et al. of their own data.

Fire does not disclose or make obvious that "the short hairpin RNA molecule is **stably expressed** in the mammalian cell in an amount sufficient to attenuate expression of the target gene in a sequence specific manner…" as required by applicants' claims. Fire does not make obvious stably expression short hairpin RNA molecules as claimed.

Combining Fire with Good and Noonberg does not remedy the deficiencies set out above regarding Fire. The Examiner states that Good teach an expression construct comprising a U6 promoter. There is no motivation to combine Fire with Good. The mere teaching of a U6 promoter and an expression construct does not remedy the issues applicants discuss above as to Fire. The Examiner also points to columns 7-8 of Noonberg which generally describes an "in vivo oligonucleotide generator." There is no disclosure of the many other claimed characteristics of the present invention in either Good or Noonberg. See Noonberg at 7:26-27. Furthermore, there is no motivation to combine the Noonberg document specifically with Fire or Good. The Examiner is using hindsight to fill in the missing gaps in Fire, namely **stable expression in a mammalian cell** sufficient to attenuate expression of the target gene.

In sum, applicants assert that the claims are not rendered obvious by the combination Fire, Good and Noonberg, that there is no motivation to combine these references, and that the evidence provided in the Declaration from Prof. Hernandez supports a finding of non-obviousness. Applicants respectfully request the Examiner to reconsider and withdraw this ground of rejection.

VIII. Rejection Under 35 U.S.C. § 103 Over Kreutzer, Lieber, Good and Noonberg

The Examiner rejected claims 50, 52, 54-60 and 62-64 as allegedly obvious in view of Kreutzer et al. (US Application No. 20040102408) ("'408 publication"), Leiber et al. (USPN 6,130,092), Good et al., and Noonberg et al.

In reply, applicants respectfully traverse the rejection. Applicants maintain their position that the '408 publication is not a proper reference under 35 U.S.C. §102(e) and thus under §103. Applicants also submit that the '408 publication in combination with Leiber and Good and Noonberg do not render the claimed invention obvious for the reasons of record and the reasons set out below.

A. The '408 Publication Is Not Proper Art Under 35 U.S.C. § 120(e)

The '408 publication is not proper prior art under 35 U.S.C. §102(e) (and thus under §103). The '408 publication is a division of USSN 09/889,802, which was filed on September 17, 2001, which was a §371 application of PCT/DE00/00244, which was filed on January 29, 2000. January 29, 2000 is prior to November 29, 2000. Therefore, the '408 publication does not arise "from an international filing date on or after November 29, 2000" as required by MPEP § 706.02(f)(I)(C).

The Examiner does not cite to any section of the MPEP in the Final Office Action, but appears to rely upon the following statement from the MPEP :

> (c) For U.S. application publications of applications that claim the benefit under 35 U.S.C. 120 or 365(c) of an international application filed prior to November 29, 2000, apply the reference under 35 U.S.C. 102(e) as of the actual filing date of the later-filed U.S. application that claimed the benefit of the international application.

See MPEP § 706.02(f)(I)(C)(3)(c).

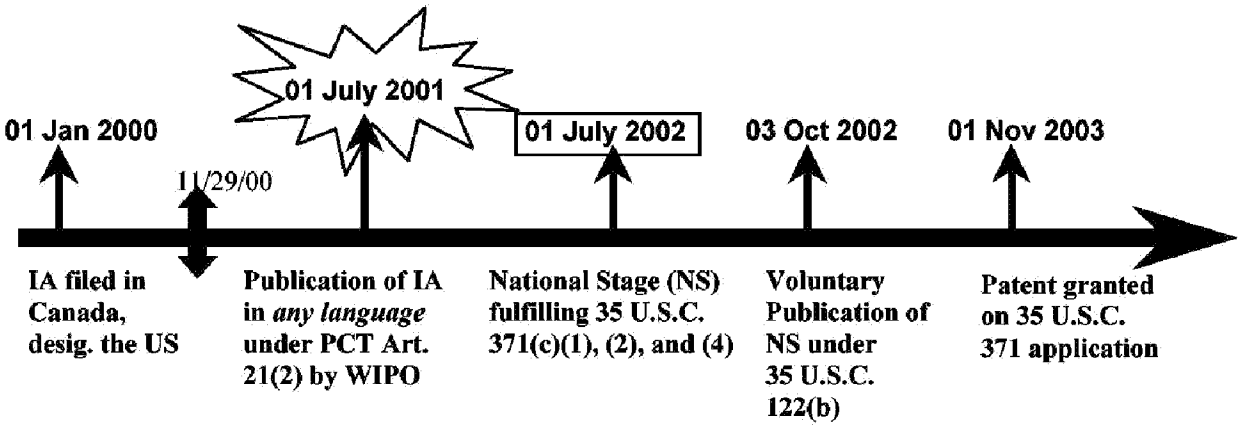
In this case, the '408 publication is not proper art under 35 U.S.C. §102(e). The '408 publication is a "U.S. application publication" that claims the benefit under § 120 of the PCT international application (the '244 PCT, filed on January 29, 2000) filed prior to November 29, 2000. The above section of the M.P.E.P. instructs Examiners to "apply the reference as of the actual filing date of the later-filed U.S. application that claimed the benefit of the international application. The '408 publication claims the benefit of the PCT under §120. (See Declaration filed in the prosecution history of the '408 publication.) Therefore, the actual filing of the U.S. application (the '408 publication) is March 6, 2003 and is the proper 102(e) date. Following this section of the M.P.E.P., the 102(e) of the '408 publication would be March 6, 2003, and therefore, it is not proper prior art against the claims of the present application.

The '408 publication is a later-filed U.S. application publication (filed on March 6, 2003) claiming benefit of the '244 PCT, which was filed on January 29, 2000, which was filed prior to November 29, 2000. The application from which the '408 publication is a divisional, the '802

application, never published and is abandoned. Applicants previously directed the Examiner to Example 6 in the M.P.E.P. and for the Examiner's convenience reproduce it here:

Example 6 : References based on the national stage (35 U.S.C. 371) of an International Application filed prior to November 29, 2000 (language of the publication under PCT Article 21(2) is not relevant).

The reference U.S. patent issued from an international application (IA) that was filed prior to November 29, 2000 has a 35 U.S.C. 102 (e) prior art date of the date of fulfillment of the requirements of 35 U.S.C. 371 (c)(1), (2) and (4). This is the pre-AIPA 35 U.S.C. 102 (e). The application publications, both the WIPO publication and the U.S. publication, published from an international application that was filed prior to November 29, 2000, do not have any 35 U.S.C. 102 (e) prior art date. According to the effective date provisions as amended by Pub. L. 107-273, the amendments to 35 U.S.C. 102 (e) and 374 are not applicable to international applications having international filing dates prior to November 29, 2000. The application publications can be applied under 35 U.S.C. 102 (a) or (b) as of their publication dates.



The 35 U.S.C. 102(e)(1) date for the IA Publication by WIPO is: None. The 35 U.S.C. 102(e)(1) date for the Publication by USPTO is: None. The 35 U.S.C. 102(e) date for the Patent is: 01 July 2002.

The IA publication by WIPO can be applied under 35 U.S.C. 102 (a) or (b) as of its publication date (01 July 2001).

Additional * Benefit Claims :

If the IA properly claimed ** > the benefit of < any earlier-filed U.S. application (whether provisional or nonprovisional), there would still be no 35 U.S.C. 102 (e)(1) date for the U.S. and WIPO application publications, and the 35 U.S.C. 102 (e) date for the patent will still be 01 July 2002 (the date of fulfillment of the requirements under 35 U.S.C. 371 (c)(1), (2) and (4)).

If a later-filed U.S. nonprovisional (35 U.S.C. 111 (a)) application claimed the benefit of the IA in the example above, the 35 U.S.C. 102 (e)(1) date of the application publication of the later-filed U.S. application would be the actual filing date of the later-filed U.S. application, and the 35 U.S.C. 102 (e) date of the patent of the later-filed U.S. application would be 01 July 2002 (the date that the earlier-filed IA fulfilled the requirements of 35 U.S.C. 371 (c)(1), (2) and (4)).

If the patent was based on a later-filed U.S. application that claimed the benefit of the international application and the later filed U.S. application's filing date is before the date the requirements of 35 U.S.C. 371 (c)(1), (2) and (4) were fulfilled (if fulfilled at all), the 35 U.S.C. 102 (e) date of the patent would be the filing date of the later-filed U.S. application that claimed the benefit of the international application.

The parent of the '408 publication was U.S. application Serial No. 09/889,802 ("the '802 application), which is now abandoned. For purposes of Example 6, the '408 publication is a "later-filed U.S. non-provisional (35 U.S.C. 111 (a)) application claim[ing] the benefit of" an international application filed before November 29, 2000. Therefore, the '408 publication falls within the category of applications treated in the box of Example 6 entitled "Additional Benefit Claims." As the emphasized section states, the publication of a later-filed application has a 102(e)(1) date of its actual filing date, not its effective filing date. Therefore, the 102(e) date of the '408 publication is its actual filing date, March 6, 2003, which is later than the January 22, 2002 priority date of the present application.

The Examiner argues that the '408 publication has a 102(e) date which is the effective filing date of its direct parent, the '802 application. However, the '802 never published and would not be "an application publication" as referred to in M.P.E.P. Example 6 and therefore would not be eligible for a 102(e) date. Accordingly, the '408 publication is not a proper reference under Section 102(e) and the rejection should be withdrawn. Should the Examiner insist that Kreutzer is prior art, Applicants request supervisory review of her interpretation of Section 102(e) in view of Example 6 of M.P.E.P. § 706.02(f)(1).

B. The '408 Publication Combined with Leiber, Good and Noonberg Do Not Make Obvious the Presently Claimed Invention

The Examiner's rejection ignores several elements of the present claims and fails to articulate any rational basis for why these missing elements would have been obvious in view of the '408 publication and other prior art cited in the office action. The '408 publication cannot make the claimed invention obvious because, for example, it does not contemplate stable expression in a mammalian cell of short hairpin RNAs comprising a double-stranded region wherein the double-stranded region consists of at least 20 nucleotides but not more than 29 nucleotides as required by the present claims. There is no motivation to combine the '408 publication with Leiber, Good and Noonberg and to do so requires hindsight in view of the claimed invention.

Applicants point out that the only mention of a hairpin in the '408 publication is in ¶ [0019], which refers to *chemically modifying* the loop region, which is a context wherein a

hairpin could *never* be expressed from a vector in a mammalian cell containing the target gene, as required by the claims. The presently claimed invention requires that the vector be expressed stably in mammalian cells and this could not be the case based on the description in the '408 publication. The Examiner does not dispute the context of ¶ [0019], but instead responds by referring to dsRNA "that is formed by a single auto complementary RNA comprising a loop", which the Examiner interprets as a hairpin RNA. The Examiner provides no citation, but presumably relies on ¶ [0017], which refers in part to "[a] region II which is complementary within the double-stranded structure is formed by…autocomplementary regions of a topologically closed RNA single strand which is preferably in circular form." An encoded hairpin RNA molecule expressed within a cell (as presently claimed) has a 5' and 3' end, is therefore topologically open. This expressed RNA is not rendered obvious by a single reference in the '408 publication to an entirely distinct molecule, a topologically closed (preferably circular) RNA. Again, the disclosure in the '408 publication cannot render obvious the claimed invention. The three other references which the Examiner cites (Leiber, Good and Noonberg) cannot remedy these deficiencies. The Examiner's interpretation of the '408 publication as referring to an expressed hairpin RNA molecule is clear factual error.

On page 13 of the Final Office Action, the Examiner refers to ¶ [0028] and takes the position that the '408 publication "teach[es] the expression in mammalian cells of dsRNA from a vector." Paragraph 28 is reproduced below:

> At least two dsRNAs which differ from each other or at least one vector encoding them can be introduced into the cell, where at least segments of one strand of each dsRNA are complementary to in each case one of at least two different target genes. This makes it possible simultaneously to inhibit the expression of at least two different target genes. In order to suppress, in the cell, the expression of a double-stranded-RNA-dependent protein kinase, PKR, one of the target genes is advantageously the PKR gene. This allows effective suppression of the PKR activity in the cell.

This passage does not describe or make obvious the expression of a short hairpin RNA, but rather describes two different dsRNAs, not hairpins. This does not address applicants' points above, that the '408 publication does not render obvious the elements of the present claims that require a short hairpin RNA comprising a double-stranded region wherein the double-stranded

region consists of at least 20 nucleotides but not more than 29 nucleotides, and where the hairpin RNA is stably expressed in a mammalian cell. For example, further missing from the '408 publication is any disclosure of a size range of the double-stranded region as presently claimed, that is, consisting of at least 20 nucleotides but not more than 29 nucleotides. Also missing from the '408 publication is any disclosure about a requirement that the double-stranded region is complementary to the target gene, or that the short hairpin RNA molecule is stably expressed in the mammalian cell.

Lieber et al., Good et al., and Noonberg et al. do not remedy the shortcomings of the '408 publication. In failing to take these missing elements into account, the Examiner has not made out a *prima facie* case, and the rejection cannot possibly articulate a rational basis for a finding of obviousness based on the '408 publication. Such a finding constitutes clear factual error.

Leiber discloses a ribozyme library comprising a collection of ribozyme genes encoding a hammerhead structure and flanking sequences of random nucleotides cloned at least once into an expression cassette for ribozyme expression (see claim 1). The ribozymes used are "from a selection of ribozymes with known stability and structure." (See Description of the Invention.) The structure of a ribozyme, having a hammerhead shape, is very different than a short hairpin RNA. The stability is likely very different. The Leiber references warns of "an unpredictable effect on the folding" that can occur from different ribozyme genes being expressed in the library. (See 3rd paragraph in Detailed Description of the Invention.) Therefore, there would be no motivation for a person of ordinary skill in the art to combine the libraries described in Leiber with the '408 publication, or let alone, any other non-ribozyme type of expression vector for fear of improper and unpredictable folding.

Good and Noonberg are discussed above, and those comments apply here as well. Neither Good nor Noonberg can remedy the deficiencies of the '408 publication, and Leiber. The combination of all four references would not have been made by one of ordinary skill in the art because there would have been no motivation to combine a reference discussing chemical modification of a nucleic acid (the '408 publication), with a ribozyme library (Leiber), with an in vivo oligonucleotide generator (Noonberg), with generally a U6 promoter (Good). The Examiner seems to have used hindsight to supplement her rejection with piecemeal references to

attempt to find all of the claimed elements of the present claims. Applicants maintain that the combination does not render obvious the claimed invention and respectfully request that the Examiner reconsider and withdraw this ground of rejection.

CONCLUSION

Consideration of this paper and allowance of this application are requested. If it would advance prosecution, the Examiner is invited to contact the undersigned to discuss the contents of this paper.

Dated: July 19, 2010

Respectfully submitted,

/Jane M. Love, Ph.D./

Jane M. Love, Ph.D.
Registration No. 42,812

Attorney for Applicants

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399 Park Avenue
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PTO/SB/22 (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless if displays a valid OMB control number.

PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a) FY 2009 *(Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).)*	Docket Number (Optional) 0287000.00130US3
Application Number 11/894,676-Conf. #8161	Filed August 20, 2007
For METHODS AND COMPOSITIONS FOR RNA INTERFERENCE	
Art Unit 1635	Examiner K. Chong

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

		Fee	Small Entity Fee	
[ ]	One month (37 CFR 1.17(a)(1))	$130	$65	$
[ ]	Two months (37 CFR 1.17(a)(2))	$490	$245	$
[x]	Three months (37 CFR 1.17(a)(3))	$1110	$555	$ 555.00
[ ]	Four months (37 CFR 1.17(a)(4))	$1730	$865	$
[ ]	Five months (37 CFR 1.17(a)(5))	$2350	$1175	$

[x] Applicant claims small entity status. See 37 CFR 1.27.

[ ] A check in the amount of the fee is enclosed.

[x] Payment by credit card. Form PTO-2038 is attached.

[ ] The Director has already been authorized to charge fees in this application to a Deposit Account.

[x] The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 08-0219 .

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

[ ] applicant/inventor.

[ ] assignee of record of the entire interest. See 37 CFR 3.71.
Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96).

[x] attorney or agent of record. Registration Number 42,812

[ ] attorney or agent under 37 CFR 1.34.
Registration number if acting under 37 CFR 1.34 _____

/Jane M. Love, Ph.D./ — Signature
July 19, 2010 — Date

Jane M. Love, Ph.D. — Typed or printed name
(212) 230-8800 — Telephone Number

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below.

[ ] Total of 1 forms are submitted.

Electronic Patent Application Fee Transmittal

Application Number:	11894676
Filing Date:	20-Aug-2007
Title of Invention:	Methods and compositions for RNA interference
First Named Inventor/Applicant Name:	Gregory J. Hannon
Filer:	Jane Maureen Love/sophie murray
Attorney Docket Number:	287000.130US3

Filed as Small Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 3 months with $0 paid	2253	1	555	555

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
			Total in USD ($)	**555**

Electronic Acknowledgement Receipt

EFS ID:	8040964
Application Number:	11894676
International Application Number:	
Confirmation Number:	8161
Title of Invention:	Methods and compositions for RNA interference
First Named Inventor/Applicant Name:	Gregory J. Hannon
Customer Number:	84834
Filer:	Jane Maureen Love/sophie murray
Filer Authorized By:	Jane Maureen Love
Attorney Docket Number:	287000.130US3
Receipt Date:	19-JUL-2010
Filing Date:	20-AUG-2007
Time Stamp:	15:26:00
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Credit Card
Payment was successfully received in RAM	$555
RAM confirmation Number	1772
Deposit Account	080219
Authorized User	LADD,CATHLEEN

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

Charge any Additional Fees required under 37 C.F.R. Section 1.16 (National application filing, search, and examination fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.17 (Patent application and reexamination processing fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.19 (Document supply fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.20 (Post Issuance fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		287000_130US3_Reponse_Final_OA_07192010.pdf	412400 4fa88e665fe07f7b0f3a73ee4fc570fb3512f60c	yes	20

Multipart Description/PDF files in .zip description

Document Description	Start	End
Amendment After Final	1	1
Claims	2	3
Applicant Arguments/Remarks Made in an Amendment	4	20

Warnings:

Information:

2	Extension of Time	287000_130US3_EOT_07192010.pdf	92945 67fe42e22d3ba3461ad8375367589c4d33d5a6fb	no	1

Warnings:

Information:

3	Fee Worksheet (PTO-875)	fee-info.pdf	30096 f765833a89d5aea9b2f71e3f66c498f3d5383f4c	no	2

Warnings:

Information:

Total Files Size (in bytes):	535441

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/31 (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

NOTICE OF APPEAL FROM THE EXAMINER TO THE BOARD OF PATENT APPEALS AND INTERFERENCES	Docket Number (Optional) 0287000.00130US3

In re Application of	Gregory J. HANNON et al.
Application Number	11/894,676-Conf. #8161
Filed	August 20, 2007
For	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE
Art Unit	1635
Examiner	K. Chong

Applicant hereby **appeals** to the Board of Patent Appeals and Interferences from the last decision of the examiner.

The fee for this Notice of Appeal is (37 CFR 41.20(b)(1)) $ 540.00

[x] Applicant claims small entity status. See 37 CFR 1.27. Therefore, the fee shown above is reduced by half, and the resulting fee is: $ 270.00

[ ] A check in the amount of the fee is enclosed.

[x] Payment by credit card. Form PTO-2038 is attached.

[ ] The Director has already been authorized to charge fees in this application to a Deposit Account.

[x] The Director is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. 08-0219 .

[ ] A petition for an extension of time under 37 CFR 1.136(a) (PTO/SB/22) is enclosed.

WARNING: INFORMATION ON THIS FORM MAY BECOME PUBLIC. CREDIT CARD INFORMATION SHOULD NOT BE INCLUDED ON THIS FORM. PROVIDE CREDIT CARD INFORMATION AND AUTHORIZATION ON PTO-2038.

I am the

[ ] applicant /inventor.

[ ] assignee of record of the entire interest. See 37 CFR 3.71. Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96)

[x] attorney or agent of record.
Registration number 42,812

[ ] attorney or agent acting under 37 CFR 1.34.
Registration number if acting under 37 CFR 1.34. _____

/Jane M. Love, Ph.D./
Signature

Jane M. Love, Ph.D.
Typed or printed name

(212) 937-7233
Telephone number

July 27, 2010
Date

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below*.

[x] *Total of 1 forms are submitted.

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office electronic filing system in accordance with § 1.6(a)(4).

Dated: July 27, 2010 Electronic Signature for Carolyn Decasseres: /Carolyn Decasseres/

Electronic Acknowledgement Receipt

EFS ID:	8095081
Application Number:	11894676
International Application Number:	
Confirmation Number:	8161
Title of Invention:	Methods and compositions for RNA interference
First Named Inventor/Applicant Name:	Gregory J. Hannon
Customer Number:	84834
Filer:	Julia Anne Grimes/Carolyn DeCasseres
Filer Authorized By:	Julia Anne Grimes
Attorney Docket Number:	287000.130US3
Receipt Date:	27-JUL-2010
Filing Date:	20-AUG-2007
Time Stamp:	15:17:26
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Notice of Appeal Filed	0287000_00130US3_Notice_of_Appeal_072710.pdf	58825 cadf40b045954486f567ea045e7decce492799bb	no	1

Warnings:

Information:

Total Files Size (in bytes):	58825

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National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Document code: WFEE

United States Patent and Trademark Office
Sales Receipt for Accounting Date: 08/09/2010

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01 FC : 1401 540.00 DA

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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Alexandria, Virginia 22313-1450
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/894,676	08/20/2007	Gregory J. Hannon	287000.130US3	8161

84834 7590 08/19/2010

WilmerHale/Cold Spring Harbor Laboratory
399 Park Avenue
New York, NY 10022

EXAMINER	
CHONG, KIMBERLY	

ART UNIT	PAPER NUMBER
1635	

NOTIFICATION DATE	DELIVERY MODE
08/19/2010	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

Teresa.carvalho@wilmerhale.com
whipusptopairs@wilmerhale.com

Interview Summary	Application No.	Applicant(s)	
	11/894,676	HANNON ET AL.	
	Examiner	Art Unit	
	KIMBERLY CHONG	1635	

All participants (applicant, applicant's representative, PTO personnel):

(1) *KIMBERLY CHONG*.

(2) *JANE LOVE, VLADIMIR DROZDOFF*.

(3)*CHRISTOPHER LOW, JOSEPH WOITACH*.

(4)*BENNETT CELSA*.

Date of Interview: *05 August 2010*.

Type: a)☐ Telephonic b)☐ Video Conference
c)☒ Personal [copy given to: 1)☐ applicant 2)☐ applicant's representative]

Exhibit shown or demonstration conducted: d)☐ Yes e)☒ No.
If Yes, brief description: _____.

Claim(s) discussed: _____.

Identification of prior art discussed: _____.

Agreement with respect to the claims f)☐ was reached. g)☐ was not reached. h)☒ N/A.

Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: *Applicant's representatives addressed rejections of record, particularly Fire et al. and Kreutzer et al. and state nothing in the record rebuts the evidence established by Professor Hernandez in the declaration of record. It is stated by Prof. Hernandez that Elbashir teach away from using long dsRNAs, such as 29mers in RNAi because they are efficiently processed and further state the teachings of Elbashir apply to hairpin RNA as claimed, thus there would be no reasonable expectation of success. The Examiner agreed to review Elbashir again and see if it applies to hairpin RNA. The Examiner also stated that if the evidence provided by Prof Hernandez is sufficient and cannot be rebutted with evidence provided by Examiner, then this would overcome the rejection of record. With regard to Kreutzer, the representatives continue to allege the reference is not prior art. This argument has been previously addressed. The representatives have also stated that the priority date cannot be confirmed because the PCT from which the parent of Kreutzer arose is in German and even if Kreutzer was prior art, it does not render the claims obvious as argued in remarks recently filed to the outstanding Office action. These arguments will be addressed in response to Applicant's remarks of record. A complete copy of the handout provided by the representatives is attached and is made of record* .

(A fuller description, if necessary, and a copy of the amendments which the examiner agreed would render the claims allowable, if available, must be attached. Also, where no copy of the amendments that would render the claims allowable is available, a summary thereof must be attached.)

THE FORMAL WRITTEN REPLY TO THE LAST OFFICE ACTION MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP Section 713.04). If a reply to the last Office action has already been filed, APPLICANT IS GIVEN A NON-EXTENDABLE PERIOD OF THE LONGER OF ONE MONTH OR THIRTY DAYS FROM THIS INTERVIEW DATE, OR THE MAILING DATE OF THIS INTERVIEW SUMMARY FORM, WHICHEVER IS LATER, TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW. See Summary of Record of Interview requirements on reverse side or on attached sheet.

	/Kimberly Chong/ Primary Examiner AU1635

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

———

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:

1) A brief description of the nature of any exhibit shown or any demonstration conducted,
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
   (The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiner's version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, "Interview Record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/894,676	08/20/2007	Gregory J. Hannon	287000.130US3	8161

84834 7590 08/30/2010

WilmerHale/Cold Spring Harbor Laboratory
399 Park Avenue
New York, NY 10022

EXAMINER
CHONG, KIMBERLY

ART UNIT	PAPER NUMBER
1635	

NOTIFICATION DATE	DELIVERY MODE
08/30/2010	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

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Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

Teresa.carvalho@wilmerhale.com
whipusptopairs@wilmerhale.com

Office Action Summary	Application No.	Applicant(s)	
	11/894,676	HANNON ET AL.	
	Examiner	Art Unit	
	KIMBERLY CHONG	1635	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *07/19/2010*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *50,52,54-60-63* is/are pending in the application.

4a) Of the above claim(s) *61* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *50,52,54-60,62 and 63* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Status of Application/Amendment/Claims

Applicant's response filed 07/19/2010 has been considered. The Finality of the previous Office action mailed 01/27/2010 has been withdrawn in view of the new rejections below. Rejections and/or objections not reiterated from the previous office action are hereby withdrawn. The following rejections and/or objections are either newly applied or are reiterated and are the only rejections and/or objections presently applied to the instant application. The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

With entry of the amendment filed on 07/19/2010, claims 50, 52, 54-60, 62 and 63 are pending in the application. Claims 61 and non-elected subject matter is withdrawn as being drawn to a non-elected invention.

Response to Declaration

The declaration filed on 11/04/2009 under 37 CFR 1.132 by Professor Hernandez does not provide sufficient evidence that a person of ordinary skill in the art would not have had a reasonable expectation of success at using a shRNA with a double stranded region of between 20 and 29 nucleotides in length.

Professor Hernandez provides evidence by way of the Elbashir et al. reference (Genes Dev 2001 of record) which teach dsRNA of 39-501 could be processed to 21-23 nt fragments while a dsRNA of 29 base pairs was slowly processed to 21-23 nt fragments (see Figure 2). Elbashir concludes that specific inhibition of target RNA

expression, in Drosophila cells in vitro, was detected for dsRNAs as short at 38 bp but dsRNAs of 29-36 bp were not effective in this process. Professor Hernandez understands the reference to include short hairpin RNA structures and states that one of ordinary skill in the art would have understood Elbashir et al. to "expressly teach away from using short hairpin RNAs having double-stranded regions of less than 30 bp, for example, 20-29 bp in length, to mediate RNAi."

Applicants argue that the Examiner does not provide any countervailing factual evidence that would rebut the statements of Professor Hernandez and does not provide “any further shred of evidence that would call into question the statements made by Prof. Hernandez."

In response, the previous Office action did in fact provide evidence that a dsRNA having a double stranded region of at least 21 bp was capable of mediating RNAi in cells which is direct evidence against the data provided by Elbashir. It is clearly shown in Kreutzer et al. (of record) that a dsRNA 21 nucleotide base paired molecule was capable of efficiently reducing gene expression in mammalian cells (see Examples). Applicant did not comment on this reference in this regard however this is direct evidence that one of ordinary skill in the art would have expected a dsRNA of less than 29 bp or having a double stranded region of at least 20 base pairs to be capable of mediating RNAi in mammalian cells.

The declaration provided by Professor Hernandez provides direct factual evidence supported by references such as Elbashir et al. but also provides opinion evidence in the conclusions stated by the Professor that one of skill in the art would

understand Elbashir et al. expressly teach away from using short hairpin RNAs having double-stranded regions of less than 30 bp, for example, 20-29 bp in length, to mediate RNAi.

Elbashir et al. does conclusively provide evidence that a dsRNA of 29 base pairs in length did not efficiently mediate RNAi in Drosophila cells in vitro. However, there is no factual evidence provided in Elbashir or any of the other references that expressly teach that dsRNAs having a duplex of less that 29 base pairs were not capable of mediating RNAi. This conclusion by Professor Hernandez appears to be opinion evidence without any factual support. MPEP 716.01(c) states:

> In assessing the probative value of an expert opinion, the examiner must consider the nature of the matter sought to be established, the strength of any opposing evidence, the interest of the expert in the outcome of the case, and the presence or absence of factual support for the expert's opinion. Ashland Oil, Inc. v. Delta Resins & Refractories, Inc., 776 F.2d 281, 227 USPQ 657 (Fed. Cir. 1985), cert. denied, 475 U.S. 1017 (1986). See also In re Oelrich, 579 F.2d 86, 198 USPQ 210 (CCPA 1978) (factually based expert opinions on the level of ordinary skill in the art were sufficient to rebut the prima facie case of obviousness); [emphasis added].

Thus, as factually supported by Professor Hernandez, a person of ordinary skill in the art would have no reasonable expectation of success at using a 29 bp dsRNA to mediate RNAi in Drosophila cells as shown by Elbashir et al., however the evidence provided by Elbashir et al. does not teach away from using a dsRNA 29 bp to mediate RNAi in mammalian cells as instantly claimed and more importantly does not teach away from using a dsRNA of less than 29 bp to mediate RNAi in any cell type.

This fact is demonstrated in the prior art as shown by Kreutzer et al. as discussed above and by Caplen et al. (PNAS Vol. 98, No. 17, August 14, 2001).

Caplen et al. demonstrates that small dsRNAs from 21-27 nucleotides in length can specifically inhibit gene expression in mammalian cells (see page 9744 and Figure 1). Caplen et al. also demonstrates that dsRNAs of 23-25 nucleotides in length can induce specific interference in cells such as C. elegans, which goes against what Professor Hernandez submits one of ordinary skill in the art would conclude from Elbashir et al: "Elbashir et al. expressly teach away from using short hairpin RNAs having double stranded regions of less than 30bp, for example 20-29 bp in length, to mediate RNAi.

Thus based on Caplen et al. one of ordinary skill in the art would clearly have a reasonable expectation of success in using a dsRNA of less than 29 bp to mediate RNAi in mammalian cells and further provides factual evidence that even in cells, such as C. elegans or Drosophila as taught by Elbashir, dsRNA of less than 29 bp are capable of efficiently mediating RNAi.

The prior art as a whole at the time of filing of the instant invention provides the skilled artisan with a reasonable expectation of success at using a dsRNA having a double stranded region of at least 20 nucleotides but not more than 29 nucleotides to mediate RNAi in mammalian cells.

A response to Applicant's argument that Kreutzer et al. (2004/0102408) is not prior art is warranted as this reference is used to dispute the evidence provided in the declaration above. Applicants continue to argue Kreutzer et al. is not a proper 102(e) reference because it claims the benefit under section 120 of the PCT/DE00/00244 which was filed prior to November 29, 2000.

Applicant is correct in that the Kreutzer et al. reference cannot use the date of the PCT as the 102(e) however the Kreutzer et al. application is a divisional of a US filed application 09/889,802 filed 09/17/2001 and for purposes of applying the reference as prior art, the filing date of the priority US filed application 09/889,802 is the proper 102(e) date of Kreutzer '408 application. Thus Kreutzer et al. '408 application has a 102(e) date which is the effective filing date of the '802 application i.e. 09/17/2001, which is before the priority date of the instant application.

MPEP 706.02(f)(1)(C):

(3) If the international application has an international filing date prior to November 29, 2000, apply the reference under the provisions of 35 U.S.C.102 and 374, prior to the AIPA amendments:

(a) For U.S. patents, apply the reference under 35 U.S.C. 102(e) as of the earlier of the date of completion of the requirements of 35 U.S.C. 371(c)(1), (2) and (4) or the filing date of the later-filed U.S. application that claimed the benefit of the international application;

(b) For U.S. application publications and WIPO publications directly resulting from international applications under PCT Article 21(2), never apply these references under 35 U.S.C. 102(e). These references may be applied as of their publication dates under 35 U.S.C. 102(a) or (b);

(c) For U.S. application publications of applications that claim the benefit under 35 U.S.C. 120 or 365(c) of an international application filed prior to November 29, 2000, apply the reference under 35 U.S.C. 102(e) as of the actual filing date of the later-filed U.S. application that claimed the benefit of the international application

In following the MPEP guidelines, because the international application PCT/DE00/00244 has an international filing date prior to November 29, 2000, section (3)(c) would apply. This section states that for US application publications (the '408

application) of applications (the ‘802 application) that claim the benefit under 35 U.S.C. 120 or 365(c) of an international application filed prior to November 29, 2000 (PCT/DE00/00244), apply the reference i.e. the '408 application, under 35 U.S.C. 102(e) as of the actual filing date of the later-filed US application that claimed the benefit of the international application i.e. the ‘802 application. Thus, the 102(e) date of the '408 reference would be 09/17/2001.

Applicant’s arguments would be correct if the ‘408 application *directly* resulted from PCT/DE00/00244 and in that case the guidelines of (3)(b) above would apply. However because the ‘408 application did not directly result from the PCT application, this guideline does not apply. The ‘408 application resulted from the ‘802 application as a divisional application.

The fact the ‘802 application was never published and would not be considered "an application publication" is not relevant to the ‘408 application claiming the benefit of the application and the IAP rules are not relevant in this instance.

On April 23, 2010, this application and Applicant’s arguments against Kreutzer et al. not being a proper prior art reference were discussed with Quality Assurance Specialist Bennett Celsa who agreed with the Examiner and stated the IAP rules and Example 6 did not apply to Kreutzer et al. ‘408 application because this reference had a proper 102(e) date using the US effective filing date of the ‘802 application.

Thus, Kreutzer et al. is available as a prior art reference and the priority application ‘802 is available in Public Pair as well as a translation of PCT/DE00/00244.

New Claim Rejections

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. See *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970);and, *In re Thorington,* 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent is shown to be commonly owned with this application. See 37 CFR 1.130(b).

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

Claims 50, 52, 54-60, 62 and 63 are provisionally rejected under the judicially created doctrine of double patenting over claims 3, 40, 42-47 and 49-51 of copending Application No. 10/997,086. This is a provisional double patenting rejection since the conflicting claims have not yet been patented.

Although the conflicting claims are not identical, they are not patentably distinct from each other because the instant claims are directed to a method of attenuating gene expression in a mammalian cell comprising introducing a library of shRNA expression constructs wherein the construct comprises a promoter and a shRNA targeted to a gene. The claims of the ’086 application are directed to a method of attenuating gene expression in a mammalian cell comprising by introducing shRNA expression constructs into a cell. The specification of the ’086 application discloses the use of a library of shRNA constructs and it would be obvious to use the shRNA of the

‘086 application in a library of shRNA constructs to attenuate expression of a target gene.

Moreover, as taught by Lieber et al. (of record), the use of inhibitory nucleic acid molecules in a library is advantages to search for a function gene. Lieber et al. teach making randomized ribozyme libraries and introducing said ribozyme libraries into mammalian cells, selecting cells into which the library expression systems were introduced and analyzing the phenotypes of the cells (see Figure 2 and columns 3 and 8 and claims 1-8). Lieber et al. teach the ribozymes are chemically synthesized by transcription using expression cassettes comprising pol II or pol III promoters (see column 3). Therefore it would have been further obvious to use the shRNA of the '086 application.

This is a provisional obviousness-type double patenting rejection.

Claim Rejections - 35 USC § 112

The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 62 and 63 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

The claims recite the "expression of the target gene is attenuated by at least 60%" or "about 60% to about 90%". These claims are indefinite because it is unclear what the decrease in expression is being measured against or compared with and

without assumption the skilled artisan would not reasonably be apprised of the scope of the invention. For purposes of examination, the claims are interpreted to mean the attenuation is being measure against a normal control cell consisting of an expression construct encoding a shRNA that does not target the target gene.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

Claims 50, 52, 54-60, 62 and 63 are rejected under 35 U.S.C. 103(a) as being unpatentable over Symonds et al. (US 2002/0160393), Lieber et al. (US Patent No. 6,130,092 cited on Applicant's IDS filed 01/10/2008), Fire et al. (US Patent Number 6,506,559 cited on Applicant's IDS filed 01/10/2008), Good et al. (Gene Therapy 1997 cited on Applicant's IDS filed 01/10/2008) and Noonberg et al. (US Patent No. 5,624,803).

The claims are drawn to a method of attenuating expression of a target gene in a mammalian cells comprising introducing into mammalian cells a library of RNA expression constructs wherein each construct comprises a promoter and a shRNA wherein the construct comprises LTR sequences and wherein the shRNA comprises at least 20 but less than 29 nucleotide double stranded region and wherein the promoter is a pol III, U6 promoter.

Symonds et al. teach a method of attenuating expression of a target gene comprising introducing a dsRNA comprising a hairpin loop wherein the hairpin RNA is encoded by a DNA vector (see at least paragraphs 0108-0114 and Figure 8A). Symonds et al. teach the hairpin RNA molecule can comprise nucleotide strands of at least 20 nucleotides in length (see paragraph 0136). The expression vector used can be retroviral vectors or adenoviral expression vectors and can be stably integrated into the host cell (see paragraph 0158). Figure 9 exemplifies retroviral expression constructs comprising LTR sequences flanking the hairpin RNA. It was well known in the art at the time of filing that dsRNAs greater than 30 base pairs were capable of activating an unwanted PKR response in cells as taught by Elbashir (Nature 2001 of record cited by Applicant in response) who found that RNAi was active in mammalian cells but was very difficult to detect if using a dsRNA >30 base pairs. Thus, the dsRNA taught by Symonds et al. would have the inherent property of not triggering the PKR response in cells. (Note: The Symonds et al. application finds support for the teachings above in priority document of the Provisional application 60/258,731 filed 12/28/2000, at least on pages 5, 11 and Figure 2A).

Symonds et al. does not specifically disclose the expression construct comprises a pol III or specifically a U6 promoter and does not specifically teach using an expression library of hairpin RNA.

Methods of attenuating expression of a target gene and searching for the function gene comprising making randomized inhibitory nucleic acid libraries were known in the art at the time of filing of the instant invention. Lieber et al. teach the use of

ribozyme libraries and introducing the ribozyme libraries into mammalian cells, selecting cells into which the library expression systems were introduced and analyzing the phenotypes of the cells (see Figure 2 and columns 3 and 8 and claims 1-8). Lieber et al. teach the ribozymes are chemically synthesized by transcription using expression cassettes comprising pol II or pol III promoters (see column 3).

Fire et al. disclose a method of attenuating expression of a target gene in mammalian cells (see column 8, lines 12-19) using a library of RNA expression constructs comprising RNAi molecules (see columns 12-13), wherein the RNA can be formed by a single self-complementary RNA i.e. a hairpin RNA (see column 7, lines 42-44).

Good et al. teach an expression construct comprising a U6 promoter and a coding sequence for a hairpin RNA wherein the expression construct is capable of efficiently expressing small hairpin RNA and LTR sequences flanking the RNA sequences (see entire document and at least Figure 1).

Likewise Noonberg et al. teach an expression construct for generation of short-sequence specific oligonucleotides for the purpose of gene regulation wherein the construct comprises a U6 promoter (see columns 7-8). Noonberg et al. teach such constructs facilitate delivery of oligonucleotides to any target cell.

It would have been obvious to one of ordinary skill in the art to use a library of RNA expression constructs capable of expression the shRNA taught by Symonds et al. and obvious to use a U6 promoter in the RNA expression construct to generate shRNA that are capable of attenuating expression of a target gene.

Lieber et al. teach identifying a gene responsible for a particular phenotype is crucial to important any biological mechanism and our understanding of disease and teach the use of a library expression system that can identify genes that are specifically involved in producing a particular phenotype by knocking down intracellular expression, one would have clearly been motivated to incorporate a shRNA in the library expression system to attenuate the expression of a target gene and identify the function of said gene.

Fire et al. also recognized the importance of identifying gene function in an organism and teach using a library of dsRNA for attenuating expression of a target gene in cells or organisms (see column 12, starting at line 17 to column 13).

Moreover it was well known in the art that pol III promoters such as U6 promoters could be used to efficiently generate inhibitory oligonucleotides as taught by Noonberg et al. and given Good et al. teach a construct comprising U6 promoters were capable of expressing shRNA, one of ordinary skill in the art would have used a U6 promoter to generate the shRNA of Symonds et al.

One would have a reasonable expectation of success at using a library of hairpin RNA constructs because Lieber et al. and Fire et al. teach efficient identification of target genes using inhibitory RNA molecules and would have expected to be able to use the shRNA of Symonds et al. in methods of attenuating expression of a target gene. One of ordinary skill in the art would have expected to be able to generate the shRNA of Symonds et al. from a RNA construct that was capable of attenuating expression of a target gene because this was taught by Good et al.

Thus in the absence of evidence to the contrary, the invention as a whole would have been prima facie obvious to one of ordinary skill in the art at the time the invention was made.

Response to Arguments

Claim Rejections - 35 USC § 112

The rejection of claim 64 under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement is moot as the claim has been canceled.

Claim Rejections - 35 USC § 102

The rejection of claim 64 under 35 U.S.C. 102(b) as being anticipated by McSwiggen et al. (US 20050277133) is moot as the claim has been canceled.

Claim Rejections - 35 USC § 103

The rejection of claims 50-59 under 35 U.S.C. 103(a) as being unpatentable over Fire et al. (US Patent Number 6,506,559 cited on Applicant's IDS filed 01/10/2008), Good et al. (Gene Therapy 1997 cited on Applicant's IDS filed 01/10/2008) and Noonberg et al. (US Patent NO. 5,624,803) is withdrawn in view of the new grounds of rejection above and therefore response to arguments is moot.

The rejection of claims 50-59 under 35 U.S.C. 103(a) as being unpatentable over Kreutzer et al. (US Application No. 20040102408), Lieber et al. (US Patent No.

6,130,092 cited on Applicant's IDS filed 01/10/2008), Good et al. (Gene Therapy 1997 cited on Applicant's IDS filed 01/10/2008) and Noonberg et al. (US Patent NO. 5,624,803) is withdrawn in view of the new grounds of rejection above and therefore response to argument is moot.

Double Patenting

The rejection of claims 50-60 under the judicially created doctrine of double patenting over claims 1, 2, 7-20, 24 and 59-63 of copending Application No. 10/350,798 is moot as this application is abandoned.

The rejection of claims 50-60 under the judicially created doctrine of double patenting over claims 1, 2, 6-7, 9-10 and 23-28 of copending Application No. 09/858,862 is withdrawn.

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Kimberly Chong whose telephone number is 571-272-3111. The examiner can normally be reached Monday thru Friday between 7-4 pm.

If attempts to reach the examiner by telephone are unsuccessful please contact Christopher Low at 571-272-0951. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Patent applicants with problems or questions regarding electronic images that can be viewed in the Patent Application Information Retrieval system (PAIR) can now contact the USPTO's Patent Electronic Business Center (Patent EBC) for assistance.

Representatives are available to answer your questions daily from 6 am to midnight (EST). The toll free number is (866) 217-9197. When calling please have your application serial or patent number, the type of document you are having an image problem with, the number of pages and the specific nature of the problem. The Patent Electronic Business Center will notify applicants of the resolution of the problem within 5-7 business days. Applicants can also check PAIR to confirm that the problem has been corrected. The USPTO's Patent Electronic Business Center is a complete service center supporting all patent business on the Internet. The USPTO's PAIR system provides Internet-based access to patent application status and history information. It also enables applicants to view the scanned images of their own application file folder(s) as well as general patent information available to the public. For more information about the PAIR system, see http://pair-direct.uspto.gov.

For all other customer support, please call the USPTO Call Center (UCC) at 800-786-9199.

/Kimberly Chong/
Primary Examiner
Art Unit 1635

Notice of References Cited	Application/Control No. 11/894,676	Applicant(s)/Patent Under Reexamination HANNON ET AL.	
	Examiner KIMBERLY CHONG	Art Unit 1635	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-2002/0160393	10-2002	Symonds et al.	435/6
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Caplen et al. Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. PNAS 2001, Vol. 98, No. 17: 9742-9747.
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Search Notes	Application/Control No.	Applicant(s)/Patent under Reexamination
	11/894,676	HANNON ET AL.
	Examiner	Art Unit
	KIMBERLY CHONG	1635

SEARCHED			
Class	Subclass	Date	Examiner

INTERFERENCE SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES (INCLUDING SEARCH STRATEGY)		
	DATE	EXMR
updated	8/15/2010	KC

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Hannon et al.	Confirmation No.:	8161
Application No:	11/894,676	Art Unit:	1635
Filed:	August 20, 2007	Examiner:	K. CHONG
Title:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT IN RESPONSE TO AUGUST 30, 2010 NON-FINAL OFFICE ACTION

This Amendment is filed in response to the August 30. 2010 Non-Final Office Action for which a response is due November 30, 2010. Applicants request a two-month extension of time to January 30, 2011, which is a Sunday. Thus, a response is due on Monday, January 31, 2011. Accordingly, this paper is being timely filed. The Commissioner is authorized to charge any fees due, or to credit any overpayment in fees, to Deposit Account No. 08-0219.

Claim Listing begin on page 2.
Remarks begin on page 4.

Claim Listing

This listing of the claims will replace all prior versions and listings of claims in the application:

1-49. (Cancelled)

50. (Currently Amended) A method for attenuating expression of a target gene in a mammalian cell, the method comprising

introducing into [[a]] mammalian cells a library of RNA expression constructs, each expression construct comprising:

(i) an RNA polymerase promoter, and

(ii) a sequence encoding a short hairpin RNA molecule comprising a double-stranded region wherein the double-stranded region consists of at least 20 nucleotides but not more than 29 nucleotides, [~~such that the short hairpin RNA does not trigger a protein kinase RNA-activated (PKR) response in the mammalian cells~~],

wherein the short hairpin RNA molecule is a substrate for Dicer-dependent cleavage and does not trigger a protein kinase RNA-activated (PKR) response in the mammalian cell,

wherein the double-stranded region of the short hairpin RNA molecule comprises a sequence that is complementary to a portion of the target gene, and

wherein the short hairpin RNA molecule is stably expressed in the mammalian cell in an amount sufficient to attenuate expression of the target gene in a sequence specific manner, and is expressed in the cell without use of a PK inhibitor, whereby expression of the target gene is inhibited.

51. (Cancelled)

52. (Previously presented) The method of claim 50, wherein the expression construct further comprises LTR sequences located 5' and 3' of the sequence encoding the short hairpin RNA molecule.

53. (Cancelled)

54. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of at least 21 nucleotides.

55. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of at least 22 nucleotides.

56. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of at least 25 nucleotides.

57. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of 29 nucleotides.

58. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule has a total length of about 70 nucleotides.

59. (Previously presented) The method of claim 50, wherein the RNA polymerase promoter comprises a pol II promoter or a pol III promoter.

60. (Previously presented) The method of claim 59, wherein the pol III promoter comprises a U6, an H1, or an SRP promoter.

61. (Withdrawn) The method of claim 59, wherein the pol II promoter comprises a U1 or a CMV promoter.

62. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule attenuates expression of the target gene in the mammalian cell by at least about 60%.

63. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule attenuates expression of the target gene in the mammalian cell by about 60% to about 90%.

64. (Cancelled)

REMARKS

Claims 50, 52, 54-60 and 62-63 were pending and under examination. Claim 50 is amended to more particularly point out the presently claimed invention. The amendment to claim 50 raises no issue of new matter. Support for the amendment to claim 50 ("wherein the short hairpin RNA molecule is a substrate for Dicer-dependent cleavage and does not trigger a protein kinase RNA-activated (PKR) response in the mammalian cells") and "is expressed in the cell without use of a PKR inhibitor") can be found throughout the priority application, U.S. Publication No. 2003/0084471. For example, support can be found in Examples 6 and 7 of the priority application, which demonstrate that "…short hairpins are highly effective in specifically suppressing gene expression." U.S. Publication No. 2003/0084471 ¶ 0251 (emphasis added); see also ¶ 0240 ("Additionally, we wanted to demonstrate that unlike long dsRNAs, short dsRNAs do not provoke a non-specific PKR or PKR-like response," *i.e.*, no PKR inhibitor is necessary for the expressed short hairpin RNAs to specifically suppress gene expression). In contrast, Example 8 discloses methods "to circumvent the PKR response in cell types in which in might be advantageous to [suppress] gene expression with long dsRNAs." U.S. Publication No. 2003/0084471 ¶ 0255. For example, "approaches include treating cells with an agent that inhibits protein kinase RNA-activated (PKR) apoptosis." Id.; see also ¶ 0107 ("As described herein, Applicants have demonstrated that the PKR response can be overcome in favor of the sequence-specific RNAi response. However in certain instances, it may be desirable to treat the cells with agents which inhibit expression of PKR.").

I. The State of the Art Prior to January 22, 2002[1]

In order to provide background to the views of a person of ordinary skill in the art, and background against which the present invention was made, Applicants provide below a brief summary of the state of the art regarding gene silencing using RNA molecules. This summary is not to be considered an admission that any reference set out below is proper prior art as to the presently claimed invention.

[1] January 22, 2002 is the filing date of the parent USSN 10/055,797 to which the present application claims priority.

A. Dr. Hannon's Goal: Exploiting RNAi to Study Gene Function in Mammalian Cells

By the invention of the short hairpin technology described in the Hannon application, Dr. Hannon and his co-inventors successfully achieved an ambitious goal of exploiting RNAi as a powerful and widely applicable genetic tool to study gene function in mammalian cells. In particular, this novel approach allowed one to use RNAi to stably attenuate expression of the target gene in a sequence specific manner in a mammalian cell, without activating a non-sequence specific PK response. To achieve this goal, Dr. Hannon and his co-inventors focused on identifying and understanding the cellular machinery that mediated RNAi in the cell. A key part of their work involved identifying and characterizing the components of the RNAi pathway. Among other things, Dr. Hannon and his co-inventors isolated and described two critical components of the RNAi machinery: the enzyme Dicer, which the inventors named and demonstrated as mediating the processing of dsRNA (Bernstein et al. Nature, 2001), and "RISC", the nuclease complex responsible for degradation of target mRNAs. Dr. Hannon concisely summarized his overall strategy in a grant proposal for the work he subsequently carried out:

> My laboratory has devoted a number of years to creating improved tools for probing gene function in cultured mammalian cells; however, our experience indicates that a facile loss-of-function tool is lacking. Unfortunately, dsRNA induces somewhat generic responses in mammalian cells. It is our hope that by understanding the mechanistic basis of dsRNA-induced silencing, we may not only unravel a mysterious and important piece of biology but also provide the means to create improved tools for analyzing gene function in diverse organisms in which traditional genetic methods are either cumbersome or unavailable. This notion that has contributed to the decision to focus substantial effort in my laboratory toward elucidating the mechanism of RNA interference…
>
> …In this application, we propose a biochemical approach to deciphering the mechanisms that underlie dsRNA-induced gene silencing. RNA-interference allows an adaptive defense against both exogenous and endogenous dsRNAs, providing something akin to a dsRNA immune response. The primary goal of the work proposed in this application is to understand the mechanisms by which a cell can raise this response. We have presented evidence that RNA interference is accomplished, at least in part, through the

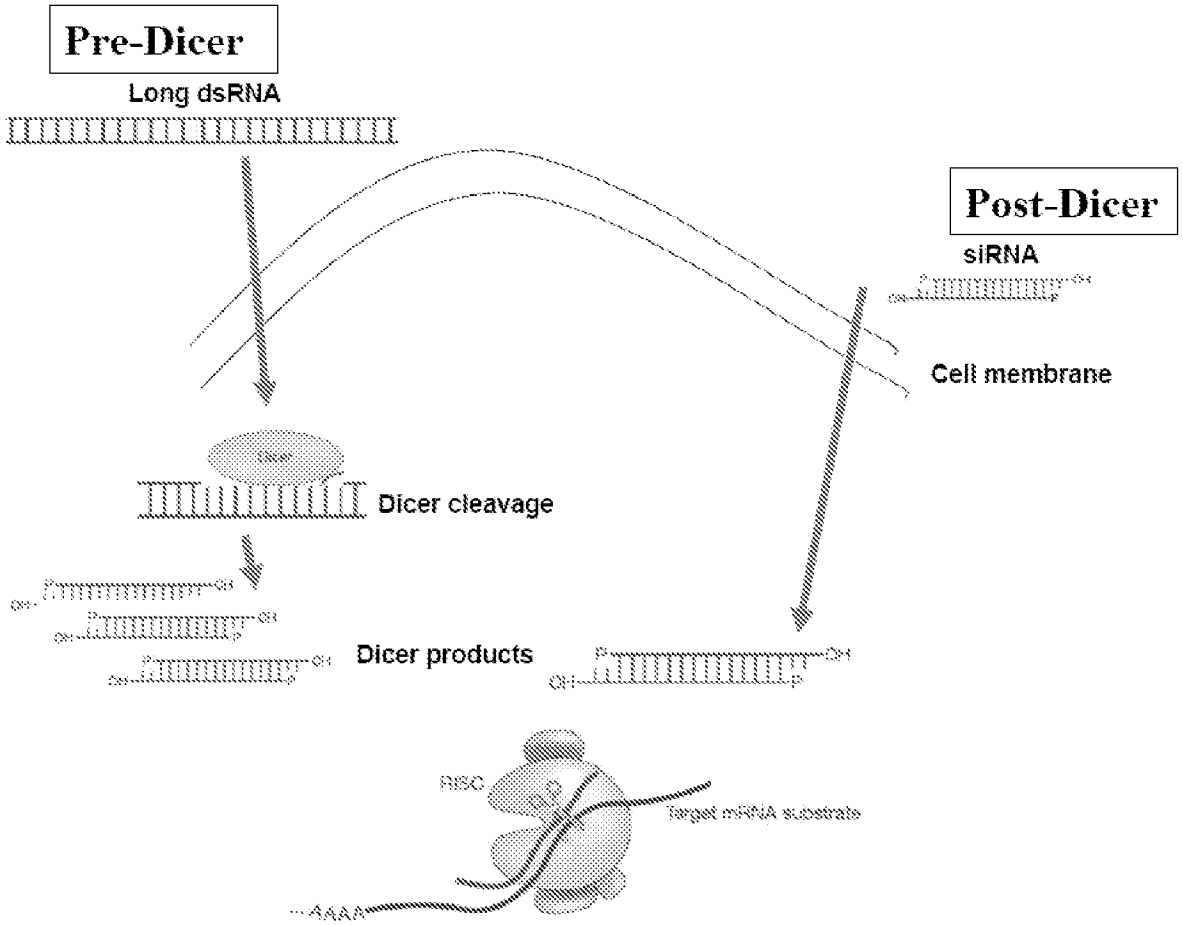
> action of a sequence-specific nuclease that is generated in response to dsRNA. Our data, and that of others (Hamilton and Baulcombe, 1999), is consistent with a model in which dsRNAs present in a cell are converted, in a manner analogous to antigen processing, into discrete, small RNAs that guide the nuclease in the choice of substrate. We propose to purify and characterize the nuclease and to clone the protein and RNA components of the enzyme. In addition, we propose to develop approaches that may allow the use of cultured Drosophila cells as a general tool for probing gene function. The combination of these studies may lead eventually to an ability to harness RNA interference as a genetic tool in other organisms, particularly mammals, in which analogous tools are presently lacking.

B. Dicer Cleaves Long dsRNA to Make Guide RNAs or siRNAs

Hannon and his co-inventors demonstrated that Dicer processes long dsRNAs into short (approximately 21-25 nt) RNAs, which are referred to as short interfering RNAs (siRNAs) or "guide" RNAs, the term coined by Dr. Hannon. Bernstein et al. Nature 409: 363-366 (2001). The siRNAs are then incorporated into a protein (nuclease) complex called the RNA-induced silencing complex (RISC) Hammond et al., Nature 404:293-296 (2000). The siRNAs function to guide the RISC/siRNA complex to specific mRNAs, which are recognized through base pairing interactions by having a complementary sequence to the siRNA, and are then destroyed by RISC. Through this process, guide RNAs or siRNAs can inhibit gene expression by targeting destruction of specific mRNAs in the cell. Notably, the ability of long dsRNAs to trigger RNAi, therefore, requires Dicer to first cleave or process the long dsRNA into guide or siRNAs. Bernstein et al. Nature 409: 363-366 (2001); Hammond et al., Nature Rev. Genetics 2:110-119.

C. Pre-Dicer and Post-Dicer Strategies to Achieve RNAi

Dr. Hannon's work in discovering Dicer and the mechanism of Dicer processing demonstrated that one could potentially intervene in the RNAi pathway in two places.



(I) A **Pre-Dicer** strategy (see Pre-Dicer Pathway in above diagram) starts with long dsRNA triggers (see "Long dsRNA" in diagram above). Fire et al. demonstrated that long dsRNAs (for example, 300-500 bp) could effect gene silencing. Once introduced into a cell, these long dsRNA triggers are cleaved into siRNAs by Dicer. The siRNAs then combine with RISC to mediate specific gene silencing.

(II) A **Post-Dicer** strategy (see Post-Dicer Pathway above) uses short RNAs that mimic the siRNA products of Dicer cleavage (i.e., 21-25 nucleotide long short RNAs with 3' overhangs). Once introduced into a cell, the siRNAs bypass the Dicer enzyme altogether. The siRNAs directly combine with RISC to effect gene silencing. Elbashir SM, Lendeckel W, Tuschl T (2001) RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev* 15(2):188–200. Elbashir et al. has been discussed by Dr. Hernandez in her Declaration filed in

this case. In this regard, Elbashir et al. included data showing that dsRNAs of 30 nucleotides in length or shorter were ineffective in mediating RNAi and would not work as Pre-Dicer triggers. Short RNAs would work only if they were designed to bypass Dicer processing.

D. Fire, Elbashir and Caplen Fail to Show Stable, Long Term Silencing

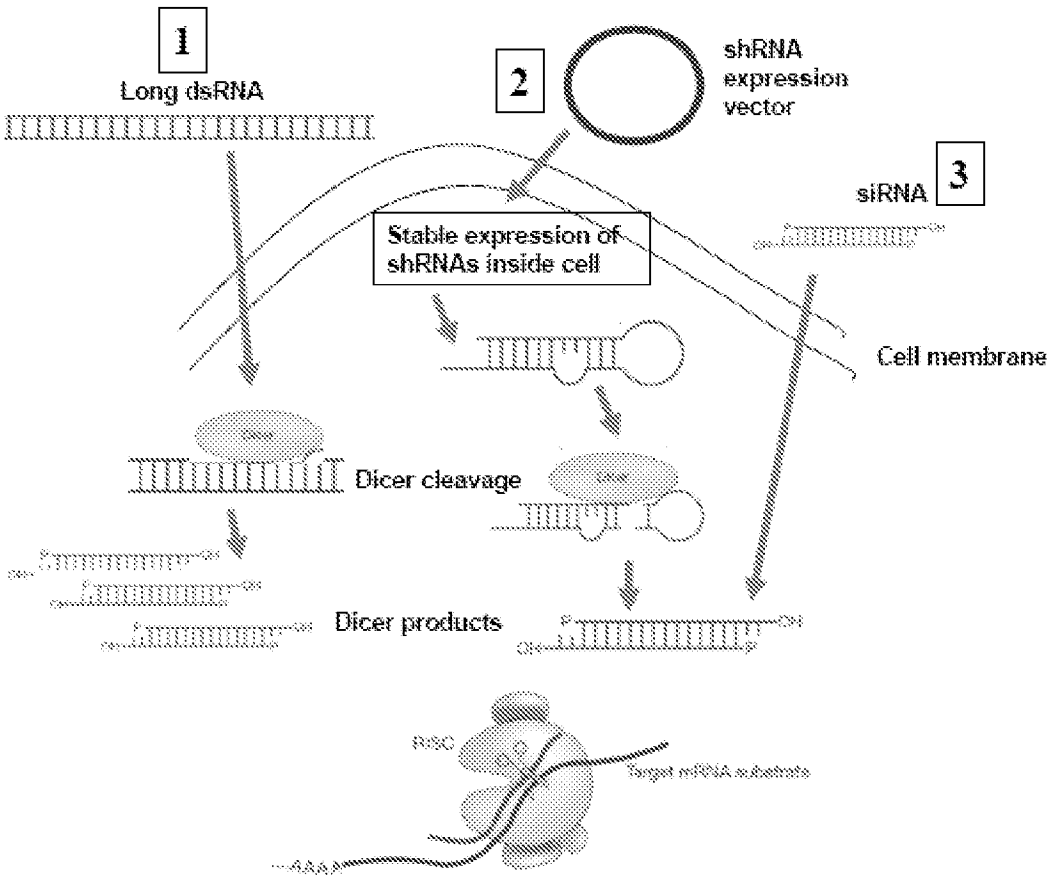
Fire's approach of using long dsRNA as a pre-Dicer trigger failed to show how one could use this strategy in mammalian cells. Of course, it was known that introducing or expressing long dsRNA in most mammalian cells would kill them by activating the anti-viral/PKR response. (Williams, B. R. Role of the double-stranded RNA-activated protein kinase (PKR) in cell regulation. *Biochem. Soc. Trans.* 25, 509–513 (1997).) This innate anti-viral pathway would have taught away from using dsRNA for silencing expression of a particular gene in a mammalian cell.

Another approach was taken by both Elbashir and Caplen -- using post-Dicer triggers, that is, siRNAs to achieve inhibition of gene expression. One primary drawback of this approach is that the effect is only transient. The application of siRNAs (see the post-Dicer pathway in the above diagram) is transitory. Once the siRNAs are applied exogenously into the cell, processed by Dicer and then complexed with RISC, there is no additional effect. This Post-Dicer approach using siRNAs will only temporarily silence genes.

These two approaches (Pre-Dicer and Post-Dicer) did not provide for stable, long term silencing in mammalian cells. Therefore, the pre-Dicer and post-Dicer approaches were of limited benefit in mammalian cells. Stable, long term silencing was necessary to carry out studies in mammalian cells to understand the genetic basis of human disease that Dr. Hannon envisioned. Before RNAi could be harnessed as a tool for silencing specific genes in mammalian systems, such as in methods claimed in the present invention, a considerable hurdle had to be overcome. The problem was how to trigger RNAi in a gene-specific manner in mammalian cells without invoking non-specific anti-viral responses to the RNAi trigger.

E. Invention of Hannon et al. Using Expressed shRNA in Mammalian Cells

Hannon demonstrated that one could actually engineer a pre-Dicer trigger that would ***not*** activate the anti-viral/PKR response, that could be ***stably expressed*** in the mammalian cell and surprisingly, would function as a potent trigger to specifically silence gene expression in mammalian cells. The presently claimed invention solves the problems of stable expression, avoidance of the PK response and sequence-specific inhibition of gene expression in mammalian cells. The diagram below illustrates the shRNA expression vector approach, which is claimed by the applicants.



The above diagram shows the introduction of shRNA expression vectors into the mammalian cell. These vectors can be ***stably expressed*** in a mammalian cell and ***don't activate the PKR response***. The vectors express a short hairpin RNA molecule which is a ***substrate for Dicer-dependent cleavage and does not activate the PKR response.*** The double-stranded region

of the short hairpin RNA molecule comprises a sequence that is complementary to a portion of the target gene.

Thus, the entirely different approach of Elbashir and Caplen - that of using **post-Dicer** triggers which could act to silence gene expression without being processed by Dicer ***taught away*** from Hannon's invention of using stable expression of short hairpin RNAs as **pre-Dicer** triggers to suppress mammalian gene expression.

F. Industry Acclaim

As evidenced by numerous awards and by the adoption of his short hairpin technology as a fundamental biomedical research tool, Dr. Hannon's pioneering work in the RNAi field has received widespread acclaim. In 2005, Dr. Hannon received the Award for Outstanding Achievement in Cancer Research from the American Association for Cancer Research (AACR), which honored Dr. Hannon "…for his work uncovering the biochemical mechanism of RNA interference of gene expression (RNAi) and his contributions to the discovery and development of short hairpin RNAs as tools for genetic manipulation of mammalian cells." (See Declaration Under 37 C.F.R. §1.131, Exhibit O). In 2007, Dr. Hannon received two more prestigious awards, the Award in Molecular Biology from the National Academy of Sciences, and the Paul Marks prize for the valuable contribution his RNAi work to cancer research from Memorial Sloan-Kettering Cancer Center (See Declaration Under 37 C.F.R. §1.131, Exhibit Q). In granting that award, MSKCC noted how Dr. Hannon had applied his research in understanding the RNAi pathway to develop this valuable new technology, and his recognition as a leader in the field:

> Dr. Hannon is a leader in the relatively new field of RNA interference (RNAi). RNAi is a naturally occurring mechanism for regulating the expression of genes (controlling which genes are turned on and turned off in cells). In the laboratory, it is used as a tool to study the function of specific genes, and it's being investigated as a therapeutic approach for treating many different diseases, including cancer.
>
> Dr. Hannon's laboratory has elucidated key biochemical details of the components of the pathways involved in RNAi and is using these findings to develop molecular tools that can be used for gene

> discovery, the evaluation of gene function, and the generation of animal models. He has developed new techniques for using RNAi to study cancer development and is investigating possible cancer therapies that make use of small interfering RNAs (siRNAs).
>
> Dr. Hannon discovered several proteins and enzymes that are an essential part of the RNAi mechanism, including Dicer, which cleaves double-stranded RNA into siRNAs; the RISC complex, which helps regulate protein translation and is involved in the body's defense against viral infections; and Argonaute2, which cleaves messenger RNA.
>
> He also has been at the forefront of adapting RNAi techniques to study genes in mammals, and using these techniques to understand the variety of pathways that can lead to the formation of tumors.

The presently claimed invention described in the Hannon application was the basis for various shRNA libraries, which have become widely used tools for genetic analysis in mammalian cells. Reflecting the valuable contribution of this technology to biomedical research, during 2002-2006, Dr. Hannon was among the top five most highly cited scientists with the highest number of high impact papers in the field of molecular biology and genetics. The 2002 Genes & Development paper, "Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells," in which Dr. Hannon reported much of the work underlying the presently claimed invention, was cited more than 500 times, including more than 100 papers in the biotechnology field (See Declaration Under 37 C.F.R. §1.131, Exhibits K, M and N).

II. Second Declaration of Professor Hernandez Under 37 C.F.R. §1.132

The Examiner has taken the position the First Hernandez Declaration "does not provide sufficient evidence that a person of ordinary skill in the art would not have had a reasonable expectation of success at using a shRNA with a double stranded region of between 20 and 29 nucleotides in length." The Examiner then takes the position that Kreutzer et al. (of record) "provide evidence that a dsRNA having a double stranded region of at least 21 bp was capable of mediating RNAi in cells which is direct evidence against the data in Elbashir." The Examiner goes on to state that Kreutzer et al. show "that a dsRNA 21 nucleotide base paired molecule was capable of efficiently reducing gene expression in mammalian cells."

The Examiner also parses the First Declaration of Professor Hernandez as including factual evidence and "opinion evidence in the conclusions stated by the Professor that one of skill in the art would understand Elbashir et al. expressly teach away from using short hairpin RNAs having double-stranded regions of less than 30 bp…."

In reply, applicants respectfully traverse the Examiner's position. Professor Hernandez has since reviewed the Office Action, the Kreutzer et al. paper and the Caplen et al. paper and has further provided testimony that neither Kreutzer et al. nor Caplen et al. would not make obvious the claimed invention and would not provide any basis for a person of ordinary skill in the art at the time (note that Prof. Hernandez ***was*** such a person of ordinary skill in this art at that time) to believe the claimed invention would have been obvious. Please see Second Declaration of Professor Hernandez Under 35 U.S.C. § 1.132 submitted herewith. See also Dr. Hernandez's Curriculum vitae attached thereto as Exhibit A.

Applicants have identified several legal and factual errors with regard to the Examiner's discussion of the First Hernandez Declaration which are important to point out. First, the Examiner has improperly discounted the "opinion" evidence provided by Professor Hernandez. The Examiner mistakenly believes Professor Hernandez is providing "expert opinion." In fact, Professor Hernandez is providing the opinion of a person of ordinary skill in the art, which is different than an expert opinions. The opinion of a person of ordinary skill in the art at the critical time is actually a fact to be considered in an obviousness analysis. Prof. Hernandez was an Investigator at the Howard Hughes Medical Institute at the time working in the area of RNA and studying RNA polymerase III. See at least ¶¶ 3- 15 of Prof. Hernandez's Second Declaration, and Exhibit A. She was aware of Elbashir et al. at the time. Unlike a retrospective expert opinion, the Declaration is based on the personal knowledge of Prof. Hernandez testifying as a person of ordinary skill in the art at that time. The statement of Professor Hernandez is therefore factual evidence that must be taken into account and not expert opinion as discussed in the passage from the MPEP relied upon by the Examiner, MPEP 716.01(c).

Second, it appears that the Examiner has impermissibly heightened the standard when carrying out a patentability assessment under 35 U.S.C. § 103. The Examiner criticizes the reliance on Elbashir et al. because "there is no factual evidence in Elbashir or any of the other

references that expressly teach that dsRNAs having a duplex of less than 29 base pairs ***were not capable of*** mediating RNAi." (Emphasis added.) There is no requirement that a reference show ***incapability*** to be considered "teaching away." Furthermore, and equally importantly, a person of ordinary skill in the art, Prof. Hernandez, has testified that the Elbashir paper teaches away by discouraging one of skill from pursuing the claimed invention. The evidence is overwhelming that Elbashir et al. teaches away from the claimed invention. See ¶¶ 9-15 of Second Hernandez Declaration.

Third, rebuttal evidence can be submitted by way of a declaration and the entire situation regarding patentability must be reviewed in view of the new evidence. See 37 C.F.R. §1.132 and M.P.E.P. § 2141. In particular, whenever an applicant submits additional evidence, the Examiner must reconsider patentability of the claimed invention, and any decision to maintain a rejection must show it was based on the totality of the evidence. *Id.* "Facts established by the rebuttal evidence must be evaluated along with the facts on which the conclusion of obviousness was reached, not against the conclusion itself." M.P.E.P. 2142 (citing *In re Eli Lilly & Co.*, 902 F.2d 943 (Fed. Cir. 1990))(emphasis added). "Consideration of rebuttal evidence and arguments requires Office personnel to weigh the proffered evidence and arguments. Office personnel should avoid giving evidence no weight, except in rare circumstances." See *In re Alton*, 76 F.3d 1168, 1174-75, 37 USPQ2d 1578, 1582-83 (Fed. Cir. 1996).

Here, applicants have met their burden by providing rebuttal evidence via the Second Declaration of Prof. Hernandez, a person of ordinary skill in the art at the time, including her testimony on how a person of ordinary skill in the art would have viewed the state of the art and the non-obviousness of the claimed invention. Instead of taking the factual evidence contained in the First Hernandez Declaration into account, the Examiner characterizes the statements made by Prof. Hernandez in her First Declaration and data cited therein as "opinion." Prof. Hernandez is not offering an expert opinion, but is offering the opinion of a person who was a person of ordinary skill in the art, and thus the opinion of Prof. Hernandez is ***a fact*** that must be considered in carrying out a patentability assessment as to obviousness. Indeed, Prof. Hernandez is not "arguing" but is rather presenting evidence as a person of ordinary skill in the art at that time for the Examiner to consider. It is error to consider the content of the First (or Second) Hernandez Declaration as "opinion." The statements therein are facts that must be taken into account.

In particular, the Examiner erroneously asserts "there is no factual evidence provided in Elbashir or any of the other references that expressly teach that dsRNAs having a duplex of less than 29 base pairs were not capable of mediating RNAi." And further, erroneously asserts that "the evidence provided by Elbashir et al. does not teach away from using a dsRNA to mediate RNAi in mammalian cells as instantly claimed and importantly does not teach away from using a dsRNA of less than 29bp to mediate RNAi in any cell type." In this regard, Figure 1b of Elbashir reports that dsRNAs of 29bp and 30bp in length failed to mediate RNAi (bars indicating that the effect of both 29bp and 30bp was equivalent to controls). Further, there is no evidence whatsoever in Elbashir that dsRNAs shorter than 29bp were effective as pre-Dicer triggers. Such an inference has no scientific support. Moreover, "the expectation of one of ordinary skill in the art at the time, for example, in view of the conservation across species of the RNAi machinery (see Bernstein et al, Nature 409, 363-366 (2001)), was that the negative results provided by Elbashir et al. in insect cells would also apply to the use of short hairpin RNA in mammalian cells. It would have been backwards and contrary to the Elbashir paper's text for a person of ordinary skill in the art to interpret the negative results in Elbashir as providing any reasonable expectation that one could have achieved gene silencing by stably expressing a short hairpin RNA in mammalian cells." (See Second Hernandez Declaration, ¶ 15.) Instead, the Elbashir et al. paper and the state of the art "would have taught away from using short hairpin RNAs in mammalian cell types." (Id.)

A. Caplen et al. Does Not Make The Claimed Invention Obvious And Does Not Contradict The Results Shown In Elbashir et al.

Although the Examiner has not formally cited Caplen et al. as a reference under 35 U.S.C. § 103, the Examiner has relied upon Caplen et al. to "demonstrate[s] that small dsRNAs from 21-27 nucleotides in length can specifically inhibit gene expression in mammalian cells (see page 9744 and Figure 1)." (See Office Action, p. 5.) The Examiner believes that Caplen et al. "goes against what Professor Hernandez submits one of ordinary skill in the art would conclude from Elbashir et al…." The Examiner states that "based on Caplen et al. one of ordinary skill in the art would clearly have a reasonable expectation of success in using a dsRNA of less than 29 bp to mediate RNAi in mammalian cells and further provides factual evidence

that even in cells, such as C. elegans or Drosophila as taught by Elbashir, dsRNA of less than 29 bp are capable of efficiently mediating RNAi." (See Office Action, p. 5.)

In reply, Applicants traverse the position taken by the Examiner. In response to the view set out by the Examiner, Professor Hernandez has reviewed the statements made by the Examiner and has reviewed the Caplen et al. reference. (See Second Hernandez Declaration, ¶¶ 23-27.) Prof. Hernandez disagrees with the Examiner's view and points out that the Caplen paper reports results with regard to small inhibitory RNAs (siRNAs) and does not address short hairpin RNA structures at all. Caplen et al. report on experiments using siRNAs that are double-stranded RNAs having specific overhang structures that are designed to mimic the processed structure of siRNAs. In fact, the approach described by Caplen is the same approach that Elbashir describes in Figure 5 which shows use of synthetic 21- and 22-nt RNAs to mediate target RNA cleavage.

Professor Hernandez points out that the last sentence of the Introduction of the Caplen paper states that "[g]iven the observations that (*i*) 21-25-nt dsRNAs with a characteristic structure can mediate RNAi in cell extracts…." Professor Hernandez notes that the "characteristic structure" referred to by Caplen et al. is a double-stranded, non-hairpin, structure with a specific overhang structure specifically designed to mimic the processed structure of siRNAs. The overhang structure of the dsRNAs used in the experiments is specified on page 9744 as "(20 and 21 nucleotides base-paired with 2-nt 3' overhangs)" and in the text below Table 1: "dsRNA molecules were formed with each strand carrying a 5'-OP_4, 3"-OH, and 2-base 3' overhangs." (See Second Hernandez Declaration, ¶ 25.)

It is the opinion of Prof. Hernandez that the siRNA having the overhang structures described in Caplen et al. would have provided no insight or expectation that a different, short hairpin RNA structure would have mediated RNAi in a mammalian cell. In fact, Prof. Hernandez is of the view that the results in Caplen et al. would have ***taught away*** from the use of short hairpin RNAs. A person of ordinary skill in the art at the time, reading Caplen et al. would have been taught to use dsRNAs with specific overhang structures mimicking siRNAs, not short hairpins.

Finally, Caplen et al. do not disclose or teach the use of a "short hairpin RNA molecule is a substrate for Dicer-dependent cleavage" as is required by the claim language as currently amended (see claim 50). The siRNA's disclosed by Caplen et al. would not be recognized by Dicer as a substrate, and indeed, are the structure of post-Dicer products. For these reasons, Applicants maintain that the presently claimed invention is not obvious.

B. *Kreutzer et al. Does Not Make The Claimed Invention Obvious And Does Not Contradict The Results Shown In Elbashir et al.*

The Examiner has relied upon Kreutzer et al. to allegedly show "that a dsRNA 21 nucleotide base paired molecule was capable of efficiently reducing gene expression in mammalian cells (see Examples)." (See Office Action, p. 3.) The Examiner goes on to state that "this is direct evidence that one of ordinary skill in the art would have expected a dsRNA of less than 29 bp or having a double stranded region of at least 20 base pairs to be capable of mediating RNAi in mammalian cells." (See Office Action, p. 3.)

In reply, applicants traverse the Examiner's position and submit that the Kreutzer et al. reference would not have made obvious the use of a short hairpin RNA structure, having a double-stranded region consisting of at least 20 nucleotides but not more than 29 nucleotides as recited in the presently claimed invention. Indeed, Professor Hernandez has reviewed the Office Action and the Kreutzer et al. reference and is of the view that Kreutzer et al. would not have given a person of ordinary skill in the art a reasonable expectation of success that stably expressing a short hairpin RNA having a double-stranded region consisting of at least 20 nucleotides but not more than 29 nucleotides would attenuate gene expression in mammalian cells. (See Second Declaration of Professor Hernandez, ¶¶ 16-22.)

Prof. Hernandez points out that the "dsRNA" that Kreutzer describes (see [0069]) is a synthetic and chemically altered RNA molecule (synthons modified by disulfide bridges) comprised of single strands linked by a disulfide bridge. Prof. Hernandez is of the opinion, as a person of ordinary skill in the art at the time, that such a chemically altered species would not have provided any reasonable expectation of success with regard to how an unmodified dsRNA, or a hairpin RNA molecule that is expressed within a cell, would have affected gene expression. (See Second Hernandez Declaration, ¶ 18.)

Kreutzer provides no evidence that the chemically modified RNA structures are even processed through the RNAi pathway. Elbashir taught that to overcome the inability of the cellular RNAi machinery to process short dsRNA molecules into the 21-23nt (guide) siRNA mediating target gene suppression, one instead could directly introduce a dsRNA mimicking an siRNAs into the cell. (See Figure 5 of Elbashir.) In view of Elbashir, one of skill would have expected that a 21 bp dsRNA could therefore serve as an RNAi trigger without the need for processing. According to Professor Hernandez, to one of skill, such a result, however, would have provided no evidence or expectation that a hairpin RNA molecule with a 21 bp double-stranded region could mediate RNAi, in particular because to mediate RNAi, the hairpin RNA would first have to be processed into a dsRNA. Prof. Hernandez reconfirms that in view of Elbashir, a short hairpin RNA (having a double-stranded region of less than 29 bp or at least 20 base pairs) was capable of acting in such a way was, in fact, surprising and unexpected. (See Second Hernandez Declaration, ¶ 19.)

Prof. Hernandez also points out that the sole references Kreutzer makes to RNA hairpin structures are made in the context of addressing the problem of degradation of the dsRNA in the cell. To afford protection from degradation, Kreutzer et al. suggested use of chemically altered dsRNAs, generated through "chemical modification" of the dsRNA or by chemically modifying the nucleotides in the loop region of an RNA hairpin loop. See, for example, paragraph 19 of Kreutzer et al. Here, Kreutzer states "an RNA hairpin loop, in particular when using a vector according to the invention. To afford protection from degradation, it is expedient for the nucleotides to be chemically modified in the loop region between the double-stranded structure." Professor Hernandez states that the vector referred to and the fact that chemical modifications are proposed both indicate that this statement refers to an RNA produced *in vitro* which would then need to be delivered into cells. Professor Hernandez concludes from this disclosure that such a synthetic structure could ***not*** be expressed in a mammalian cell from the proposed vector. (See Second Hernandez Declaration, ¶ 20.)

Further, Professor Hernandez states that Kreutzer et al. would not have provided any reasonable expectation that one could have used the presently claimed methods to successfully suppress gene expression in a mammalian cell. (See Second Hernandez Declaration, ¶ 21.) The Examples in Kreutzer et al. do not show expression of a short hairpin RNA in mammalian cells.

Instead, the Example 1 shows *in vitro* transcription (*e.g.*, starting at paragraph 44); generation of double-stranded RNA by *in vitro* hybridization (*e.g.*, starting at paragraph 46). Similarly, Example 2 shows transfection (not stable expression) of dsRNA having a length of 315 bp (see Seq. I.D. No. 5 and paragraph 66) and microinjection of a chemically modified, synthetic, dsRNA of 21 bp (see Seq I.D. No. 8 and paragraph 69) into a murine cell line. The 21 bp dsRNA was not a hairpin, and was chemically modified and synthesized using solid state chemistry. In paragraph 69, Kreutzer et al. state: "A dsRNA linked chemically at the 3' end of the RNA as shown in sequence listing No. 8 to the 5' end of the complementary RNA via a C18 linker group was prepared (L-dsRNA). To this end, synthons modified by disulfide bridges were used." The paragraph goes on to describe solid support chemical methods used to carry out the chemical reactions needed to obtain the L-dsRNA. Professor Hernandez, after reviewing the above disclosures, is of the opinion that the disclosure of Kreutzer et al. would not have taught or made obvious to a person of ordinary skill in the art at the time the claimed methods of Hannon et al. because the Hannon methods require *in vivo* stable expression of a construct to express a short hairpin RNA having a double-stranded region consisting of at least 20 nucleotides but not more than 29 nucleotides.

Finally, Professor Hernandez notes that the final sentence of Kreutzer et al. clarifies the meaning of the results presented in Example 2. She points out that Kreutzer et al. state in paragraph 76 "[t]his result demonstrates that even shorter dsRNAs can be used for specifically inhibiting gene expression in mammals when the double strands are stabilized by chemically linking the single strands." Professor Hernandez, as a person of ordinary skill in the art concludes that, in this statement, a person of ordinary skill in the art at the time would have understood that: (1) *in vitro* transcription of single stranded RNAs was required by the method of Kreutzer et al.; (2) that solid state chemical modification of those single strands was required by the method of Kreutzer et al. (also a set of *in vitro* chemical steps); and (3) microinjection of chemically modified dsRNAs into mammalian cells was required. Professor Hernandez states that that none of these teachings would have made obvious the methods of Hannon et al.

In conclusion, applicants respectfully traverse the statements made by the Examiner as to the Caplen et al. reference and the Kreutzer et al. reference. These references do not contradict the teachings of Elbashir et al., and indeed are consistent with the teachings of Elbashir et al. (see

Figure 5 of Elbashir et al.). Kreutzer et al. and Caplen et al. would not make obvious the presently claimed invention which requires a very different structure -- a short hairpin RNA molecule wherein the double-stranded region consists of at least 20 nucleotides but not more than 29 nucleotides and wherein the short hairpin RNA molecule is a substrate for Dicer-dependent cleavage. Neither Kreutzer et al. nor Caplen et al. teach or make obvious such a hairpin structure.

III. Declaration Under 37 C.F.R. §1.131

Although it is the applicants position that none of the cited references anticipate or make obvious the claimed invention, the applicants have also submitted here with a Declaration Under 37 C.F.R. §1.131 which establishes conception of the invention at least prior to the dates of Caplen et al., Kreutzer et al., and Symonds et al., coupled with due diligence through to a subsequent reduction to practice. The invention claimed was conceived and reduced to practice at least prior to August 14, 2001, the publication date of Caplen et al., *PNAS*, Vol. 98, No. 17, pp. 9742-9747, which is also prior to December 28, 2001, *i.e.*, the filing date of U.S. Publication No. US 2002/0160393, Symonds et al., U.S. Serial No. 10/035,098 and which is also prior to the date of filing of the parent application of Kreutzer et al. (U.S. Serial No. 09/889,802, filed September 17, 2001).

A. §131 Declaration Establishes Invention Prior to August 14, 2001

Applicants have submitted a Declaration which establishes that the claimed invention was conceived prior to August 14, 2001 and was diligently reduced to practice. See attached Declaration Under 37 C.F.R. §1.131 and Exhibits A – Q.

Briefly, the Declaration presents evidence that the claimed invention was conceived in connection with work to identify a practical loss-of-function tools for probing gene function in mammalian cells. The work proposed by the inventors began with work to elucidate the mechanism of RNA interference. (See 131 Declaration, Exhibits A and B.) Certain aspects of this work were reported in Bernstein et al. *Nature* 409: 363-366 (2001) (131 Declaration, Exhibit C) in a paper entitled "Role for a bidentate ribonuclease in the initiation step of RNA

interference." This paper describes the identification and cloning of the enzyme, which the inventors named "Dicer."

In another grant, the inventors (131 Declaration, Exhibit D) set out experimental procedures for creating stable, loss-of-function mutations in embryonic cells using RNAi. On page 14, the grant states that "[w]e have chosen to approach this goal by encoding dsRNA in the form of an inverted repeat or hairpin that can be expressed from a promoter of choice." The grant also states that the inventors goal was " to devise strategies for presentation of the dsRNA trigger that allow it to elude PKR surveillance." The grant application (Exhibit D) on page 19 describes two methods for modifying the approach described in Aim 1 to "create hairpins with significantly shorter loops." The first is "to simply clone short hairpin sequences [either] as single, synthetic DNA fragments, and the second is to clone "in two steps if hairpin formation in such synthetic oligonucleotides competes too vigorously with intermolecular hybridization to produce clonable fragments." Furthermore, Figure 7 on page 21 of Exhibit D depicts the use of libraries of expression vectors expressing an encoded "dsRNA cassette" to carry out functional screens in cultured cells.

Illustrating the second cloning strategy are oligonucleotides that were ordered for use as PCR primers, which would generate an amplified PCR product comprising a sequence encoding a short hairpin (See 131 Declaration, Exhibit E). The amplified PCR product resulting from the PCR reaction is a double-stranded nucleic acid product that has a 28 nucleotide region of the target gene sequence, followed by a Hpa I restriction enzyme cleavage site, followed by the Zeomycin gene, followed by another Hpa I cleavage site, followed by the reverse complement of the 28 nucleotide region of the target gene. The PCR product is then cloned into an expression vector using Zeomycin selection. The vector is then digested using the HpaI restriction enzyme, resulting in a vector encoding a short hairpin consisting of (a) the target gene sequence, (b) a loop consisting of a HpaI restriction enzyme cleavage site and (c) the reverse complement of the target gene sequence. The HpaI site facilitates selection of positive bacterial clones, *i.e.*, those transformed with the expression vector, from which the desired expression vector may be purified according to standard plasmid purification methods. Notably, among the target sequences shown in these examples are those directed to a human gene, human hypoxanthine—

guanine phosphoribosyl transferase (HGPRT1 and HGPRT2 primers (See 131 Declaration, Exhibits D-F.)

The 131 Declaration includes experimental results that assessed the ability of various short hairpin RNAs to specifically suppress gene expression in mammalian cells known to exhibit a PKR response, but without provoking a PKR response. (See 131 Declaration, Exhibit G.) The bar graph shows a short hairpin RNA that has a double stranded region of 25 nucleotides in length inhibits target gene expression in human cells. As the nomenclature indicates, the double-stranded region of this short hairpin RNA molecule has a sequence that is complementary to a portion of the target gene, firefly luciferase. The bar graph shows, as a result of the experiment, a specific suppression of firefly luciferase gene expression in the 293T cells. See the bar labeled "SHP 25 luc hp" on the graph in Exhibit G. The 131 Declaration includes similar results in human HeLa cells (Exhibit H), *Drosophila* S2 cells (Exhibit I), and human 293T cells (Exhibit J).

The work described in the 131 Declaration was published. One paper was published in *Genes and Development* in March 2002 entitled "Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells." (See Exhibit K.)

Finally, as mentioned above in this response, since the inventors made the claimed invention and published Paddison et al., (Exhibit K), the invention of using stably expressed short hairpin RNAs to inhibit gene expression in mammalian cells has been recognized by industry organizations. (See 131 Declaration, Exhibits M-Q.)

B. Caplen et al., Kreutzer et al. and Symonds et al. Not Prior Art

Accordingly, the Caplen et al. reference is not prior art and should be withdrawn as a reference. It has a publication date on its face of August 14, 2001, and the §131 Declaration establishes a reduction to practice at least prior to this date.

Similarly, the Kreutzer et al. publication, which the Examiner has alleged has a 102(e) date of September 17, 2001 is not prior art. Applicants respectfully request the Examiner to withdraw any reliance on this reference.

Finally, the Symonds et al. publication, which is only entitled to the effective date of December 28, 2001, as discussed more fully hereinbelow, is not prior art. Applicants respectfully request that the Examiner withdraw any reliance on this reference.

IV. Obviousness-Type Double Patenting

The Examiner rejected the pending claims over co-pending application U.S. Serial No. 10/997,086.

In reply, applicants request that the Examiner hold this rejection in abeyance since the '086 application is not yet allowed. Applicants will provide a Terminal Disclaimer when one of the applications, either the '086 or the '676 is deemed allowed by the Examiner.

V. Rejection Under 35 U.S.C. § 103 over Symonds, Lieber, Fire, Good and Noonberg

The Examiner rejected claims 50, 52, 54-60 and 62 and 63 as allegedly obvious in view of Symonds et al. (US 2002/0160393, "the Symonds '393 publication"), Lieber et al., Fire et al. (USPN 6,506,599), Good et al., and Noonberg et al. The Examiner has added the Symonds '393 publication as the primary reference in this obviousness rejection.

In reply, applicants respectfully traverse the rejection. Applicants request reconsideration of the Examiner's position alleging that the claims are obvious. The Examiner (1) bases her rejections on clear errors of fact in the technical differences between the cited art and the claimed invention, (2) ignores critical evidence presented in the First Declaration of Prof. Hernandez, and (3) lacks a proper basis for finding a reasonable expectation of success, based on the totality of the evidence in the record. The rejection under 35 U.S.C. §103 is discussed below. In addition, a Second Declaration from Prof. Hernandez is submitted which addresses the Examiner's comments and the references relied upon by the Examiner. Finally, the applicants have submitted a Declaration Under 37 C.F.R. §1.131 which shows that the Symonds '393 publication is not proper prior art.

Applicants have shown above that the Symonds '393 publication is not proper prior art because Applicants have shown their invention was made prior to the effective date of Symonds

et al. (i.e., the filing date of the Symonds '393 publication which is December 28, 2001). Assuming *arguendo* that the Symonds '393 publication is properly citable as a basis for a rejection under 35 U.S.C. §103, Applicants have set out below reasons for non-obviousness based on the reference itself. The combination of the Symonds '393 publication, Lieber, Fire, Good and Noonberg would not make the claimed invention obvious to one of ordinary skill in the art at the time. Additionally, there is no motivation to combine the Symonds '393 publication with Leiber, Fire, Good and Noonberg since the Symonds '393 publication refers to RNA molecules including ribozymes or sequence encoding HIV Tat protein as discussed more fully hereinbelow.

A. The Claimed Invention

The claims are directed to methods as illustrated by claim 50 reproduced below (incorporating the amendments introduced in this paper). Applicants have bolded and underlined certain sections of the claim in order to emphasize parts of the claim which are discussed in the response below:

> 50. A method for attenuating expression of a target gene in a mammalian cell, the method comprising
>
> introducing into mammalian cells a library of RNA expression constructs, each expression construct comprising:
>
> (i) an RNA polymerase promoter, and
>
> (ii) a sequence encoding a short hairpin RNA molecule comprising **a double-stranded region** wherein the double-stranded region **consists of**[2] **at least 20 nucleotides but not more than 29 nucleotides**,
>
> **wherein the short hairpin RNA molecule is a substrate for Dicer-dependent cleavage** and does not trigger a protein kinase RNA-activated (PKR) response in the mammalian cell,

[2] Applicants note that, on page 10 of the August 30, 2010 Office Action in the last paragraph, the Examiner summarizes the claimed invention. In that summary, the Examiner states that the claims are drawn to a method of attenuating expression of a target gene "wherein the shRNA **comprises** at least 20 but less than 29 nucleotide double stranded region…." (Emphasis added.) Applicants note that the language in the claim was and is "consists of" and not "comprises."

> wherein the double-stranded region of the short hairpin RNA molecule comprises a sequence that is complementary to a portion of the target gene, and
>
> wherein the **short hairpin RNA molecule is stably expressed in the mammalian cell in an amount sufficient** to attenuate expression of the target gene in a sequence specific manner, **and is expressed in the cell without use of a PK inhibitor,** whereby expression of the target gene is inhibited.

Applicants traverse the rejection, and summarize below the reasons for non-obviousness which are each discussed more fully below:

(1) the Symonds '393 publication is ***not entitled to claim priority*** to the December 28, 2000 filing date of the two provisional applications (USSN 60/258,731 provisional and USSN 60/258,733 provisional);

(2) the Symonds '393 publication is ***not proper prior art*** in view of the Declaration Under 37 C.F.R. §1.131 submitted herewith;

(3) the '731 Symonds provisional, upon which the Examiner relies, does not disclose a vector expressing a short hairpin RNA molecule ***without*** the presence of an RNA molecule encoding HIV Tat protein;

(4) the Symonds '393 publication does not disclose or make obvious the required structure of the shRNA of the claimed invention, *i.e.*, that the shRNA comprises "a double-stranded region wherein the double-stranded region ***consists of at least 20 nucleotides but not more than 29 nucleotides***…;"

(5) the Symonds '393 publication does not teach or make obvious a short hairpin RNA molecule as claimed by applicants wherein the shRNA is ***a substrate for Dicer-dependent cleavage***; and

(6) the Symonds '393 publication is ***not enabled***.

B. The Symonds '393 Publication Is Not Entitled to It's Provisional Priority Date

The Examiner relies upon the Symonds '393 publication as a primary reference to allegedly support an obviousness rejection. The Symonds '393 publication has a filing date of December 28, 2001, and claims priority to two provisional applications both filed on December 28, 2000. The Examiner specifically relies upon paragraphs 108-114, Fig. 8A, paragraph 136 and Fig. 9 of this publication. The Examiner asserts that the disclosure in the priority application USSN 60/258,731, filed December 28, 2000 at least on pages 5, 11 and Figure 2A provides the same teachings and therefore the effective date of the '393 Symonds publication is December 28, 2000. Applicants respectfully disagree.

The '731 application does not contain supporting description and therefore, the disclosure upon which the Examiner relies in the Symonds '393 publication (paragraphs 108-114, 136 and 158 and Figure 8A) does not have proper priority to the provisional application. The Examiner relies on pages 5, 11 and Figure 2A of the '731 application. Pages 5 and 11 recite generally "a linear RNA molecule" which includes "a portion encoding HIV Tat protein." See lines 3-5 on page 5 of the '731 provisional. The lower portion of page 5 recites a composition that requires "(a) an RNA molecule encoding HIV Tat protein" and (b) a linear RNA molecule…." The '731 provisional only discloses a linear RNA molecule as ***always requiring*** an RNA encoding HIV Tat protein.

Figure 2A appears to be the same cartoon as in Figure 8A of the published application. However, if one looks through the '731 provisional application to determine what the "instant RNA molecule" is, one finds that the intervening sequence shown in Figure 2A of the provisional is described to have certain characteristics far afield from the invention claimed in this application. The instant RNA molecule is always described as including or associated with RNA encoding HIV Tat protein.

In particular, page 5 of the '731 application recites the "Summary of the Invention" and recites a "linear RNA molecule" that requires "a portion encoding HIV Tat protein." The present invention has nothing to do with HIV Tat protein. Symonds does not describe the presently claimed short hairpin RNAs. Moreover, as presently claimed, the short hairpin RNA is stably expressed in the cell without use of a PKR inhibitor, such as, for example, HIV Tat protein.

Furthermore, on page 11 the '731 application there is more disclosure of "linear RNA molecules" which require "a portion encoding HIV Tat protein." Page 11 also states that "[t]he invention is based on the ability of HIV Tat protein to inhibit the cellular breakdown of double-stranded RNA complexes…." This is not relevant to the presently claimed invention. The last paragraph on page 11 refers to "the length of the instant linear RNA molecule…" which "must be sufficient to give rise to a dsRNA complex that is at least about 20 nucleotides in length." A "dsRNA complex" is defined on page 9 and this can be formed by either two RNA molecules or one RNA molecule. The preferred embodiment is the complex formed from two separate molecules. The lengths of the "instant linear RNA molecule" as described at the bottom of page 11 all require the presence of "a portion encoding HIV Tat protein" and therefore are teaching away from the claimed invention in this application. The length of the "dsRNA complex" is (a) a very large range of lengths "at least about 20 nucleotides in length, and (b) requires that the RNA encode HIV Tat protein. This is a clear difference in the disclosure and is not a proper priority document for the '393 Symonds publication. Indeed, the title of the provisional application is "TAT-based Methods for Facilitating Double-Stranded RNA Mediated Gene Suppression." The claimed invention has nothing to do with "TAT-based methods."

The '733 provisional also does not provide support. For example, on page 9 of the '733 application there is a recitation at lines 18-22 which require that the "the third sequence situated between the first and second sequences so as to permit the first and second sequences to hybridize with each other, which third sequence comprises (i) a ribozyme and (ii) a target sequence which is specifically recognized by the ribozyme and is absent in the first and second sequences…." There is no description in this '733 provisional application that provides for a hairpin loop structure where the hairpin does not have a ribozyme characteristic. This entire disclosure is about "ribozyme-containing RNA molecules." The Examiner neglects to appreciate this overall aspect of the disclosure of the Symonds '733 provisional application. The disclosure in the published Symonds et al. application upon which the Examine relies in paragraphs 108-114, 136 and 158 does not find support in the '733 provisional application as filed on December 28, 2000.

Professor Hernandez has reviewed the two provisional applications and the Symonds '393 publication and concludes that disclosure in the '393 publication does not find support in either of the two provisional applications. See Second Hernandez Declaration, ¶ 37.

Applicants submit that neither of the priority applications USSN 60/258,731 or USSN 60/258,733 supports the disclosure upon which the Examiner relies in her rejection which appears in the U.S. Pub. No. 2002/0160393 and therefore is not entitled to the priority date of December 28, 2000.

C. Symonds et al. Is Not Prior Art In View of §131 Declaration

As discussed above, the proper effective date of the disclosure of the '393 Symonds publication is December 28, 2001. Therefore, based on the Declaration Under 37 C.F.R. § 1.131 submitted herewith which establishes conception of the invention prior to December 28, 2001 coupled with due diligence from prior to said date to a subsequent reduction to practice, Symonds et al. is not prior art and should be withdrawn as a reference.

D. Symonds et al. Does Not Make Obvious the Invention Claimed

Although it is Applicants' position that Symonds et al. is not entitled to the benefit of the '731 or '733 provisional filing date for the reasons stated above, Applicants also submit that the Symonds '393 publication does not make obvious the claimed invention.

First, the '731 and '733 Symonds provisional applications do not disclose a vector expressing a short hairpin RNA molecule ***without the presence of a PK inhibitor***, such as an RNA molecule encoding HIV Tat protein or a ribozyme structure. Each of the provisional applications ('731 and '733) require there to be some additional structure as part of or associated with a "linear RNA molecule." The '731 provisional only discloses use of a linear RNA molecule that has as part of the molecule RNA that encoding HIV Tat protein, or has in association with a linear RNA molecule another RNA molecule that encodes HIV Tat protein. There is no disclosure of a short hairpin RNA and use of a short hairpin RNA as presently claimed. The presently claimed invention requires that the "short hairpin RNA molecule is a substrate for Dicer-dependent cleavage…" (See claim 50.) The linear RNA molecule encoding

HIV Tat or including a ribozyme type structure as disclosed by Symonds would not be a suitable substrate for Dicer-dependent cleavage.

Second, the Symonds '393 publication does not disclose or make obvious the required structure of the shRNA of the claimed invention, *i.e.*, that the shRNA comprise "a double-stranded region wherein the double-stranded region ***consists of at least 20 nucleotides but not more than 29 nucleotides***…." (See claim 50.) The '393 publication at ¶ 136 states "[t]he length of the instant linear RNA molecule must be sufficient to give rise to a dsRNA complex that is at least about 20 nucleotides in length." The definition of "double-stranded RNA complex" in the publication requires that the "two portions of a linear RNA molecule which are complementary to, and are capable of or have therefore hybridized to, each other." (See ¶ 0095 of the '393 publication.) The definition of "hybridizing conditions" in the publication "shall mean conditions permitting hybridization between two complementary strands of RNA having a length of at least seven nucleotides." (See ¶ 0096 of the '393 publication.) Therefore, the range of lengths of dsRNA complexes encompassed by the '393 publication is large in that it only requires there to be 7 nt's that are hybridizing to meet the term double-stranded RNA complex. Apparently, there can be much non-hybridization in the dsRNA complex if the lengths are as long as disclosed in ¶ 00136. In these cases, the lack of hybridization along the double-stranded region would likely cause the dsRNA complex of Symonds to ***not be recognized by Dicer as a substrate***. Therefore, the Symonds '393 publication does not make obvious the specific length requirement of the double-stranded region of the claimed invention, nor does it make obvious the structural requirement that the shRNA be a suitable substrate for Dicer-dependent cleavage. In particular, given the state of the art, the skilled artisan would not have expected that an RNA hairpin having a double-stranded region of 20 to 29 nucleotides in length would undergo processing to an siRNA or would be effective in triggering sequence specific gene attenuation through RNAi.

E. Professor Hernandez, as a Person of Ordinary Skill in the Art as of January 22, 2002, States that Symonds Would Not Have Made Obvious the Claimed Invention Alone or Combined with the Other Cited References

As evidence of the non-obviousness of the claimed invention, Applicants have submitted a Second Declaration under 37 C.F.R. § 1.132 from Professor Hernandez. Prof. Hernandez has

reviewed the previous Office Action and the Symonds '393 publication, including the two Symonds provisional applications. See Second Hernandez Declaration, ¶¶ 28-43. As Prof. Hernandez states, it would not have been obvious to one of ordinary skill in the art at the time of the invention that one could attenuate target gene expression in a mammalian cell by introducing an expression construct encoding a short hairpin RNA molecule having a double-stranded region of 20-29 nucleotides in view of these documents. Indeed, according to Prof. Hernandez it was unexpected in view of the state of the art at the time that the claimed method would result in effective target gene attenuation, and one of ordinary skill at the time of the invention would have had no reasonable expectation that it would do so.

Professor Hernandez concludes that a person of ordinary skill in the art would have believed that the Symonds provisional applications and the '393 publication would teach away from invention claimed by Hannon et al. because Symonds require the presence of a PK inhibitor, such as HIV Tat protein or a ribozyme structure. Indeed, the shRNA approach claimed in the present Hannon application requires that the vectors are expressed in a mammalian cell ***without the use of a PK inhibitor***. Therefore, Symonds et al. teach away from the invention claimed by Hannon et al. Additionally, Professor Hernandez states that there are a vast number of lengths of the first and second strands of the dsRNA complex as set out by Symonds and there is no reason to choose one length over any other length. Accordingly, it is Professor Hernandez's opinion that the Symonds '393 publication would not make the claimed invention obvious to a person of ordinary skill in the art.

Professor Hernandez states that the claimed requirement that the short hairpin RNA molecule is a substrate for Dicer-dependent cleavage is not described in the '733 application and that the linear molecules described in the '733 application include a sequence corresponding to a ribozyme and would not make obvious the invention of Hannon et al. Indeed, Prof. Hernandez concludes that Symonds teaches away from the claimed invention. (See Second Hernandez Declaration ¶ 30.) As to the '731 provisional application, Prof. Hernandez states that this provisional application describing use of Tat protein of HIV to suppress or inhibit the PKR pathway is "entirely different from and teaches away from the solution described in Dr. Hannon's application in expressing short hairpin RNAs that do not elicit a PK response." (See Second Hernandez Declaration, ¶ ¶31-33.) Prof. Hernandez states that the disclosure of the '731

application upon which the Examiner relies provides "no qualification about whether any one length would work better than any other length, so it would not have been obvious to a person of ordinary skill in the art at the time which length to choose." (See Second Hernandez Declaration, ¶ 34.) In addition, Prof. Hernandez states in para. 35 of her Second Declaration that the definition of "hybridizing conditions" in the '731 application "adds to the vast number of choices for length of the first and second strands provided for in the '731 application." Prof. Hernandez concludes again that "this disclosure does not teach nor make obvious to a person of ordinary skill in the art the invention claimed by Dr. Hannon et al."

Professor Hernandez has reviewed the two Symonds provisional applications and the '393 publication and she sets out specific reasoning in paragraphs 28-43 for why the '393 publication fails to describe the short hairpin approach claimed by Dr. Hannon et al. and that the '393 publication teaches away from claimed methods.

F. The Symonds '393 Publication Is Not An Enabling Disclosure for the Claims Recited Therein

The Symonds '393 publication is the published application of USSN 10/035,098. The '098 application is abandoned and there are no continuations or divisionals filed which claim priority to it. Symonds received a Non-Final Office Action and a Final Office Action both of which set out rejections as the invention not being enabled by the application. Symonds et al. did not reply to the Final Office Action and permitted the application to become abandoned without arguing further against the rejection set out by the Patent Office that the specification was not enabling for the claims. The Symonds '393 publication would not have taught one of skill in the art that the method of negatively altering gene expression is operable. (See discussion in the Final Office Action from the '098 file history, at page 4.) Thus, the Symonds '393 publication is not an enabling disclosure for the claims recited therein.

G. Secondary references Lieber, Fire, Good and Noonberg Do Not Remedy the Deficiencies of the Symonds '393 Publication Primary Reference

The Examiner has combined the Symonds '393 publication with four secondary references in order to set out the rejection of the claims under 35 U.S.C. § 103. None of the secondary references alone or in combination with any other, or all other, of the secondary

references remedy the deficiencies of the Symonds '393 publication. There is no motivation to combine these specific five references together. The Examiner has implemented a hindsight analysis in order to group together references that, in a piecemeal way, allegedly disclose disparate elements of the claimed invention. Indeed, as discussed above there are a number of requirements of the claimed invention that the Symonds '393 publication do not disclose or make obvious. The combination of the Symonds '393 publication with Lieber, Fire, Good and Noonberg does not remedy these deficiencies.

Leiber discloses a ribozyme library comprising a collection of ribozyme genes encoding a hammerhead structure and flanking sequences of random nucleotides cloned at least once into an expression cassette for ribozyme expression (see claim 1). The ribozymes used are "from a selection of ribozymes with known stability and structure." (See Description of the Invention.) The structure of a ribozyme, having a hammerhead shape, is very different than a short hairpin RNA. A ribozyme structure would not be a substrate that would be recognized by the Dicer enzyme and thus would not make obvious the requirement of the presently pending claims. Indeed, the Leiber references warns of "an unpredictable effect on the folding" that can occur from different ribozyme genes being expressed in the library. (See 3rd paragraph in Detailed Description of the Invention.) Therefore, there would be no motivation for a person of ordinary skill in the art to combine the libraries described in Leiber with the Symonds '393 publication since unpredictable folding would result in unpredictable structures in the end.

Just as the Symonds '393 publication teaches away from the presently claimed invention due to the focus on ribozyme-like structures and association with RNA encoding the HIV Tat protein, the combination of Symonds '393 publication with Leiber enhances that teaching away since this combination teaches even more so the use of a ribozyme-like structure. Such a structure would not make obvious, and indeed teach away from, at least the following requirements of the claimed invention. This combination would teach away from the requirement of "wherein the short hairpin RNA molecule is ***a substrate for Dicer-dependent cleavage***…" (emphasis added). A ribozyme would not be cleaved by Dicer. (See Chakravarthy et al. (Epub 2010 Oct 13) "Substrate-specific kinetics of dicer-catalyzed RNA processing," *J Mol Biol.* 2010 Dec 3;404(3):392-402.) The combination would also teach away from the claim requirement that the shRNA is expressed ***without use of a PK inhibitor***. The use of a ribozyme

or HIV Tat protein as taught by Symonds teaches away from this aspect of the claimed invention as well.

The addition of Fire to the combination of the Symonds '393 publication and Lieber does not remedy the above-mentioned deficiencies. The combination including Fire does not make obvious shRNAs with the double-strand region length requirement that is presently claimed. The combination including Fire does not disclose or make obvious that the "the short hairpin RNA molecule is ***stably expressed*** in the mammalian cell in an amount sufficient to attenuate expression of the target gene in a sequence specific manner…" (emphasis added) as required by applicants' claims.

Combining the Symonds '393 publication with Lieber, Fire, Good and Noonberg does not remedy the deficiencies set out above. The Examiner states that Good teach an expression construct comprising a U6 promoter. The mere teaching of a U6 promoter and an expression construct does not remedy the issues applicants discuss above as to Fire, Lieber and the '393 Symonds publication. The Examiner also points to columns 7-8 of Noonberg which generally describes an "in vivo oligonucleotide generator." There is no disclosure of the many other claimed characteristics of the present invention in either Good or Noonberg. See Noonberg at 7:26-27. Furthermore, there is no motivation to combine either the Noonberg or Good document specifically with any of the other four references.

The Examiner is using hindsight to fill in the missing gaps in the Symonds '393 publication. The five references together do not disclose or make obvious the specific requirements of the claimed invention. For example, the five references together do not make obvious ***stable expression*** in a mammalian cell of short hairpin RNAs comprising a double-stranded region wherein the double-stranded region ***consists of at least 20 nucleotides but not more than 29 nucleotides*** as required by the present claims. Nor do they made obvious the use of a shRNA that is ***a substrate for Dicer***. Furthermore, they do not make obvious where the shRNA is expressed ***without a PK inhibitor***. Indeed, the combination of all five references would not have been made by one of ordinary skill in the art because there would have been no motivation to combine a reference discussing ribozyme or HIV Tat encoding dsRNAs (the '393 Symonds publication), with a ribozyme library (Leiber), with attenuating gene expression with

dsRNA generally (Fire), with an *in vivo* oligonucleotide generator (Noonberg), and with generally a U6 promoter (Good). The Examiner seems to have used hindsight to supplement her rejection with piecemeal references to attempt to find all of the claimed elements of the present claims. Applicants maintain that the combination does not set out a prima facie case of obviousness, nor does it render obvious the claimed invention. Prof. Hernandez has also reviewed these secondary references states that the combination of Symonds, Lieber, Fire, Good and Noonberg would not have made the claimed methods obvious to a person of ordinary skill in the art at the time with a reasonable expectation of success. See Second Hernandez Declaration at paras. 42 and 43. Applicants respectfully request that the Examiner reconsider and withdraw this ground of rejection.

In sum, applicants assert that the claims are not rendered obvious by the combination the Symonds '393 publication, Lieber, Fire, Good and Noonberg, that there is no motivation to combine these references, and that the evidence provided in the First and Second Declarations from Prof. Hernandez supports a finding of non-obviousness. Applicants respectfully request the Examiner to reconsider and withdraw this ground of rejection.

CONCLUSION

Consideration of this paper and allowance of this application are requested. If it would advance prosecution, the Examiner is invited to contact the undersigned to discuss the contents of this paper.

Dated: January 31, 2011

Respectfully submitted,

/Jane M. Love, Ph.D./

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Hannon et al.	Confirmation No.:	8161
Application No:	11/894,676	Art Unit:	1635
Filed:	August 20, 2007	Examiner:	K. CHONG
Title:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Docket No.: 287000-130-US3

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Declaration Under 37 C.F.R. §1.131

We, Gregory J. Hannon, Patrick J. Paddison, Scott Hammond, Amy Caudy and Emily Bernstein, Douglas Conklin hereby declare as follows:

1. We are the inventors of the above-referenced patent application.

2. All the work described within this declaration was performed in the United States.

3. All of the work described within this declaration was performed by us, or on our behalf and under our direction.

4. We have reviewed our records, including the slides documents submitted herewith, and declare that the claimed invention, which is

> a method for attenuating expression of a target gene in a mammalian cell, the method comprising introducing into a mammalian cell a library of RNA expression constructs, each expression construct comprising:
>
> (i) an RNA polymerase promoter, and

> (ii) a sequence encoding a short hairpin RNA molecule comprising a double-stranded region wherein the double-stranded region consists of at least 20 nucleotides but not more than 29 nucleotides,
>
> wherein the short hairpin RNA molecule is a substrate for Dicer-dependent cleavage and does not trigger a protein kinase RNA-activated (PKR) response in the mammalian cells,
>
> wherein the double-stranded region of the short hairpin RNA molecule comprises a sequence that is complementary to a portion of the target gene, and
>
> wherein the short hairpin RNA molecule is stably expressed in the mammalian cell in an amount sufficient to attenuate expression of the target gene in a sequence specific manner, and is expressed in the cell without use of a PK inhibitor,
>
> whereby expression of the target gene is inhibited

including original (and amended) claims 50, 52, 54-63 was conceived and reduced to practice at least prior to August 14, 2001, the publication date of Caplen et al., *PNAS*, Vol. 98, No. 17, pp. 9742-9747, which is also prior to December 28, 2001, *i.e.*, the filing date of U.S. Publication No. US 2002/0160393, Symonds et al., U.S. Serial No. 10/035,098 and which is also prior to the date of filing of the parent application of Kreutzer et al. (U.S. Serial No. 09/889,802, filed September 17, 2001).

A. Hannon Draft Grant Application

5. We attach a copy of a draft grant application (**Exhibit A**) which was prepared prior to August 14, 2001. A review of email indicates that this draft grant application was prepared at least by sometime in January 2000. The specific aims, as indicated on the first page of the draft grant application (**Exhibit A**, page 12), were directed to identifying and characterizing the critical components of the RNA interference (RNAi) machinery. The "Preliminary Results" this page refers to (see 4th paragraph on page 12) were reported in Hammond et al., *Nature* 404:293-

296 (2000) (**Exhibit B**) in a paper entitled "An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophia* cells."

6. In particular, one aspect of the proposed work was directed to isolating and cloning the protein and RNA components of the RISC complex (RNA-induced silencing complex), the nuclease complex responsible for degradation of target mRNAs, and characterizing it's function, both *in vitro* and *in vivo*. To allow us to carry out such studies, we established a model system using cultured *Drosophila* cells that provided a readily available source of material in sufficient quantities for the necessary biochemical studies.

7. The Summary on page 15 provides the rationale for the proposed work:

> My laboratory has devoted a number of years to creating improved tools for probing gene function in cultured mammalian cells; however, our experience indicates that a facile loss-of-function tool is lacking. Unfortunately, dsRNA induces somewhat generic responses in mammalian cells. It is our hope that by understanding the mechanistic basis of dsRNA-induced silencing, we may not only unravel a mysterious and important piece of biology but also provide the means to create improved tools for analyzing gene function in diverse organisms in which traditional genetic methods are either cumbersome or unavailable. This notion that has contributed to the decision to focus substantial effort in my laboratory toward elucidating the mechanism of RNA interference.

The final paragraph on page 36 further elaborates on this rationale:

> In this application, we propose a biochemical approach to deciphering the mechanisms that underlie dsRNA-induced gene silencing. RNA-interference allows an adaptive defense against both exogenous and endogenous dsRNAs, providing something akin to a dsRNA immune response. The primary goal of the work proposed in this application is to understand the mechanisms by which a cell can raise this response. We have presented evidence that RNA interference is accomplished, at least in part, through the action of a sequence-specific nuclease that is generated in response to dsRNA. Our data, and that of others (Hamilton and Baulcombe, 1999), is consistent with a model in which dsRNAs present in a cell are converted, in a manner analogous to antigen processing, into discrete, small RNAs that guide the nuclease in the choice of substrate.

> We propose to purify and characterize the nuclease and to clone the protein and RNA components of the enzyme. In addition, we propose to develop approaches that may allow the use of cultured *Drosophila* cells as a general tool for probing gene function. The combination of these studies may lead eventually to an ability to harness RNA interference as a genetic tool in other organisms, particularly mammals, in which analogous tools are presently lacking.

8. At that time, there was a lack of available practical loss-of-function tools for probing gene function in mammalian cells. The work proposed in this draft application to elucidate the mechanism of RNA interference was intended to develop such tools. In other words, by understanding the mechanistic basis of RNA interference, we could use that understanding to exploit the RNAi pathway and create new tools to study gene function and the lack of certain gene function in mammalian cells.

9. The work proposed in this application to identify and characterize components of the RNAi cellular machinery was carried out by us prior to August 14, 2001. Certain aspects of this work were reported in Bernstein et al. *Nature* 409: 363-366 (2001) (**Exhibit C**) in a paper entitled "Role for a bidentate ribonuclease in the initiation step of RNA interference." This paper describes the identification and cloning of the enzyme, which we named "Dicer." The paper describes how this enzyme is evolutionarily conserved in worms, flies, plants, fungi and mammals, and the paper reports the role of this newly discovered enzyme in the RNAi pathway in cells. In particular, these results indicated that the process of gene silencing through the RNAi pathway could be divided into at least two distinct steps. In the first step, long dsRNA (double-stranded RNA) is processed by Dicer into approximately 22 nt (nucleotide) "guide" sequences. In the second step, these guide RNAs are incorporated into a distinct nuclease complex we first called the "RNA-induced silencing complex" or RISC. The RISC complex uses the guide sequences to specifically identify and destroy homologous mRNAs. We named the RNAs that

were processed by Dicer "guide sequences" or "guide RNAs" based on their role in targeting RISC to specific mRNAs based on sequence. The results and work described in Bernstein et al. (2001) were included in this patent application, U.S. Serial No. 11/894,676, and also in the related application U.S. Serial No. 10/055,797, such as in Example 2.

B. Draft SBIR Grant Application

10. We attach as **Exhibit D** a copy of a draft grant application to SBIR (Small Business Innovation Research) which was prepared prior to August 14, 2001.[1]

11. The first page of this draft grant lists three Aims directed toward achieving stable gene silencing in mammalian cells. Aim 1 is the "creation of stable, loss-of-function mutations in embryonic cells using RNAi." Aim 2 is the "creation of stable loss-of-function mutations in non-embryonic cell types," which proposes "numerous strategies for bypassing [the] problem" that "long dsRNAs provoke a PKR response in differentiated cell types."

12. Attached pages 13-25 of **Exhibit D** provide more detail regarding each of these Aims. Starting on page 13, the grant application describes the Experimental Procedures for Aim 1. Aim 1 is defined as "Creation of stable, loss-of-function mutations in embryonic cells using RNAi." On page 14, the grant states that "[w]e have chosen to approach this goal by encoding dsRNA in the form of an inverted repeat or hairpin that can be expressed from a promoter of choice." Regarding this objective, on page 14 the draft grant states that "[w]e have achieved the goal of simplified hairpin construction by dividing the process into two steps (Fig. 6)." Figure 6 is on page 15 and depicts a "strategy for the creation of hairpin RNAs for stable expression of dsRNA" and illustrates that "expression of a GFP hairpin RNA induced stable silencing of an

[1] For convenience, we have added page numbers to this document.

exogenous GFP reporter in [mouse embryonic] P19 cells." The use of the strategy and also the results described in Aim 1 are described in Example 3 (entitled "A Simplified Method for the Creation of Hairpin Constructs for RNA Interference") and Fig. 27, and in Example 4 (entitled "Long dsRNAs Suppress Gene Expression in Mammalian Cells") and Figs. 28-34 of the parent application, U.S. Serial No. 10/055,797. Aim 1 also describes silencing mammalian genes for which assays are available to allow "positive selection for loss-or function" in mammalian cells, *e.g.*, HPRT and TK. (See 2nd paragraph on page 16 of **Exhibit D**.)

13. The grant application states the goals for Aim 2 on the top of page 18 of **Exhibit D**: "our goal is to devise strategies for presentation of the dsRNA trigger that allow it to elude PKR surveillance." The "Expression Strategies" provided in the grant state that "PKR requires approximately 30 bp of contiguous double-stranded sequence to trigger dimerization and activation of the enzyme." (See first paragraph under "Expression Strategies" on page 18. The third paragraph in that section on page 18 describes expression of hairpin RNAs in various mammalian cells: "NIH 3T3, 293, HeLa, U2OS, Rat 1 and C2C12" and various expression vectors incorporating various promoters, including U1, U6 and CMV.

14. In the section entitled "Short RNA hairpins" on page 19 of **Exhibit D**, the grant application describes use of short RNA hairpins that are "below the cut-off for triggering RNA for investigating "whether the expression of short RNA hairpins can be used to induce efficient silencing." The research plan here also refers to "short synthetic RNAs that mimic our Dicer products." In other words, this refers to RNAs that have a double-stranded region of 20 to 22 base pairs. It further states that "short synthetic hairpins directed against GFP, TK and HPRT will be expressed from CMV, U1 and U6 promoter vectors in the cell types noted above." (See page 19.)

15. The grant application on page 19 describes two methods for modifying the approach described in Aim 1 to "create hairpins with significantly shorter loops." The first is "to simply clone short hairpin sequences [either] as single, synthetic DNA fragments, and the second is to clone "in two steps if hairpin formation in such synthetic oligonucleotides competes too vigorously with intermolecular hybridization to produce clonable fragments." Furthermore, Figure 7 on page 21 of **Exhibit D** depicts the use of libraries of expression vectors expressing an encoded "dsRNA cassette" to carry out functional screens in cultured cells.

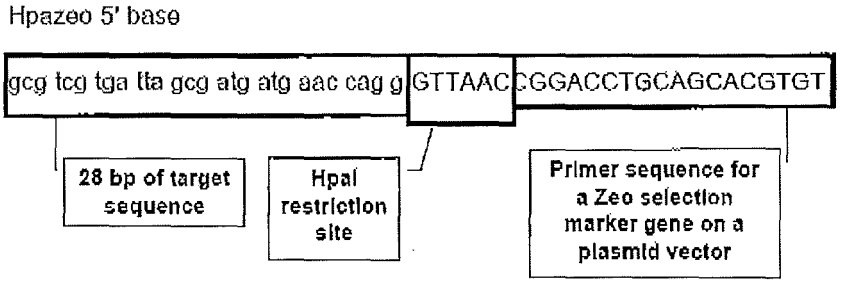
C. Primer Order to Invitrogen

16. Attached at **Exhibit E** is a copy of an email that was sent to Invitrogen to order oligonucleotide primers. The email was sent prior to August 14, 2001.

17. The email lists a number of pairs of oligonucleotide primers which were to be synthesized for use in cloning a sequence encoding a short hairpin RNA into a plasmid expression vector in order to obtain a short hairpin expression product as shown in Figure 37 of the parent application, U.S. Serial No. 10/055,797. These oligonucleotides requested through this e-mail order are examples of oligonucleotide primers designed for cloning such an expression vector using a two step cloning method, as referred to at paragraph 15 above and described in the grant application (**Exhibit D**) on the bottom of page 19. Note that the nucleic acids are synthesized in pairs (5' and 3') for use as 5' and 3' primers in a PCR amplification. For example, this is indicated by a "5" or a "3" at the end of each label, e.g., as in the first primer pair listed in the e-mail, "HPRTHpaZeo1 – 5" and "HPRThpazeo 1 – 3.".

18. Each primer consists of (a) a 28 nucleotide region of the target gene, followed by (b) a Hpa I restriction site (GTTAAC), followed by (c) a primer sequence for a Zeomycin selection

marker gene (Zeo) on a plasmid. Both a 5' nucleic acid and a 3' nucleic acid with these elements were to be synthesized as shown by the pairs of nucleic acids listed in **Exhibit E**. The elements of the first-listed nucleic acid in **Exhibit E** are labeled below:



19. The sequence of these nucleic acids reflects a two step cloning strategy for generating a DNA expression vector capable of expressing a short hairpin RNA having a double-stranded region of 28 base pairs. The nucleic acid pairs as indicated in **Exhibit E** are used as primers for a PCR reaction, using a Zeo selection marker gene as the PCR template. The amplified PCR product resulting from that PCR reaction is a double-stranded nucleic acid product that has a 28 nucleotide region of the target gene sequence, followed by a Hpa I restriction enzyme cleavage site, followed by the Zeomycin gene, followed by another Hpa I cleavage site, followed by the reverse complement of the 28 nucleotide region of the target gene.

20. In the first cloning step, the PCR product is cloned into an expression vector using Zeomycin selection. In the second cloning step, the vector is then digested using the HpaI restriction enzyme, resulting in a vector encoding a short hairpin consisting of (a) the target gene sequence, (b) a loop consisting of a HpaI restriction enzyme cleavage site and (c) the reverse complement of the target gene sequence. When transformed into bacterial cells, the HpaI site

facilitates selection of positive bacterial clones, i.e., those transformed with the expression vector. The draft SBIR Grant Application (**Exhibit D**) refers to such a two step cloning strategy at the bottom of page 19. s

21. The resulting expression vector constructed through this two step strategy encodes a short hairpin having a 28 base pair double-stranded region and an intervening loop consisting of an HpaI site. The short RNA hairpin encoded by an expression vector constructed using the primers listed in the Primer Order to Invitrogen (**Exhibit E**) has the same hairpin structure as shown in Figure 37 of the '797 application (see also **Exhibit F**).

22. The target genes referred to in **Exhibit E** and in **Exhibit D** include: human hypoxanthine—guanine phosphoribosyl transferase (HGPRT1 and HGPRT2 primers) and the mouse tyrosinase gene (tyro1 and tyro2 primers). The indicated target genes therefore indicate the resulting encoded short RNA hairpins (and expression constructs) are directed to silencing their corresponding target gene in mammalian cells, in particular, human cells and mouse cells. Additionally, as indicated in the Draft SBIR Grant Application (**Exhibit D**) on page 16 (second paragraph), HGRPT gene is directed to a gene target "for which exists a positive selection for loss-of-function" upon stable expression of the hairpin RNA in the cell.

D. Luciferase Simple Hairpin

23. Attached at **Exhibit F** is a copy of a slide dated at least by December 28, 2001. Information in this slide is also shown in Figure 37 in the parent application U.S. Serial No. 10/055,797. The slide illustrates two short hairpin RNA molecules. The second hairpin, the "Luciferase simple hairpin" has a double-stranded region consisting of 28 base pairs in length. .

The double-stranded region is highlighted. The double-stranded region of the short hairpin RNA molecule has a sequence that is complementary to a portion of the target gene, firefly luciferase.

24. The loop region of the hairpin on **Exhibit F** contains the sequence GUUAAC which is a HpaI restriction site. This is an example of a cloned simple hairpin that would be obtained using the methods described above in **Exhibit D** (specifically, the two-step method of hairpin cloning referred to here at paragraph 15) and using the PCR primers listed in **Exhibit E**.

E. Short Hairpin RNA Experiment in Human 293 T Cells

25. Attached at **Exhibit G** is a copy of a slide dated at least as early as October 2001. The title of the slide is "SHP 293T" indicating that this data is from an experiment using short hairpin RNA in 293T cells, a line of human embryonic kidney cells. This experiment assessed the ability of various short hairpin RNAs to specifically suppress gene expression in these cells, without provoking a PKR response. The 293T cells were co-transfected with a plasmid expressing the target gene, firefly luciferase, a plasmid expressing Renilla luciferase and one of various test hairpin RNAs. Subsequent to transfection, the level of expression of both luciferase proteins was measured. In the slide, the different test hairpin RNAs are indicated on the X axis of the slide underneath each of the bars. The respective bars indicate the degree to which the various introduced RNAs, including short hairpin RNAs, suppressed expression of the target firefly luciferase gene, as assayed by the ratio of firely luciferase to Renilla luciferase expression. As indicated in the slide, these results demonstrated that short RNA hairpins specifically suppressed expression of their target gene without provoking a PKR response in the cells.

26. For example, the nomenclature "SHP 25 luc hp" indicates a short hairpin RNA that has a double stranded region of 25 nucleotides in length. As the nomenclature indicates, the double-

stranded region of this short hairpin RNA molecule has a sequence that is complementary to a portion of the target gene, firefly luciferase. The bar graph shows, as a result of the experiment, a specific suppression of firefly luciferase gene expression in the 293T cells. See the bar labeled "SHP 25 luc hp" on the graph.

27. In the slide, the nomenclature "SHP 33 luc hp mism ngl3" indicates a short hairpin RNA that has a double stranded region of 33 nucleotides in length and has a mismatch in the sequence so that the sequence is not fully complementary to the sequence of the luciferase target gene. This bar of the bar graph shows, as a result of the experiment using a mismatched hairpin sequence, no specific suppression of firefly luciferase gene expression. The slide shows that short hairpin constructs with double-stranded regions of 32 nucleotides, 33 nucleotides, 34 nucleotides and 35 nucleotides did not exhibit attenuation of luciferase gene expression.

28. This slide shows an example of a short hairpin with a double-stranded region of 25 nucleotides in length, which did not trigger a protein kinase RNA-activated (PKR) response in the mammalian cell, and which did attenuate expression of the target gene, luciferase, in a sequence specific manner in the mammalian cells, 293T. The information in this slide was also included as Figure 39 of the parent application U.S. Serial No. 10/055,797.

F. Short Hairpin RNA Experiment in Human HeLa Cells

29. Attached at **Exhibit H** is a copy of a slide dated at least as early as October 2001. The slide shows data from an experiment using human HeLa cells (a cell line derived from human cervical cancer cells). We knew at the time of this experiment that long dsRNA initiates a PKR response in these cells. Using the same protocol as the experiment discussed above (**E**), this

experiment similarly assessed the ability of various short hairpin RNAs to specifically suppress gene expression in HeLa cells, without provoking a PKR response.

30. As indicated in the slide, these results demonstrated that short RNA hairpins specifically suppressed expression of their target gene without provoking a PKR response in the cells. For example, introducing a short hairpin RNA having a double-stranded region of 25 base pairs ("SHP 25 Luc hp") into the cells specifically suppressed expression of the firefly luciferase target gene. Longer double-stranded regions or mismatched target sequences did not result in suppression of target gene expression. The information in this slide was also included as Figure 40 of the parent application U.S. Serial No. 10/055,797.

G. Short Hairpin RNA Experiment in *Drosophila* S2 Cells

31. Attached at **Exhibit I** is a copy of a slide dated at least as early as October 2001. The data in this slide was generated using the same type of experimental procedure as discussed above in **Exhibits G and H**. The data in this slide indicates that short hairpin with a double-stranded region of 25 nucleotides ("SHP 25 luc hp") functioned to specifically inhibit expression of the target gene in the cells. The information in this slide was also included as Figure 38 of the parent application U.S. Serial No. 10/055,797.

H. Expression of Encoded Short Hairpins Specifically Suppress Gene Expression in Mammalian Cells

32. Attached at **Exhibit J** is a copy of a slide dated as least by January 2002 which shows results from an experiment which was included as Figure 42 (bottom) of the parent application U.S. Serial No. 10/055,797. The description of this experiment and the data can be found on page 17 of the '797 application. The results of this experiment demonstrate that expression of

encoded short hairpin RNAs effectively and specifically suppressed expression of a target gene in 293T cells, without provoking a PKR response. .

I. Paddison et al., *Genes Dev.* 2002, 16:948-958

33. The work described above culminated in several publications. One paper was published in *Genes and Development* in March 2002 entitled "Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells." (See **Exhibit K.**) This paper reports that "short hairpin RNAs (shRNAs) can be engineered to suppress the expression of desired genes in culture *Drosophila* and mammalian cells. shRNA can be synthesized exogenously or can be transcribed from RNA polymerase III promoters *in vivo*, thus permitting the construction of continuous cell lines or transgenic animals in which RNAi enforces stable and heritable gene silencing." (See Abstract of **Exhibit K.**)

34. A copy of a manuscript of the Paddison et al. paper (**Exhibit K**) that was prepared prior to publication and no later than January 31, 2002, as indicated by e-mails to which the manuscript was attached, is attached at **Exhibit L**.

35. Results of additional representative experiments, conducted similarly to the experiment referred to here in part **H**, "Expression of Encoded Short Hairpins Specifically Suppress Gene Expression in Mammalian Cells," are also reported in Paddison et al., among other places, at Fig. 4. (**Exhibit K**). Results of additional representative experiments conducted similarly to the Short Hairpin RNA Experiment in *Drosophila* S2 Cells (**G**), the Short Hairpin RNA Experiment in Human 293 T Cells (**E**) and the Short Hairpin RNA Experiment in Human HeLa Cells (**F**) are reported in Paddison et al., among other places, at Figs. 1 through 3. (**Exhibit K**). Figures 44A and 44B of the '676 application correspond to Figure 6A and 6B of Paddison et al. (**Exhibit K**).

J. Industry Awards

36. During 2002-2006, Paddison et al. (**Exhibit K**), having been cited by more than 500 subsequently published scientific papers, was therefore among the most highly cited "high impact" papers in the fields of molecular biology and genetics, as indicated by an analysis published by ScienceWatch.com (**Exhibit M**, see Table 2). A citation history summary for Paddison et al. (**Exhibit K**) is shown in **Exhibit N**.

37. Since we made the claimed invention and published Paddison et al., (**Exhibit K**), the invention of using stably expressed short hairpin RNAs to inhibit gene expression in mammalian cells has been recognized by industry organizations. For example, in 2005, Dr. Hannon received the Award for Outstanding Achievement in Cancer Research from the American Association for Cancer Research (AACR), which honored Dr. Hannon "...for his work uncovering the biochemical mechanism of RNA interference of gene expression (RNAi) and his contributions to the discovery and development of short hairpin RNAs as tools for genetic manipulation of mammalian cells." (**Exhibit O**).

38. In 2007, Dr. Hannon received two more prestigious awards, the Award in Molecular Biology from the National Academy of Sciences (**Exhibit P**), and the Paul Marks prize for the valuable contribution his RNAi work to cancer research from Memorial Sloan-Kettering Cancer Center (**Exhibit Q**). In granting that award, MSKCC noted how Dr. Hannon had applied his research in understanding the RNAi pathway to develop this valuable new technology, and his recognition as a leader in the field:

> Dr. Hannon is a leader in the relatively new field of RNA interference (RNAi). RNAi is a naturally occurring mechanism for regulating the expression of genes (controlling which genes are turned on and turned off in cells). In the laboratory, it is used as a

tool to study the function of specific genes, and it's being investigated as a therapeutic approach for treating many different diseases, including cancer.

Dr. Hannon's laboratory has elucidated key biochemical details of the components of the pathways involved in RNAi and is using these findings to develop molecular tools that can be used for gene discovery, the evaluation of gene function, and the generation of animal models. He has developed new techniques for using RNAi to study cancer development and is investigating possible cancer therapies that make use of small interfering RNAs (siRNAs).

Dr. Hannon discovered several proteins and enzymes that are an essential part of the RNAi mechanism, including Dicer, which cleaves double-stranded RNA into siRNAs; the RISC complex, which helps regulate protein translation and is involved in the body's defense against viral infections; and Argonaute2, which cleaves messenger RNA.

He also has been at the forefront of adapting RNAi techniques to study genes in mammals, and using these techniques to understand the variety of pathways that can lead to the formation of tumors.

K. Conclusion

39. The documents attached hereto as **Exhibits A - M** demonstrate that that the invention claimed, including claims 50, 52, 54-60, 62 and 63, was conceived at least as early as August 14, 2001, which is prior to the effective filing date of Caplen et al., Symonds et al., and Kreutzer et al. These documents and our declaration also show diligence and reduction(s) to practice.

40. We further declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that the making of willfully false statements and the like is punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United

States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed: _____
Gregory J. Hannon

Dated: _____

Signed: _____
Patrick J. Paddison

Dated: _____

Signed: _____
Scott Hammond

Dated: _____

Signed: _____
Amy Caudy

Dated: January 25, 2011

Signed: _____
Emily Bernstein

Dated: _____

Signed: _____
Douglas Conklin

Dated: _____

Exhibits to Declaration Under 37 C.F.R. §1.131

Exhibit	Title
A	Hannon Draft Grant Application
B	Hammond et al., *Nature* 404:293-296 (2000)
C	Bernstein et al. *Nature* 409: 363-366 (2001)
D	Draft grant application to SBIR (Small Business Innovation Research)
E	Email of Primer Order to Invitrogen
F	Luciferase Simple Hairpin Slide
G	Short Hairpin RNA Experiment in Human 293 T Cells Slide
H	Short Hairpin RNA Experiment in Human HeLa Cells Slide
I	Short Hairpin RNA Experiment in *Drosophila* S2 Cells Slide
J	Short Hairpins Specifically Suppress Gene Expression Slide
K	Paddison et al., *Genes Dev.* 2002, 16:948-958
L	Manuscript of Paddison et al.
M	ScienceWatch Biology's Hottest 2002-2006
N	Paddison et al. Citations
O	2005 Award for Outstanding Achievement in Cancer Research from AACR
P	2007 Award in Molecular Biology from the National Academy of Sciences
Q	2007 Paul Marks Prize from Memorial Sloan-Kettering Cancer Center

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Hannon et al.	Confirmation No.:	8161
Application No:	11/894,676	Art Unit:	1635
Filed:	August 20, 2007	Examiner:	K. CHONG
Title:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Docket No.: 287000-130-US3

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Declaration Under 37 C.F.R. §1.131

We, Gregory J. Hannon, Patrick J. Paddison, Scott Hammond, Amy Caudy and Emily Bernstein, Douglas Conklin hereby declare as follows:

1. We are the inventors of the above-referenced patent application.

2. All the work described within this declaration was performed in the United States.

3. All of the work described within this declaration was performed by us, or on our behalf and under our direction.

4. We have reviewed our records, including the slides documents submitted herewith, and declare that the claimed invention, which is

> a method for attenuating expression of a target gene in a mammalian cell, the method comprising introducing into a mammalian cell a library of RNA expression constructs, each expression construct comprising:
>
> (i) an RNA polymerase promoter, and

> (ii) a sequence encoding a short hairpin RNA molecule comprising a double-stranded region wherein the double-stranded region consists of at least 20 nucleotides but not more than 29 nucleotides,
>
> wherein the short hairpin RNA molecule is a substrate for Dicer-dependent cleavage and does not trigger a protein kinase RNA-activated (PKR) response in the mammalian cells,
>
> wherein the double-stranded region of the short hairpin RNA molecule comprises a sequence that is complementary to a portion of the target gene, and
>
> wherein the short hairpin RNA molecule is stably expressed in the mammalian cell in an amount sufficient to attenuate expression of the target gene in a sequence specific manner, and is expressed in the cell without use of a PK inhibitor,
>
> whereby expression of the target gene is inhibited

including original (and amended) claims 50, 52, 54-63 was conceived and reduced to practice at least prior to August 14, 2001, the publication date of Caplen et al., *PNAS*, Vol. 98, No. 17, pp. 9742-9747, which is also prior to December 28, 2001, *i.e.*, the filing date of U.S. Publication No. US 2002/0160393, Symonds et al., U.S. Serial No. 10/035,098 and which is also prior to the date of filing of the parent application of Kreutzer et al. (U.S. Serial No. 09/889,802, filed September 17, 2001).

A. Hannon Draft Grant Application

5. We attach a copy of a draft grant application (**Exhibit A**) which was prepared prior to August 14, 2001. A review of email indicates that this draft grant application was prepared at least by sometime in January 2000. The specific aims, as indicated on the first page of the draft grant application (**Exhibit A**, page 12), were directed to identifying and characterizing the critical components of the RNA interference (RNAi) machinery. The "Preliminary Results" this page refers to (see 4th paragraph on page 12) were reported in Hammond et al., *Nature* 404:293-

296 (2000) (**Exhibit B**) in a paper entitled "An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophia* cells."

6. In particular, one aspect of the proposed work was directed to isolating and cloning the protein and RNA components of the RISC complex (RNA-induced silencing complex), the nuclease complex responsible for degradation of target mRNAs, and characterizing it's function, both *in vitro* and *in vivo*. To allow us to carry out such studies, we established a model system using cultured *Drosophila* cells that provided a readily available source of material in sufficient quantities for the necessary biochemical studies.

7. The Summary on page 15 provides the rationale for the proposed work:

> My laboratory has devoted a number of years to creating improved tools for probing gene function in cultured mammalian cells; however, our experience indicates that a facile loss-of-function tool is lacking. Unfortunately, dsRNA induces somewhat generic responses in mammalian cells. It is our hope that by understanding the mechanistic basis of dsRNA-induced silencing, we may not only unravel a mysterious and important piece of biology but also provide the means to create improved tools for analyzing gene function in diverse organisms in which traditional genetic methods are either cumbersome or unavailable. This notion that has contributed to the decision to focus substantial effort in my laboratory toward elucidating the mechanism of RNA interference.

The final paragraph on page 36 further elaborates on this rationale:

> In this application, we propose a biochemical approach to deciphering the mechanisms that underlie dsRNA-induced gene silencing. RNA-interference allows an adaptive defense against both exogenous and endogenous dsRNAs, providing something akin to a dsRNA immune response. The primary goal of the work proposed in this application is to understand the mechanisms by which a cell can raise this response. We have presented evidence that RNA interference is accomplished, at least in part, through the action of a sequence-specific nuclease that is generated in response to dsRNA. Our data, and that of others (Hamilton and Baulcombe, 1999), is consistent with a model in which dsRNAs present in a cell are converted, in a manner analogous to antigen processing, into discrete, small RNAs that guide the nuclease in the choice of substrate.

> We propose to purify and characterize the nuclease and to clone the protein and RNA components of the enzyme. In addition, we propose to develop approaches that may allow the use of cultured *Drosophila* cells as a general tool for probing gene function. The combination of these studies may lead eventually to an ability to harness RNA interference as a genetic tool in other organisms, particularly mammals, in which analogous tools are presently lacking.

8. At that time, there was a lack of available practical loss-of-function tools for probing gene function in mammalian cells. The work proposed in this draft application to elucidate the mechanism of RNA interference was intended to develop such tools. In other words, by understanding the mechanistic basis of RNA interference, we could use that understanding to exploit the RNAi pathway and create new tools to study gene function and the lack of certain gene function in mammalian cells.

9. The work proposed in this application to identify and characterize components of the RNAi cellular machinery was carried out by us prior to August 14, 2001. Certain aspects of this work were reported in Bernstein et al. *Nature* 409: 363-366 (2001) (**Exhibit C**) in a paper entitled "Role for a bidentate ribonuclease in the initiation step of RNA interference." This paper describes the identification and cloning of the enzyme, which we named "Dicer." The paper describes how this enzyme is evolutionarily conserved in worms, flies, plants, fungi and mammals, and the paper reports the role of this newly discovered enzyme in the RNAi pathway in cells. In particular, these results indicated that the process of gene silencing through the RNAi pathway could be divided into at least two distinct steps. In the first step, long dsRNA (double-stranded RNA) is processed by Dicer into approximately 22 nt (nucleotide) "guide" sequences. In the second step, these guide RNAs are incorporated into a distinct nuclease complex we first called the "RNA-induced silencing complex" or RISC. The RISC complex uses the guide sequences to specifically identify and destroy homologous mRNAs. We named the RNAs that

were processed by Dicer "guide sequences" or "guide RNAs" based on their role in targeting RISC to specific mRNAs based on sequence. The results and work described in Bernstein et al. (2001) were included in this patent application, U.S. Serial No. 11/894,676, and also in the related application U.S. Serial No. 10/055,797, such as in Example 2.

B. Draft SBIR Grant Application

10. We attach as **Exhibit D** a copy of a draft grant application to SBIR (Small Business Innovation Research) which was prepared prior to August 14, 2001.[1]

11. The first page of this draft grant lists three Aims directed toward achieving stable gene silencing in mammalian cells. Aim 1 is the "creation of stable, loss-of-function mutations in embryonic cells using RNAi." Aim 2 is the "creation of stable loss-of-function mutations in non-embryonic cell types," which proposes "numerous strategies for bypassing [the] problem" that "long dsRNAs provoke a PKR response in differentiated cell types."

12. Attached pages 13-25 of **Exhibit D** provide more detail regarding each of these Aims. Starting on page 13, the grant application describes the Experimental Procedures for Aim 1. Aim 1 is defined as "Creation of stable, loss-of-function mutations in embryonic cells using RNAi." On page 14, the grant states that "[w]e have chosen to approach this goal by encoding dsRNA in the form of an inverted repeat or hairpin that can be expressed from a promoter of choice." Regarding this objective, on page 14 the draft grant states that "[w]e have achieved the goal of simplified hairpin construction by dividing the process into two steps (Fig. 6)." Figure 6 is on page 15 and depicts a "strategy for the creation of hairpin RNAs for stable expression of dsRNA" and illustrates that "expression of a GFP hairpin RNA induced stable silencing of an

[1] For convenience, we have added page numbers to this document.

exogenous GFP reporter in [mouse embryonic] P19 cells." The use of the strategy and also the results described in Aim 1 are described in Example 3 (entitled "A Simplified Method for the Creation of Hairpin Constructs for RNA Interference") and Fig. 27, and in Example 4 (entitled "Long dsRNAs Suppress Gene Expression in Mammalian Cells") and Figs. 28-34 of the parent application, U.S. Serial No. 10/055,797. Aim 1 also describes silencing mammalian genes for which assays are available to allow "positive selection for loss-or function" in mammalian cells, *e.g.*, HPRT and TK. (See 2nd paragraph on page 16 of **Exhibit D**.)

13. The grant application states the goals for Aim 2 on the top of page 18 of **Exhibit D**: "our goal is to devise strategies for presentation of the dsRNA trigger that allow it to elude PKR surveillance." The "Expression Strategies" provided in the grant state that "PKR requires approximately 30 bp of contiguous double-stranded sequence to trigger dimerization and activation of the enzyme." (See first paragraph under "Expression Strategies" on page 18. The third paragraph in that section on page 18 describes expression of hairpin RNAs in various mammalian cells: "NIH 3T3, 293, HeLa, U2OS, Rat 1 and C2C12" and various expression vectors incorporating various promoters, including U1, U6 and CMV.

14. In the section entitled "Short RNA hairpins" on page 19 of **Exhibit D**, the grant application describes use of short RNA hairpins that are "below the cut-off for triggering RNA for investigating "whether the expression of short RNA hairpins can be used to induce efficient silencing." The research plan here also refers to "short synthetic RNAs that mimic our Dicer products." In other words, this refers to RNAs that have a double-stranded region of 20 to 22 base pairs. It further states that "short synthetic hairpins directed against GFP, TK and HPRT will be expressed from CMV, U1 and U6 promoter vectors in the cell types noted above." (See page 19.)

15. The grant application on page 19 describes two methods for modifying the approach described in Aim 1 to "create hairpins with significantly shorter loops." The first is "to simply clone short hairpin sequences [either] as single, synthetic DNA fragments, and the second is to clone "in two steps if hairpin formation in such synthetic oligonucleotides competes too vigorously with intermolecular hybridization to produce clonable fragments." Furthermore, Figure 7 on page 21 of **Exhibit D** depicts the use of libraries of expression vectors expressing an encoded "dsRNA cassette" to carry out functional screens in cultured cells.

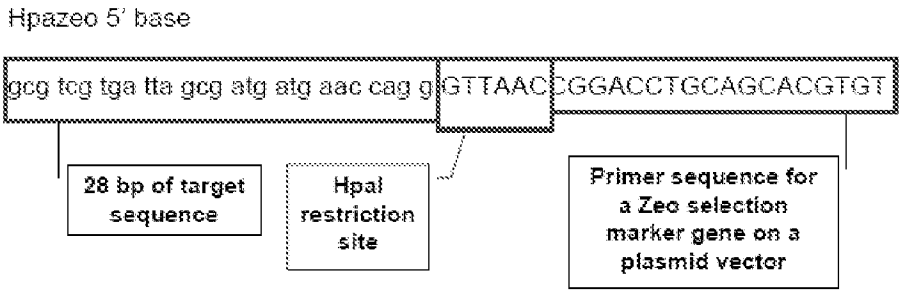
C. Primer Order to Invitrogen

16. Attached at **Exhibit E** is a copy of an email that was sent to Invitrogen to order oligonucleotide primers. The email was sent prior to August 14, 2001.

17. The email lists a number of pairs of oligonucleotide primers which were to be synthesized for use in cloning a sequence encoding a short hairpin RNA into a plasmid expression vector in order to obtain a short hairpin expression product as shown in Figure 37 of the parent application, U.S. Serial No. 10/055,797. These oligonucleotides requested through this e-mail order are examples of oligonucleotide primers designed for cloning such an expression vector using a two step cloning method, as referred to at paragraph 15 above and described in the grant application (**Exhibit D**) on the bottom of page 19. Note that the nucleic acids are synthesized in pairs (5' and 3') for use as 5' and 3' primers in a PCR amplification. For example, this is indicated by a "5" or a "3" at the end of each label, e.g., as in the first primer pair listed in the e-mail, "HPRTHpaZeo1 – 5" and "HPRThpazeo 1 – 3.".

18. Each primer consists of (a) a 28 nucleotide region of the target gene, followed by (b) a Hpa I restriction site (GTTAAC), followed by (c) a primer sequence for a Zeomycin selection

marker gene (Zeo) on a plasmid. Both a 5' nucleic acid and a 3' nucleic acid with these elements were to be synthesized as shown by the pairs of nucleic acids listed in **Exhibit E**. The elements of the first-listed nucleic acid in **Exhibit E** are labeled below:



19. The sequence of these nucleic acids reflects a two step cloning strategy for generating a DNA expression vector capable of expressing a short hairpin RNA having a double-stranded region of 28 base pairs. The nucleic acid pairs as indicated in **Exhibit E** are used as primers for a PCR reaction, using a Zeo selection marker gene as the PCR template. The amplified PCR product resulting from that PCR reaction is a double-stranded nucleic acid product that has a 28 nucleotide region of the target gene sequence, followed by a Hpa I restriction enzyme cleavage site, followed by the Zeomycin gene, followed by another Hpa I cleavage site, followed by the reverse complement of the 28 nucleotide region of the target gene.

20. In the first cloning step, the PCR product is cloned into an expression vector using Zeomycin selection. In the second cloning step, the vector is then digested using the HpaI restriction enzyme, resulting in a vector encoding a short hairpin consisting of (a) the target gene sequence, (b) a loop consisting of a HpaI restriction enzyme cleavage site and (c) the reverse complement of the target gene sequence. When transformed into bacterial cells, the HpaI site

facilitates selection of positive bacterial clones, i.e., those transformed with the expression vector. The draft SBIR Grant Application (**Exhibit D**) refers to such a two step cloning strategy at the bottom of page 19. s

21. The resulting expression vector constructed through this two step strategy encodes a short hairpin having a 28 base pair double-stranded region and an intervening loop consisting of an HpaI site. The short RNA hairpin encoded by an expression vector constructed using the primers listed in the Primer Order to Invitrogen (**Exhibit E**) has the same hairpin structure as shown in Figure 37 of the '797 application (see also **Exhibit F**).

22. The target genes referred to in **Exhibit E** and in **Exhibit D** include: human hypoxanthine—guanine phosphoribosyl transferase (HGPRT1 and HGPRT2 primers) and the mouse tyrosinase gene (tyro1 and tyro2 primers). The indicated target genes therefore indicate the resulting encoded short RNA hairpins (and expression constructs) are directed to silencing their corresponding target gene in mammalian cells, in particular, human cells and mouse cells. Additionally, as indicated in the Draft SBIR Grant Application (**Exhibit D**) on page 16 (second paragraph), HGRPT gene is directed to a gene target "for which exists a positive selection for loss-of-function" upon stable expression of the hairpin RNA in the cell.

D. Luciferase Simple Hairpin

23. Attached at **Exhibit F** is a copy of a slide dated at least by December 28, 2001. Information in this slide is also shown in Figure 37 in the parent application U.S. Serial No. 10/055,797. The slide illustrates two short hairpin RNA molecules. The second hairpin, the "Luciferase simple hairpin" has a double-stranded region consisting of 28 base pairs in length. .

The double-stranded region is highlighted. The double-stranded region of the short hairpin RNA molecule has a sequence that is complementary to a portion of the target gene, firefly luciferase.

24. The loop region of the hairpin on **Exhibit F** contains the sequence GUUAAC which is a HpaI restriction site. This is an example of a cloned simple hairpin that would be obtained using the methods described above in **Exhibit D** (specifically, the two-step method of hairpin cloning referred to here at paragraph 15) and using the PCR primers listed in **Exhibit E**.

E. Short Hairpin RNA Experiment in Human 293 T Cells

25. Attached at **Exhibit G** is a copy of a slide dated at least as early as October 2001. The title of the slide is "SHP 293T" indicating that this data is from an experiment using short hairpin RNA in 293T cells, a line of human embryonic kidney cells. This experiment assessed the ability of various short hairpin RNAs to specifically suppress gene expression in these cells, without provoking a PKR response. The 293T cells were co-transfected with a plasmid expressing the target gene, firefly luciferase, a plasmid expressing Renilla luciferase and one of various test hairpin RNAs. Subsequent to transfection, the level of expression of both luciferase proteins was measured. In the slide, the different test hairpin RNAs are indicated on the X axis of the slide underneath each of the bars. The respective bars indicate the degree to which the various introduced RNAs, including short hairpin RNAs, suppressed expression of the target firefly luciferase gene, as assayed by the ratio of firely luciferase to Renilla luciferase expression. As indicated in the slide, these results demonstrated that short RNA hairpins specifically suppressed expression of their target gene without provoking a PKR response in the cells.

26. For example, the nomenclature "SHP 25 luc hp" indicates a short hairpin RNA that has a double stranded region of 25 nucleotides in length. As the nomenclature indicates, the double-

stranded region of this short hairpin RNA molecule has a sequence that is complementary to a portion of the target gene, firefly luciferase. The bar graph shows, as a result of the experiment, a specific suppression of firefly luciferase gene expression in the 293T cells. See the bar labeled "SHP 25 luc hp" on the graph.

27. In the slide, the nomenclature "SHP 33 luc hp mism ngl3" indicates a short hairpin RNA that has a double stranded region of 33 nucleotides in length and has a mismatch in the sequence so that the sequence is not fully complementary to the sequence of the luciferase target gene. This bar of the bar graph shows, as a result of the experiment using a mismatched hairpin sequence, no specific suppression of firefly luciferase gene expression. The slide shows that short hairpin constructs with double-stranded regions of 32 nucleotides, 33 nucleotides, 34 nucleotides and 35 nucleotides did not exhibit attenuation of luciferase gene expression.

28. This slide shows an example of a short hairpin with a double-stranded region of 25 nucleotides in length, which did not trigger a protein kinase RNA-activated (PKR) response in the mammalian cell, and which did attenuate expression of the target gene, luciferase, in a sequence specific manner in the mammalian cells, 293T. The information in this slide was also included as Figure 39 of the parent application U.S. Serial No. 10/055,797.

F. Short Hairpin RNA Experiment in Human HeLa Cells

29. Attached at **Exhibit H** is a copy of a slide dated at least as early as October 2001. The slide shows data from an experiment using human HeLa cells (a cell line derived from human cervical cancer cells). We knew at the time of this experiment that long dsRNA initiates a PKR response in these cells. Using the same protocol as the experiment discussed above (**E**), this

experiment similarly assessed the ability of various short hairpin RNAs to specifically suppress gene expression in HeLa cells, without provoking a PKR response.

30. As indicated in the slide, these results demonstrated that short RNA hairpins specifically suppressed expression of their target gene without provoking a PKR response in the cells. For example, introducing a short hairpin RNA having a double-stranded region of 25 base pairs ("SHP 25 Luc hp") into the cells specifically suppressed expression of the firefly luciferase target gene. Longer double-stranded regions or mismatched target sequences did not result in suppression of target gene expression. The information in this slide was also included as Figure 40 of the parent application U.S. Serial No. 10/055,797.

G. Short Hairpin RNA Experiment in *Drosophila* S2 Cells

31. Attached at **Exhibit I** is a copy of a slide dated at least as early as October 2001. The data in this slide was generated using the same type of experimental procedure as discussed above in **Exhibits G and H**. The data in this slide indicates that short hairpin with a double-stranded region of 25 nucleotides ("SHP 25 luc hp") functioned to specifically inhibit expression of the target gene in the cells. The information in this slide was also included as Figure 38 of the parent application U.S. Serial No. 10/055,797.

H. Expression of Encoded Short Hairpins Specifically Suppress Gene Expression in Mammalian Cells

32. Attached at **Exhibit J** is a copy of a slide dated as least by January 2002 which shows results from an experiment which was included as Figure 42 (bottom) of the parent application U.S. Serial No. 10/055,797. The description of this experiment and the data can be found on page 17 of the '797 application. The results of this experiment demonstrate that expression of

encoded short hairpin RNAs effectively and specifically suppressed expression of a target gene in 293T cells, without provoking a PKR response. .

I. Paddison et al., *Genes Dev.* 2002, 16:948-958

33. The work described above culminated in several publications. One paper was published in *Genes and Development* in March 2002 entitled "Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells." (See **Exhibit K**.) This paper reports that "short hairpin RNAs (shRNAs) can be engineered to suppress the expression of desired genes in culture *Drosophila* and mammalian cells. shRNA can be synthesized exogenously or can be transcribed from RNA polymerase III promoters *in vivo*, thus permitting the construction of continuous cell lines or transgenic animals in which RNAi enforces stable and heritable gene silencing." (See Abstract of **Exhibit K**.)

34. A copy of a manuscript of the Paddison et al. paper (**Exhibit K**) that was prepared prior to publication and no later than January 31, 2002, as indicated by e-mails to which the manuscript was attached, is attached at **Exhibit L**.

35. Results of additional representative experiments, conducted similarly to the experiment referred to here in part **H**, "Expression of Encoded Short Hairpins Specifically Suppress Gene Expression in Mammalian Cells," are also reported in Paddison et al., among other places, at Fig. 4. (**Exhibit K**). Results of additional representative experiments conducted similarly to the Short Hairpin RNA Experiment in *Drosophila* S2 Cells (**G**), the Short Hairpin RNA Experiment in Human 293 T Cells (**E**) and the Short Hairpin RNA Experiment in Human HeLa Cells (**F**) are reported in Paddison et al., among other places, at Figs. 1 through 3. (**Exhibit K**). Figures 44A and 44B of the '676 application correspond to Figure 6A and 6B of Paddison et al. (**Exhibit K**).

J. Industry Awards

36. During 2002-2006, Paddison et al. (**Exhibit K**), having been cited by more than 500 subsequently published scientific papers, was therefore among the most highly cited "high impact" papers in the fields of molecular biology and genetics, as indicated by an analysis published by ScienceWatch.com (**Exhibit M**, see Table 2). A citation history summary for Paddison et al. (**Exhibit K)** is shown in **Exhibit N**.

37. Since we made the claimed invention and published Paddison et al., (**Exhibit K**), the invention of using stably expressed short hairpin RNAs to inhibit gene expression in mammalian cells has been recognized by industry organizations. For example, in 2005, Dr. Hannon received the Award for Outstanding Achievement in Cancer Research from the American Association for Cancer Research (AACR), which honored Dr. Hannon "…for his work uncovering the biochemical mechanism of RNA interference of gene expression (RNAi) and his contributions to the discovery and development of short hairpin RNAs as tools for genetic manipulation of mammalian cells." (**Exhibit O**).

38. In 2007, Dr. Hannon received two more prestigious awards, the Award in Molecular Biology from the National Academy of Sciences (**Exhibit P**), and the Paul Marks prize for the valuable contribution his RNAi work to cancer research from Memorial Sloan-Kettering Cancer Center (**Exhibit Q**). In granting that award, MSKCC noted how Dr. Hannon had applied his research in understanding the RNAi pathway to develop this valuable new technology, and his recognition as a leader in the field:

> Dr. Hannon is a leader in the relatively new field of RNA interference (RNAi). RNAi is a naturally occurring mechanism for regulating the expression of genes (controlling which genes are turned on and turned off in cells). In the laboratory, it is used as a

tool to study the function of specific genes, and it's being investigated as a therapeutic approach for treating many different diseases, including cancer.

Dr. Hannon's laboratory has elucidated key biochemical details of the components of the pathways involved in RNAi and is using these findings to develop molecular tools that can be used for gene discovery, the evaluation of gene function, and the generation of animal models. He has developed new techniques for using RNAi to study cancer development and is investigating possible cancer therapies that make use of small interfering RNAs (siRNAs).

Dr. Hannon discovered several proteins and enzymes that are an essential part of the RNAi mechanism, including Dicer, which cleaves double-stranded RNA into siRNAs; the RISC complex, which helps regulate protein translation and is involved in the body's defense against viral infections; and Argonaute2, which cleaves messenger RNA.

He also has been at the forefront of adapting RNAi techniques to study genes in mammals, and using these techniques to understand the variety of pathways that can lead to the formation of tumors.

K. Conclusion

39. The documents attached hereto as **Exhibits A - M** demonstrate that that the invention claimed, including claims 50, 52, 54-60, 62 and 63, was conceived at least as early as August 14, 2001, which is prior to the effective filing date of Caplen et al., Symonds et al., and Kreutzer et al. These documents and our declaration also show diligence and reduction(s) to practice.

40. We further declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that the making of willfully false statements and the like is punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United

States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed: _____
Gregory J. Hannon

Dated: _____

Signed: _____
Patrick J. Paddison

Dated: _____

Signed: _____
Scott Hammond

Dated: _____

Signed: _____
Amy Caudy

Dated: _____

Signed: _____
Emily Bernstein

Dated: _____

Signed: _____
Douglas Conklin

Dated: 20 January 2011

Exhibits to Declaration Under 37 C.F.R. §1.131

Exhibit	Title
A	Hannon Draft Grant Application
B	Hammond et al., *Nature* 404:293-296 (2000)
C	Bernstein et al. *Nature* 409: 363-366 (2001)
D	Draft grant application to SBIR (Small Business Innovation Research)
E	Email of Primer Order to Invitrogen
F	Luciferase Simple Hairpin Slide
G	Short Hairpin RNA Experiment in Human 293 T Cells Slide
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K	Paddison et al., *Genes Dev.* 2002, 16:948-958
L	Manuscript of Paddison et al.
M	ScienceWatch Biology's Hottest 2002-2006
N	Paddison et al. Citations
O	2005 Award for Outstanding Achievement in Cancer Research from AACR
P	2007 Award in Molecular Biology from the National Academy of Sciences
Q	2007 Paul Marks Prize from Memorial Sloan-Kettering Cancer Center

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Hannon et al.	Confirmation No.:	8161
Application No:	11/894,676	Art Unit:	1635
Filed:	August 20, 2007	Examiner:	K. CHONG
Title:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Docket No.: 287000-130-US3

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Declaration Under 37 C.F.R. §1.131

We, Gregory J. Hannon, Patrick J. Paddison, Scott Hammond, Amy Caudy and Emily Bernstein, Douglas Conklin hereby declare as follows:

1. We are the inventors of the above-referenced patent application.

2. All the work described within this declaration was performed in the United States.

3. All of the work described within this declaration was performed by us, or on our behalf and under our direction.

4. We have reviewed our records, including the slides documents submitted herewith, and declare that the claimed invention, which is

> a method for attenuating expression of a target gene in a mammalian cell, the method comprising introducing into a mammalian cell a library of RNA expression constructs, each expression construct comprising:
>
> (i) an RNA polymerase promoter, and

> (ii) a sequence encoding a short hairpin RNA molecule comprising a double-stranded region wherein the double-stranded region consists of at least 20 nucleotides but not more than 29 nucleotides,
>
> wherein the short hairpin RNA molecule is a substrate for Dicer-dependent cleavage and does not trigger a protein kinase RNA-activated (PKR) response in the mammalian cells,
>
> wherein the double-stranded region of the short hairpin RNA molecule comprises a sequence that is complementary to a portion of the target gene, and
>
> wherein the short hairpin RNA molecule is stably expressed in the mammalian cell in an amount sufficient to attenuate expression of the target gene in a sequence specific manner, and is expressed in the cell without use of a PK inhibitor,
>
> whereby expression of the target gene is inhibited

including original (and amended) claims 50, 52, 54-63 was conceived and reduced to practice at least prior to August 14, 2001, the publication date of Caplen et al., *PNAS*, Vol. 98, No. 17, pp. 9742-9747, which is also prior to December 28, 2001, *i.e.*, the filing date of U.S. Publication No. US 2002/0160393, Symonds et al., U.S. Serial No. 10/035,098 and which is also prior to the date of filing of the parent application of Kreutzer et al. (U.S. Serial No. 09/889,802, filed September 17, 2001).

A. Hannon Draft Grant Application

5. We attach a copy of a draft grant application (**Exhibit A**) which was prepared prior to August 14, 2001. A review of email indicates that this draft grant application was prepared at least by sometime in January 2000. The specific aims, as indicated on the first page of the draft grant application (**Exhibit A**, page 12), were directed to identifying and characterizing the critical components of the RNA interference (RNAi) machinery. The "Preliminary Results" this page refers to (see 4th paragraph on page 12) were reported in Hammond et al., *Nature* 404:293-

296 (2000) (**Exhibit B**) in a paper entitled "An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophia* cells."

6. In particular, one aspect of the proposed work was directed to isolating and cloning the protein and RNA components of the RISC complex (RNA-induced silencing complex), the nuclease complex responsible for degradation of target mRNAs, and characterizing it's function, both *in vitro* and *in vivo*. To allow us to carry out such studies, we established a model system using cultured *Drosophila* cells that provided a readily available source of material in sufficient quantities for the necessary biochemical studies.

7. The Summary on page 15 provides the rationale for the proposed work:

> My laboratory has devoted a number of years to creating improved tools for probing gene function in cultured mammalian cells; however, our experience indicates that a facile loss-of-function tool is lacking. Unfortunately, dsRNA induces somewhat generic responses in mammalian cells. It is our hope that by understanding the mechanistic basis of dsRNA-induced silencing, we may not only unravel a mysterious and important piece of biology but also provide the means to create improved tools for analyzing gene function in diverse organisms in which traditional genetic methods are either cumbersome or unavailable. This notion that has contributed to the decision to focus substantial effort in my laboratory toward elucidating the mechanism of RNA interference.

The final paragraph on page 36 further elaborates on this rationale:

> In this application, we propose a biochemical approach to deciphering the mechanisms that underlie dsRNA-induced gene silencing. RNA-interference allows an adaptive defense against both exogenous and endogenous dsRNAs, providing something akin to a dsRNA immune response. The primary goal of the work proposed in this application is to understand the mechanisms by which a cell can raise this response. We have presented evidence that RNA interference is accomplished, at least in part, through the action of a sequence-specific nuclease that is generated in response to dsRNA. Our data, and that of others (Hamilton and Baulcombe, 1999), is consistent with a model in which dsRNAs present in a cell are converted, in a manner analogous to antigen processing, into discrete, small RNAs that guide the nuclease in the choice of substrate.

> We propose to purify and characterize the nuclease and to clone the protein and RNA components of the enzyme. In addition, we propose to develop approaches that may allow the use of cultured *Drosophila* cells as a general tool for probing gene function. The combination of these studies may lead eventually to an ability to harness RNA interference as a genetic tool in other organisms, particularly mammals, in which analogous tools are presently lacking.

8. At that time, there was a lack of available practical loss-of-function tools for probing gene function in mammalian cells. The work proposed in this draft application to elucidate the mechanism of RNA interference was intended to develop such tools. In other words, by understanding the mechanistic basis of RNA interference, we could use that understanding to exploit the RNAi pathway and create new tools to study gene function and the lack of certain gene function in mammalian cells.

9. The work proposed in this application to identify and characterize components of the RNAi cellular machinery was carried out by us prior to August 14, 2001. Certain aspects of this work were reported in Bernstein et al. *Nature* 409: 363-366 (2001) (**Exhibit C**) in a paper entitled "Role for a bidentate ribonuclease in the initiation step of RNA interference." This paper describes the identification and cloning of the enzyme, which we named "Dicer." The paper describes how this enzyme is evolutionarily conserved in worms, flies, plants, fungi and mammals, and the paper reports the role of this newly discovered enzyme in the RNAi pathway in cells. In particular, these results indicated that the process of gene silencing through the RNAi pathway could be divided into at least two distinct steps. In the first step, long dsRNA (double-stranded RNA) is processed by Dicer into approximately 22 nt (nucleotide) "guide" sequences. In the second step, these guide RNAs are incorporated into a distinct nuclease complex we first called the "RNA-induced silencing complex" or RISC. The RISC complex uses the guide sequences to specifically identify and destroy homologous mRNAs. We named the RNAs that

were processed by Dicer "guide sequences" or "guide RNAs" based on their role in targeting RISC to specific mRNAs based on sequence. The results and work described in Bernstein et al. (2001) were included in this patent application, U.S. Serial No. 11/894,676, and also in the related application U.S. Serial No. 10/055,797, such as in Example 2.

B. Draft SBIR Grant Application

10. We attach as **Exhibit D** a copy of a draft grant application to SBIR (Small Business Innovation Research) which was prepared prior to August 14, 2001.[1]

11. The first page of this draft grant lists three Aims directed toward achieving stable gene silencing in mammalian cells. Aim 1 is the "creation of stable, loss-of-function mutations in embryonic cells using RNAi." Aim 2 is the "creation of stable loss-of-function mutations in non-embryonic cell types," which proposes "numerous strategies for bypassing [the] problem" that "long dsRNAs provoke a PKR response in differentiated cell types."

12. Attached pages 13-25 of **Exhibit D** provide more detail regarding each of these Aims. Starting on page 13, the grant application describes the Experimental Procedures for Aim 1. Aim 1 is defined as "Creation of stable, loss-of-function mutations in embryonic cells using RNAi." On page 14, the grant states that "[w]e have chosen to approach this goal by encoding dsRNA in the form of an inverted repeat or hairpin that can be expressed from a promoter of choice." Regarding this objective, on page 14 the draft grant states that "[w]e have achieved the goal of simplified hairpin construction by dividing the process into two steps (Fig. 6)." Figure 6 is on page 15 and depicts a "strategy for the creation of hairpin RNAs for stable expression of dsRNA" and illustrates that "expression of a GFP hairpin RNA induced stable silencing of an

[1] For convenience, we have added page numbers to this document.

exogenous GFP reporter in [mouse embryonic] P19 cells." The use of the strategy and also the results described in Aim 1 are described in Example 3 (entitled "A Simplified Method for the Creation of Hairpin Constructs for RNA Interference") and Fig. 27, and in Example 4 (entitled "Long dsRNAs Suppress Gene Expression in Mammalian Cells") and Figs. 28-34 of the parent application, U.S. Serial No. 10/055,797. Aim 1 also describes silencing mammalian genes for which assays are available to allow "positive selection for loss-or function" in mammalian cells, *e.g.*, HPRT and TK. (See 2nd paragraph on page 16 of **Exhibit D**.)

13. The grant application states the goals for Aim 2 on the top of page 18 of **Exhibit D**: "our goal is to devise strategies for presentation of the dsRNA trigger that allow it to elude PKR surveillance." The "Expression Strategies" provided in the grant state that "PKR requires approximately 30 bp of contiguous double-stranded sequence to trigger dimerization and activation of the enzyme." (See first paragraph under "Expression Strategies" on page 18. The third paragraph in that section on page 18 describes expression of hairpin RNAs in various mammalian cells: "NIH 3T3, 293, HeLa, U2OS, Rat 1 and C2C12" and various expression vectors incorporating various promoters, including U1, U6 and CMV.

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15. The grant application on page 19 describes two methods for modifying the approach described in Aim 1 to "create hairpins with significantly shorter loops." The first is "to simply clone short hairpin sequences [either] as single, synthetic DNA fragments, and the second is to clone "in two steps if hairpin formation in such synthetic oligonucleotides competes too vigorously with intermolecular hybridization to produce clonable fragments." Furthermore, Figure 7 on page 21 of **Exhibit D** depicts the use of libraries of expression vectors expressing an encoded "dsRNA cassette" to carry out functional screens in cultured cells.

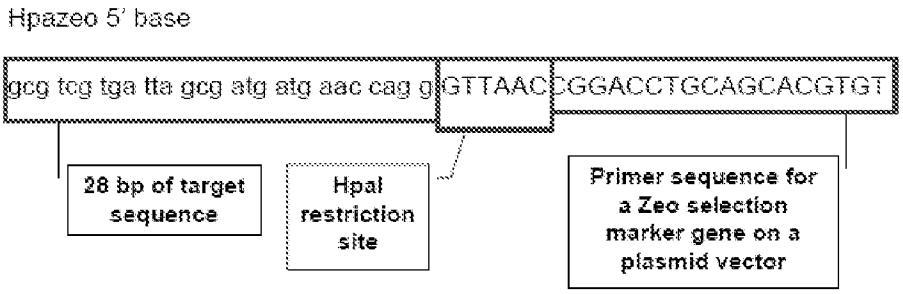
C. Primer Order to Invitrogen

16. Attached at **Exhibit E** is a copy of an email that was sent to Invitrogen to order oligonucleotide primers. The email was sent prior to August 14, 2001.

17. The email lists a number of pairs of oligonucleotide primers which were to be synthesized for use in cloning a sequence encoding a short hairpin RNA into a plasmid expression vector in order to obtain a short hairpin expression product as shown in Figure 37 of the parent application, U.S. Serial No. 10/055,797. These oligonucleotides requested through this e-mail order are examples of oligonucleotide primers designed for cloning such an expression vector using a two step cloning method, as referred to at paragraph 15 above and described in the grant application (**Exhibit D**) on the bottom of page 19. Note that the nucleic acids are synthesized in pairs (5' and 3') for use as 5' and 3' primers in a PCR amplification. For example, this is indicated by a "5" or a "3" at the end of each label, e.g., as in the first primer pair listed in the e-mail, "HPRTHpaZeo1 – 5" and "HPRThpazeo 1 – 3.".

18. Each primer consists of (a) a 28 nucleotide region of the target gene, followed by (b) a Hpa I restriction site (GTTAAC), followed by (c) a primer sequence for a Zeomycin selection

marker gene (Zeo) on a plasmid. Both a 5' nucleic acid and a 3' nucleic acid with these elements were to be synthesized as shown by the pairs of nucleic acids listed in **Exhibit E**. The elements of the first-listed nucleic acid in **Exhibit E** are labeled below:



19. The sequence of these nucleic acids reflects a two step cloning strategy for generating a DNA expression vector capable of expressing a short hairpin RNA having a double-stranded region of 28 base pairs. The nucleic acid pairs as indicated in **Exhibit E** are used as primers for a PCR reaction, using a Zeo selection marker gene as the PCR template. The amplified PCR product resulting from that PCR reaction is a double-stranded nucleic acid product that has a 28 nucleotide region of the target gene sequence, followed by a Hpa I restriction enzyme cleavage site, followed by the Zeomycin gene, followed by another Hpa I cleavage site, followed by the reverse complement of the 28 nucleotide region of the target gene.

20. In the first cloning step, the PCR product is cloned into an expression vector using Zeomycin selection. In the second cloning step, the vector is then digested using the HpaI restriction enzyme, resulting in a vector encoding a short hairpin consisting of (a) the target gene sequence, (b) a loop consisting of a HpaI restriction enzyme cleavage site and (c) the reverse complement of the target gene sequence. When transformed into bacterial cells, the HpaI site

facilitates selection of positive bacterial clones, i.e., those transformed with the expression vector. The draft SBIR Grant Application (**Exhibit D**) refers to such a two step cloning strategy at the bottom of page 19. s

21. The resulting expression vector constructed through this two step strategy encodes a short hairpin having a 28 base pair double-stranded region and an intervening loop consisting of an HpaI site. The short RNA hairpin encoded by an expression vector constructed using the primers listed in the Primer Order to Invitrogen (**Exhibit E**) has the same hairpin structure as shown in Figure 37 of the '797 application (see also **Exhibit F**).

22. The target genes referred to in **Exhibit E** and in **Exhibit D** include: human hypoxanthine—guanine phosphoribosyl transferase (HGPRT1 and HGPRT2 primers) and the mouse tyrosinase gene (tyro1 and tyro2 primers). The indicated target genes therefore indicate the resulting encoded short RNA hairpins (and expression constructs) are directed to silencing their corresponding target gene in mammalian cells, in particular, human cells and mouse cells. Additionally, as indicated in the Draft SBIR Grant Application (**Exhibit D**) on page 16 (second paragraph), HGRPT gene is directed to a gene target "for which exists a positive selection for loss-of-function" upon stable expression of the hairpin RNA in the cell.

D. Luciferase Simple Hairpin

23. Attached at **Exhibit F** is a copy of a slide dated at least by December 28, 2001. Information in this slide is also shown in Figure 37 in the parent application U.S. Serial No. 10/055,797. The slide illustrates two short hairpin RNA molecules. The second hairpin, the "Luciferase simple hairpin" has a double-stranded region consisting of 28 base pairs in length. .

The double-stranded region is highlighted. The double-stranded region of the short hairpin RNA molecule has a sequence that is complementary to a portion of the target gene, firefly luciferase.

24. The loop region of the hairpin on **Exhibit F** contains the sequence GUUAAC which is a HpaI restriction site. This is an example of a cloned simple hairpin that would be obtained using the methods described above in **Exhibit D** (specifically, the two-step method of hairpin cloning referred to here at paragraph 15) and using the PCR primers listed in **Exhibit E**.

E. Short Hairpin RNA Experiment in Human 293 T Cells

25. Attached at **Exhibit G** is a copy of a slide dated at least as early as October 2001. The title of the slide is "SHP 293T" indicating that this data is from an experiment using short hairpin RNA in 293T cells, a line of human embryonic kidney cells. This experiment assessed the ability of various short hairpin RNAs to specifically suppress gene expression in these cells, without provoking a PKR response. The 293T cells were co-transfected with a plasmid expressing the target gene, firefly luciferase, a plasmid expressing Renilla luciferase and one of various test hairpin RNAs. Subsequent to transfection, the level of expression of both luciferase proteins was measured. In the slide, the different test hairpin RNAs are indicated on the X axis of the slide underneath each of the bars. The respective bars indicate the degree to which the various introduced RNAs, including short hairpin RNAs, suppressed expression of the target firefly luciferase gene, as assayed by the ratio of firely luciferase to Renilla luciferase expression. As indicated in the slide, these results demonstrated that short RNA hairpins specifically suppressed expression of their target gene without provoking a PKR response in the cells.

26. For example, the nomenclature "SHP 25 luc hp" indicates a short hairpin RNA that has a double stranded region of 25 nucleotides in length. As the nomenclature indicates, the double-

stranded region of this short hairpin RNA molecule has a sequence that is complementary to a portion of the target gene, firefly luciferase. The bar graph shows, as a result of the experiment, a specific suppression of firefly luciferase gene expression in the 293T cells. See the bar labeled "SHP 25 luc hp" on the graph.

27. In the slide, the nomenclature "SHP 33 luc hp mism ngl3" indicates a short hairpin RNA that has a double stranded region of 33 nucleotides in length and has a mismatch in the sequence so that the sequence is not fully complementary to the sequence of the luciferase target gene. This bar of the bar graph shows, as a result of the experiment using a mismatched hairpin sequence, no specific suppression of firefly luciferase gene expression. The slide shows that short hairpin constructs with double-stranded regions of 32 nucleotides, 33 nucleotides, 34 nucleotides and 35 nucleotides did not exhibit attenuation of luciferase gene expression.

28. This slide shows an example of a short hairpin with a double-stranded region of 25 nucleotides in length, which did not trigger a protein kinase RNA-activated (PKR) response in the mammalian cell, and which did attenuate expression of the target gene, luciferase, in a sequence specific manner in the mammalian cells, 293T. The information in this slide was also included as Figure 39 of the parent application U.S. Serial No. 10/055,797.

F. Short Hairpin RNA Experiment in Human HeLa Cells

29. Attached at **Exhibit H** is a copy of a slide dated at least as early as October 2001. The slide shows data from an experiment using human HeLa cells (a cell line derived from human cervical cancer cells). We knew at the time of this experiment that long dsRNA initiates a PKR response in these cells. Using the same protocol as the experiment discussed above (**E**), this

experiment similarly assessed the ability of various short hairpin RNAs to specifically suppress gene expression in HeLa cells, without provoking a PKR response.

30. As indicated in the slide, these results demonstrated that short RNA hairpins specifically suppressed expression of their target gene without provoking a PKR response in the cells. For example, introducing a short hairpin RNA having a double-stranded region of 25 base pairs ("SHP 25 Luc hp") into the cells specifically suppressed expression of the firefly luciferase target gene. Longer double-stranded regions or mismatched target sequences did not result in suppression of target gene expression. The information in this slide was also included as Figure 40 of the parent application U.S. Serial No. 10/055,797.

G. Short Hairpin RNA Experiment in *Drosophila* S2 Cells

31. Attached at **Exhibit I** is a copy of a slide dated at least as early as October 2001. The data in this slide was generated using the same type of experimental procedure as discussed above in **Exhibits G and H**. The data in this slide indicates that short hairpin with a double-stranded region of 25 nucleotides ("SHP 25 luc hp") functioned to specifically inhibit expression of the target gene in the cells. The information in this slide was also included as Figure 38 of the parent application U.S. Serial No. 10/055,797.

H. Expression of Encoded Short Hairpins Specifically Suppress Gene Expression in Mammalian Cells

32. Attached at **Exhibit J** is a copy of a slide dated as least by January 2002 which shows results from an experiment which was included as Figure 42 (bottom) of the parent application U.S. Serial No. 10/055,797. The description of this experiment and the data can be found on page 17 of the '797 application. The results of this experiment demonstrate that expression of

encoded short hairpin RNAs effectively and specifically suppressed expression of a target gene in 293T cells, without provoking a PKR response. .

I. Paddison et al., *Genes Dev.* 2002, 16:948-958

33. The work described above culminated in several publications. One paper was published in *Genes and Development* in March 2002 entitled "Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells." (See **Exhibit K**.) This paper reports that "short hairpin RNAs (shRNAs) can be engineered to suppress the expression of desired genes in culture *Drosophila* and mammalian cells. shRNA can be synthesized exogenously or can be transcribed from RNA polymerase III promoters *in vivo*, thus permitting the construction of continuous cell lines or transgenic animals in which RNAi enforces stable and heritable gene silencing." (See Abstract of **Exhibit K**.)

34. A copy of a manuscript of the Paddison et al. paper (**Exhibit K**) that was prepared prior to publication and no later than January 31, 2002, as indicated by e-mails to which the manuscript was attached, is attached at **Exhibit L**.

35. Results of additional representative experiments, conducted similarly to the experiment referred to here in part **H**, "Expression of Encoded Short Hairpins Specifically Suppress Gene Expression in Mammalian Cells," are also reported in Paddison et al., among other places, at Fig. 4. (**Exhibit K**). Results of additional representative experiments conducted similarly to the Short Hairpin RNA Experiment in *Drosophila* S2 Cells (**G**), the Short Hairpin RNA Experiment in Human 293 T Cells (**E**) and the Short Hairpin RNA Experiment in Human HeLa Cells (**F**) are reported in Paddison et al., among other places, at Figs. 1 through 3. (**Exhibit K**). Figures 44A and 44B of the '676 application correspond to Figure 6A and 6B of Paddison et al. (**Exhibit K**).

J. Industry Awards

36. During 2002-2006, Paddison et al. (**Exhibit K**), having been cited by more than 500 subsequently published scientific papers, was therefore among the most highly cited "high impact" papers in the fields of molecular biology and genetics, as indicated by an analysis published by ScienceWatch.com (**Exhibit M**, see Table 2). A citation history summary for Paddison et al. (**Exhibit K)** is shown in **Exhibit N**.

37. Since we made the claimed invention and published Paddison et al., (**Exhibit K**), the invention of using stably expressed short hairpin RNAs to inhibit gene expression in mammalian cells has been recognized by industry organizations. For example, in 2005, Dr. Hannon received the Award for Outstanding Achievement in Cancer Research from the American Association for Cancer Research (AACR), which honored Dr. Hannon "…for his work uncovering the biochemical mechanism of RNA interference of gene expression (RNAi) and his contributions to the discovery and development of short hairpin RNAs as tools for genetic manipulation of mammalian cells." (**Exhibit O**).

38. In 2007, Dr. Hannon received two more prestigious awards, the Award in Molecular Biology from the National Academy of Sciences (**Exhibit P**), and the Paul Marks prize for the valuable contribution his RNAi work to cancer research from Memorial Sloan-Kettering Cancer Center (**Exhibit Q**). In granting that award, MSKCC noted how Dr. Hannon had applied his research in understanding the RNAi pathway to develop this valuable new technology, and his recognition as a leader in the field:

> Dr. Hannon is a leader in the relatively new field of RNA interference (RNAi). RNAi is a naturally occurring mechanism for regulating the expression of genes (controlling which genes are turned on and turned off in cells). In the laboratory, it is used as a

tool to study the function of specific genes, and it's being investigated as a therapeutic approach for treating many different diseases, including cancer.

Dr. Hannon's laboratory has elucidated key biochemical details of the components of the pathways involved in RNAi and is using these findings to develop molecular tools that can be used for gene discovery, the evaluation of gene function, and the generation of animal models. He has developed new techniques for using RNAi to study cancer development and is investigating possible cancer therapies that make use of small interfering RNAs (siRNAs).

Dr. Hannon discovered several proteins and enzymes that are an essential part of the RNAi mechanism, including Dicer, which cleaves double-stranded RNA into siRNAs; the RISC complex, which helps regulate protein translation and is involved in the body's defense against viral infections; and Argonaute2, which cleaves messenger RNA.

He also has been at the forefront of adapting RNAi techniques to study genes in mammals, and using these techniques to understand the variety of pathways that can lead to the formation of tumors.

K. Conclusion

39. The documents attached hereto as **Exhibits A - M** demonstrate that that the invention claimed, including claims 50, 52, 54-60, 62 and 63, was conceived at least as early as August 14, 2001, which is prior to the effective filing date of Caplen et al., Symonds et al., and Kreutzer et al. These documents and our declaration also show diligence and reduction(s) to practice.

40. We further declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that the making of willfully false statements and the like is punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United

States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed: _____
Gregory J. Hannon
Dated: _____

Signed: _____
Patrick J. Paddison
Dated: _____

Signed: _____
Scott Hammond
Dated: 1/18/11

Signed: _____
Amy Caudy
Dated: _____

Signed: _____
Emily Bernstein
Dated: _____

Signed: _____
Douglas Conklin
Dated: _____

Exhibits to Declaration Under 37 C.F.R. §1.131

Exhibit	Title
A	Hannon Draft Grant Application
B	Hammond et al., *Nature* 404:293-296 (2000)
C	Bernstein et al. *Nature* 409: 363-366 (2001)
D	Draft grant application to SBIR (Small Business Innovation Research)
E	Email of Primer Order to Invitrogen
F	Luciferase Simple Hairpin Slide
G	Short Hairpin RNA Experiment in Human 293 T Cells Slide
H	Short Hairpin RNA Experiment in Human HeLa Cells Slide
I	Short Hairpin RNA Experiment in *Drosophila* S2 Cells Slide
J	Short Hairpins Specifically Suppress Gene Expression Slide
K	Paddison et al., *Genes Dev.* 2002, 16:948-958
L	Manuscript of Paddison et al.
M	ScienceWatch Biology's Hottest 2002-2006
N	Paddison et al. Citations
O	2005 Award for Outstanding Achievement in Cancer Research from AACR
P	2007 Award in Molecular Biology from the National Academy of Sciences
Q	2007 Paul Marks Prize from Memorial Sloan-Kettering Cancer Center

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Hannon et al.	Confirmation No.:	8161
Application No:	11/894,676	Art Unit:	1635
Filed:	August 20, 2007	Examiner:	K. CHONG

Title: METHODS AND COMPOSITIONS FOR RNA INTERFERENCE

Docket No.: 287000-130-US3

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Declaration Under 37 C.F.R. §1.131

We, Gregory J. Hannon, Patrick J. Paddison, Scott Hammond, Amy Caudy and Emily Bernstein, Douglas Conklin hereby declare as follows:

1. We are the inventors of the above-referenced patent application.

2. All the work described within this declaration was performed in the United States.

3. All of the work described within this declaration was performed by us, or on our behalf and under our direction.

4. We have reviewed our records, including the slides documents submitted herewith, and declare that the claimed invention, which is

> a method for attenuating expression of a target gene in a mammalian cell, the method comprising introducing into a mammalian cell a library of RNA expression constructs, each expression construct comprising:
>
> (i) an RNA polymerase promoter, and

> (ii) a sequence encoding a short hairpin RNA molecule comprising a double-stranded region wherein the double-stranded region consists of at least 20 nucleotides but not more than 29 nucleotides,
>
> wherein the short hairpin RNA molecule is a substrate for Dicer-dependent cleavage and does not trigger a protein kinase RNA-activated (PKR) response in the mammalian cells,
>
> wherein the double-stranded region of the short hairpin RNA molecule comprises a sequence that is complementary to a portion of the target gene, and
>
> wherein the short hairpin RNA molecule is stably expressed in the mammalian cell in an amount sufficient to attenuate expression of the target gene in a sequence specific manner, and is expressed in the cell without use of a PK inhibitor,
>
> whereby expression of the target gene is inhibited

including original (and amended) claims 50, 52, 54-63 was conceived and reduced to practice at least prior to August 14, 2001, the publication date of Caplen et al., *PNAS*, Vol. 98, No. 17, pp. 9742-9747, which is also prior to December 28, 2001, *i.e.*, the filing date of U.S. Publication No. US 2002/0160393, Symonds et al., U.S. Serial No. 10/035,098 and which is also prior to the date of filing of the parent application of Kreutzer et al. (U.S. Serial No. 09/889,802, filed September 17, 2001).

A. Hannon Draft Grant Application

5. We attach a copy of a draft grant application (**Exhibit A**) which was prepared prior to August 14, 2001. A review of email indicates that this draft grant application was prepared at least by sometime in January 2000. The specific aims, as indicated on the first page of the draft grant application (**Exhibit A**, page 12), were directed to identifying and characterizing the critical components of the RNA interference (RNAi) machinery. The "Preliminary Results" this page refers to (see 4th paragraph on page 12) were reported in Hammond et al., *Nature* 404:293-

296 (2000) (**Exhibit B**) in a paper entitled "An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophia* cells."

6. In particular, one aspect of the proposed work was directed to isolating and cloning the protein and RNA components of the RISC complex (RNA-induced silencing complex), the nuclease complex responsible for degradation of target mRNAs, and characterizing it's function, both *in vitro* and *in vivo*. To allow us to carry out such studies, we established a model system using cultured *Drosophila* cells that provided a readily available source of material in sufficient quantities for the necessary biochemical studies.

7. The Summary on page 15 provides the rationale for the proposed work:

> My laboratory has devoted a number of years to creating improved tools for probing gene function in cultured mammalian cells; however, our experience indicates that a facile loss-of-function tool is lacking. Unfortunately, dsRNA induces somewhat generic responses in mammalian cells. It is our hope that by understanding the mechanistic basis of dsRNA-induced silencing, we may not only unravel a mysterious and important piece of biology but also provide the means to create improved tools for analyzing gene function in diverse organisms in which traditional genetic methods are either cumbersome or unavailable. This notion that has contributed to the decision to focus substantial effort in my laboratory toward elucidating the mechanism of RNA interference.

The final paragraph on page 36 further elaborates on this rationale:

> In this application, we propose a biochemical approach to deciphering the mechanisms that underlie dsRNA-induced gene silencing. RNA-interference allows an adaptive defense against both exogenous and endogenous dsRNAs, providing something akin to a dsRNA immune response. The primary goal of the work proposed in this application is to understand the mechanisms by which a cell can raise this response. We have presented evidence that RNA interference is accomplished, at least in part, through the action of a sequence-specific nuclease that is generated in response to dsRNA. Our data, and that of others (Hamilton and Baulcombe, 1999), is consistent with a model in which dsRNAs present in a cell are converted, in a manner analogous to antigen processing, into discrete, small RNAs that guide the nuclease in the choice of substrate.

> We propose to purify and characterize the nuclease and to clone the protein and RNA components of the enzyme. In addition, we propose to develop approaches that may allow the use of cultured *Drosophila* cells as a general tool for probing gene function. The combination of these studies may lead eventually to an ability to harness RNA interference as a genetic tool in other organisms, particularly mammals, in which analogous tools are presently lacking.

8. At that time, there was a lack of available practical loss-of-function tools for probing gene function in mammalian cells. The work proposed in this draft application to elucidate the mechanism of RNA interference was intended to develop such tools. In other words, by understanding the mechanistic basis of RNA interference, we could use that understanding to exploit the RNAi pathway and create new tools to study gene function and the lack of certain gene function in mammalian cells.

9. The work proposed in this application to identify and characterize components of the RNAi cellular machinery was carried out by us prior to August 14, 2001. Certain aspects of this work were reported in Bernstein et al. *Nature* 409: 363-366 (2001) (**Exhibit C**) in a paper entitled "Role for a bidentate ribonuclease in the initiation step of RNA interference." This paper describes the identification and cloning of the enzyme, which we named "Dicer." The paper describes how this enzyme is evolutionarily conserved in worms, flies, plants, fungi and mammals, and the paper reports the role of this newly discovered enzyme in the RNAi pathway in cells. In particular, these results indicated that the process of gene silencing through the RNAi pathway could be divided into at least two distinct steps. In the first step, long dsRNA (double-stranded RNA) is processed by Dicer into approximately 22 nt (nucleotide) "guide" sequences. In the second step, these guide RNAs are incorporated into a distinct nuclease complex we first called the "RNA-induced silencing complex" or RISC. The RISC complex uses the guide sequences to specifically identify and destroy homologous mRNAs. We named the RNAs that

were processed by Dicer "guide sequences" or "guide RNAs" based on their role in targeting RISC to specific mRNAs based on sequence. The results and work described in Bernstein et al. (2001) were included in this patent application, U.S. Serial No. 11/894,676, and also in the related application U.S. Serial No. 10/055,797, such as in Example 2.

B. Draft SBIR Grant Application

10. We attach as **Exhibit D** a copy of a draft grant application to SBIR (Small Business Innovation Research) which was prepared prior to August 14, 2001.[1]

11. The first page of this draft grant lists three Aims directed toward achieving stable gene silencing in mammalian cells. Aim 1 is the "creation of stable, loss-of-function mutations in embryonic cells using RNAi." Aim 2 is the "creation of stable loss-of-function mutations in non-embryonic cell types," which proposes "numerous strategies for bypassing [the] problem" that "long dsRNAs provoke a PKR response in differentiated cell types."

12. Attached pages 13-25 of **Exhibit D** provide more detail regarding each of these Aims. Starting on page 13, the grant application describes the Experimental Procedures for Aim 1. Aim 1 is defined as "Creation of stable, loss-of-function mutations in embryonic cells using RNAi." On page 14, the grant states that "[w]e have chosen to approach this goal by encoding dsRNA in the form of an inverted repeat or hairpin that can be expressed from a promoter of choice." Regarding this objective, on page 14 the draft grant states that "[w]e have achieved the goal of simplified hairpin construction by dividing the process into two steps (Fig. 6)." Figure 6 is on page 15 and depicts a "strategy for the creation of hairpin RNAs for stable expression of dsRNA" and illustrates that "expression of a GFP hairpin RNA induced stable silencing of an

[1] For convenience, we have added page numbers to this document.

exogenous GFP reporter in [mouse embryonic] P19 cells." The use of the strategy and also the results described in Aim 1 are described in Example 3 (entitled "A Simplified Method for the Creation of Hairpin Constructs for RNA Interference") and Fig. 27, and in Example 4 (entitled "Long dsRNAs Suppress Gene Expression in Mammalian Cells") and Figs. 28-34 of the parent application, U.S. Serial No. 10/055,797. Aim 1 also describes silencing mammalian genes for which assays are available to allow "positive selection for loss-or function" in mammalian cells, *e.g.*, HPRT and TK. (See 2nd paragraph on page 16 of **Exhibit D**.)

13. The grant application states the goals for Aim 2 on the top of page 18 of **Exhibit D**: "our goal is to devise strategies for presentation of the dsRNA trigger that allow it to elude PKR surveillance." The "Expression Strategies" provided in the grant state that "PKR requires approximately 30 bp of contiguous double-stranded sequence to trigger dimerization and activation of the enzyme." (See first paragraph under "Expression Strategies" on page 18. The third paragraph in that section on page 18 describes expression of hairpin RNAs in various mammalian cells: "NIH 3T3, 293, HeLa, U2OS, Rat 1 and C2C12" and various expression vectors incorporating various promoters, including U1, U6 and CMV.

14. In the section entitled "Short RNA hairpins" on page 19 of **Exhibit D**, the grant application describes use of short RNA hairpins that are "below the cut-off for triggering RNA for investigating "whether the expression of short RNA hairpins can be used to induce efficient silencing." The research plan here also refers to "short synthetic RNAs that mimic our Dicer products." In other words, this refers to RNAs that have a double-stranded region of 20 to 22 base pairs. It further states that "short synthetic hairpins directed against GFP, TK and HPRT will be expressed from CMV, U1 and U6 promoter vectors in the cell types noted above." (See page 19.)

15. The grant application on page 19 describes two methods for modifying the approach described in Aim 1 to "create hairpins with significantly shorter loops." The first is "to simply clone short hairpin sequences [either] as single, synthetic DNA fragments, and the second is to clone "in two steps if hairpin formation in such synthetic oligonucleotides competes too vigorously with intermolecular hybridization to produce clonable fragments." Furthermore, Figure 7 on page 21 of **Exhibit D** depicts the use of libraries of expression vectors expressing an encoded "dsRNA cassette" to carry out functional screens in cultured cells.

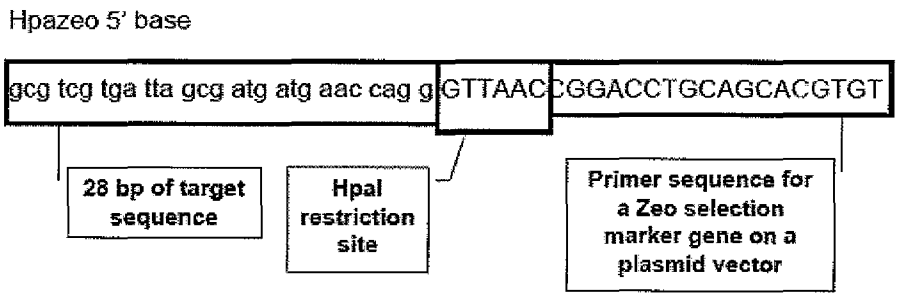
C. Primer Order to Invitrogen

16. Attached at **Exhibit E** is a copy of an email that was sent to Invitrogen to order oligonucleotide primers. The email was sent prior to August 14, 2001.

17. The email lists a number of pairs of oligonucleotide primers which were to be synthesized for use in cloning a sequence encoding a short hairpin RNA into a plasmid expression vector in order to obtain a short hairpin expression product as shown in Figure 37 of the parent application, U.S. Serial No. 10/055,797. These oligonucleotides requested through this e-mail order are examples of oligonucleotide primers designed for cloning such an expression vector using a two step cloning method, as referred to at paragraph 15 above and described in the grant application (**Exhibit D**) on the bottom of page 19. Note that the nucleic acids are synthesized in pairs (5' and 3') for use as 5' and 3' primers in a PCR amplification. For example, this is indicated by a "5" or a "3" at the end of each label, e.g., as in the first primer pair listed in the e-mail, "HPRTHpaZeo1 – 5" and "HPRThpazeo 1 – 3.".

18. Each primer consists of (a) a 28 nucleotide region of the target gene, followed by (b) a Hpa I restriction site (GTTAAC), followed by (c) a primer sequence for a Zeomycin selection

marker gene (Zeo) on a plasmid. Both a 5' nucleic acid and a 3' nucleic acid with these elements were to be synthesized as shown by the pairs of nucleic acids listed in **Exhibit E**. The elements of the first-listed nucleic acid in **Exhibit E** are labeled below:



19. The sequence of these nucleic acids reflects a two step cloning strategy for generating a DNA expression vector capable of expressing a short hairpin RNA having a double-stranded region of 28 base pairs. The nucleic acid pairs as indicated in **Exhibit E** are used as primers for a PCR reaction, using a Zeo selection marker gene as the PCR template. The amplified PCR product resulting from that PCR reaction is a double-stranded nucleic acid product that has a 28 nucleotide region of the target gene sequence, followed by a Hpa I restriction enzyme cleavage site, followed by the Zeomycin gene, followed by another Hpa I cleavage site, followed by the reverse complement of the 28 nucleotide region of the target gene.

20. In the first cloning step, the PCR product is cloned into an expression vector using Zeomycin selection. In the second cloning step, the vector is then digested using the HpaI restriction enzyme, resulting in a vector encoding a short hairpin consisting of (a) the target gene sequence, (b) a loop consisting of a HpaI restriction enzyme cleavage site and (c) the reverse complement of the target gene sequence. When transformed into bacterial cells, the HpaI site

facilitates selection of positive bacterial clones, i.e., those transformed with the expression vector. The draft SBIR Grant Application (**Exhibit D**) refers to such a two step cloning strategy at the bottom of page 19. s

21. The resulting expression vector constructed through this two step strategy encodes a short hairpin having a 28 base pair double-stranded region and an intervening loop consisting of an HpaI site. The short RNA hairpin encoded by an expression vector constructed using the primers listed in the Primer Order to Invitrogen (**Exhibit E**) has the same hairpin structure as shown in Figure 37 of the '797 application (see also **Exhibit F**).

22. The target genes referred to in **Exhibit E** and in **Exhibit D** include: human hypoxanthine—guanine phosphoribosyl transferase (HGPRT1 and HGPRT2 primers) and the mouse tyrosinase gene (tyro1 and tyro2 primers). The indicated target genes therefore indicate the resulting encoded short RNA hairpins (and expression constructs) are directed to silencing their corresponding target gene in mammalian cells, in particular, human cells and mouse cells. Additionally, as indicated in the Draft SBIR Grant Application (**Exhibit D**) on page 16 (second paragraph), HGRPT gene is directed to a gene target "for which exists a positive selection for loss-of-function" upon stable expression of the hairpin RNA in the cell.

D. Luciferase Simple Hairpin

23. Attached at **Exhibit F** is a copy of a slide dated at least by December 28, 2001. Information in this slide is also shown in Figure 37 in the parent application U.S. Serial No. 10/055,797. The slide illustrates two short hairpin RNA molecules. The second hairpin, the "Luciferase simple hairpin" has a double-stranded region consisting of 28 base pairs in length. .

The double-stranded region is highlighted. The double-stranded region of the short hairpin RNA molecule has a sequence that is complementary to a portion of the target gene, firefly luciferase.

24. The loop region of the hairpin on **Exhibit F** contains the sequence GUUAAC which is a HpaI restriction site. This is an example of a cloned simple hairpin that would be obtained using the methods described above in **Exhibit D** (specifically, the two-step method of hairpin cloning referred to here at paragraph 15) and using the PCR primers listed in **Exhibit E**.

E. Short Hairpin RNA Experiment in Human 293 T Cells

25. Attached at **Exhibit G** is a copy of a slide dated at least as early as October 2001. The title of the slide is "SHP 293T" indicating that this data is from an experiment using short hairpin RNA in 293T cells, a line of human embryonic kidney cells. This experiment assessed the ability of various short hairpin RNAs to specifically suppress gene expression in these cells, without provoking a PKR response. The 293T cells were co-transfected with a plasmid expressing the target gene, firefly luciferase, a plasmid expressing Renilla luciferase and one of various test hairpin RNAs. Subsequent to transfection, the level of expression of both luciferase proteins was measured. In the slide, the different test hairpin RNAs are indicated on the X axis of the slide underneath each of the bars. The respective bars indicate the degree to which the various introduced RNAs, including short hairpin RNAs, suppressed expression of the target firefly luciferase gene, as assayed by the ratio of firely luciferase to Renilla luciferase expression. As indicated in the slide, these results demonstrated that short RNA hairpins specifically suppressed expression of their target gene without provoking a PKR response in the cells.

26. For example, the nomenclature "SHP 25 luc hp" indicates a short hairpin RNA that has a double stranded region of 25 nucleotides in length. As the nomenclature indicates, the double-

stranded region of this short hairpin RNA molecule has a sequence that is complementary to a portion of the target gene, firefly luciferase. The bar graph shows, as a result of the experiment, a specific suppression of firefly luciferase gene expression in the 293T cells. See the bar labeled "SHP 25 luc hp" on the graph.

27. In the slide, the nomenclature "SHP 33 luc hp mism ngl3" indicates a short hairpin RNA that has a double stranded region of 33 nucleotides in length and has a mismatch in the sequence so that the sequence is not fully complementary to the sequence of the luciferase target gene. This bar of the bar graph shows, as a result of the experiment using a mismatched hairpin sequence, no specific suppression of firefly luciferase gene expression. The slide shows that short hairpin constructs with double-stranded regions of 32 nucleotides, 33 nucleotides, 34 nucleotides and 35 nucleotides did not exhibit attenuation of luciferase gene expression.

28. This slide shows an example of a short hairpin with a double-stranded region of 25 nucleotides in length, which did not trigger a protein kinase RNA-activated (PKR) response in the mammalian cell, and which did attenuate expression of the target gene, luciferase, in a sequence specific manner in the mammalian cells, 293T. The information in this slide was also included as Figure 39 of the parent application U.S. Serial No. 10/055,797.

F. Short Hairpin RNA Experiment in Human HeLa Cells

29. Attached at **Exhibit H** is a copy of a slide dated at least as early as October 2001. The slide shows data from an experiment using human HeLa cells (a cell line derived from human cervical cancer cells). We knew at the time of this experiment that long dsRNA initiates a PKR response in these cells. Using the same protocol as the experiment discussed above (**E**), this

experiment similarly assessed the ability of various short hairpin RNAs to specifically suppress gene expression in HeLa cells, without provoking a PKR response.

30. As indicated in the slide, these results demonstrated that short RNA hairpins specifically suppressed expression of their target gene without provoking a PKR response in the cells. For example, introducing a short hairpin RNA having a double-stranded region of 25 base pairs ("SHP 25 Luc hp") into the cells specifically suppressed expression of the firefly luciferase target gene. Longer double-stranded regions or mismatched target sequences did not result in suppression of target gene expression. The information in this slide was also included as Figure 40 of the parent application U.S. Serial No. 10/055,797.

G. Short Hairpin RNA Experiment in *Drosophila* S2 Cells

31. Attached at **Exhibit I** is a copy of a slide dated at least as early as October 2001. The data in this slide was generated using the same type of experimental procedure as discussed above in **Exhibits G and H**. The data in this slide indicates that short hairpin with a double-stranded region of 25 nucleotides ("SHP 25 luc hp") functioned to specifically inhibit expression of the target gene in the cells. The information in this slide was also included as Figure 38 of the parent application U.S. Serial No. 10/055,797.

H. Expression of Encoded Short Hairpins Specifically Suppress Gene Expression in Mammalian Cells

32. Attached at **Exhibit J** is a copy of a slide dated as least by January 2002 which shows results from an experiment which was included as Figure 42 (bottom) of the parent application U.S. Serial No. 10/055,797. The description of this experiment and the data can be found on page 17 of the '797 application. The results of this experiment demonstrate that expression of

encoded short hairpin RNAs effectively and specifically suppressed expression of a target gene in 293T cells, without provoking a PKR response. .

I. Paddison et al., *Genes Dev.* 2002, 16:948-958

33. The work described above culminated in several publications. One paper was published in *Genes and Development* in March 2002 entitled "Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells." (See **Exhibit K.**) This paper reports that "short hairpin RNAs (shRNAs) can be engineered to suppress the expression of desired genes in culture *Drosophila* and mammalian cells. shRNA can be synthesized exogenously or can be transcribed from RNA polymerase III promoters *in vivo*, thus permitting the construction of continuous cell lines or transgenic animals in which RNAi enforces stable and heritable gene silencing." (See Abstract of **Exhibit K.**)

34. A copy of a manuscript of the Paddison et al. paper (**Exhibit K**) that was prepared prior to publication and no later than January 31, 2002, as indicated by e-mails to which the manuscript was attached, is attached at **Exhibit L.**

35. Results of additional representative experiments, conducted similarly to the experiment referred to here in part **H**, "Expression of Encoded Short Hairpins Specifically Suppress Gene Expression in Mammalian Cells," are also reported in Paddison et al., among other places, at Fig. 4. (**Exhibit K**). Results of additional representative experiments conducted similarly to the Short Hairpin RNA Experiment in *Drosophila* S2 Cells (**G**), the Short Hairpin RNA Experiment in Human 293 T Cells (**E**) and the Short Hairpin RNA Experiment in Human HeLa Cells (**F**) are reported in Paddison et al., among other places, at Figs. 1 through 3. (**Exhibit K**). Figures 44A and 44B of the '676 application correspond to Figure 6A and 6B of Paddison et al. (**Exhibit K**).

J. Industry Awards

36. During 2002-2006, Paddison et al. (**Exhibit K**), having been cited by more than 500 subsequently published scientific papers, was therefore among the most highly cited "high impact" papers in the fields of molecular biology and genetics, as indicated by an analysis published by ScienceWatch.com (**Exhibit M**, see Table 2). A citation history summary for Paddison et al. (**Exhibit K**) is shown in **Exhibit N**.

37. Since we made the claimed invention and published Paddison et al., (**Exhibit K**), the invention of using stably expressed short hairpin RNAs to inhibit gene expression in mammalian cells has been recognized by industry organizations. For example, in 2005, Dr. Hannon received the Award for Outstanding Achievement in Cancer Research from the American Association for Cancer Research (AACR), which honored Dr. Hannon "...for his work uncovering the biochemical mechanism of RNA interference of gene expression (RNAi) and his contributions to the discovery and development of short hairpin RNAs as tools for genetic manipulation of mammalian cells." (**Exhibit O**).

38. In 2007, Dr. Hannon received two more prestigious awards, the Award in Molecular Biology from the National Academy of Sciences (**Exhibit P**), and the Paul Marks prize for the valuable contribution his RNAi work to cancer research from Memorial Sloan-Kettering Cancer Center (**Exhibit Q**). In granting that award, MSKCC noted how Dr. Hannon had applied his research in understanding the RNAi pathway to develop this valuable new technology, and his recognition as a leader in the field:

> Dr. Hannon is a leader in the relatively new field of RNA interference (RNAi). RNAi is a naturally occurring mechanism for regulating the expression of genes (controlling which genes are turned on and turned off in cells). In the laboratory, it is used as a

tool to study the function of specific genes, and it's being investigated as a therapeutic approach for treating many different diseases, including cancer.

Dr. Hannon's laboratory has elucidated key biochemical details of the components of the pathways involved in RNAi and is using these findings to develop molecular tools that can be used for gene discovery, the evaluation of gene function, and the generation of animal models. He has developed new techniques for using RNAi to study cancer development and is investigating possible cancer therapies that make use of small interfering RNAs (siRNAs).

Dr. Hannon discovered several proteins and enzymes that are an essential part of the RNAi mechanism, including Dicer, which cleaves double-stranded RNA into siRNAs; the RISC complex, which helps regulate protein translation and is involved in the body's defense against viral infections; and Argonaute2, which cleaves messenger RNA.

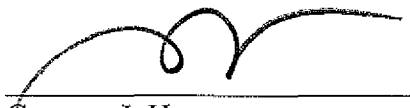
He also has been at the forefront of adapting RNAi techniques to study genes in mammals, and using these techniques to understand the variety of pathways that can lead to the formation of tumors.

K. Conclusion

39. The documents attached hereto as **Exhibits A - M** demonstrate that that the invention claimed, including claims 50, 52, 54-60, 62 and 63, was conceived at least as early as August 14, 2001, which is prior to the effective filing date of Caplen et al., Symonds et al., and Kreutzer et al. These documents and our declaration also show diligence and reduction(s) to practice.

40. We further declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that the making of willfully false statements and the like is punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United

States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed: 
Gregory J. Hannon

Dated: 1/31/11

Signed: _____
Patrick J. Paddison

Dated: _____

Signed: _____
Scott Hammond

Dated: _____

Signed: _____
Amy Caudy

Dated: _____

Signed: _____
Emily Bernstein

Dated: _____

Signed: _____
Douglas Conklin

Dated: _____

Exhibits to Declaration Under 37 C.F.R. §1.131

Exhibit	Title
A	Hannon Draft Grant Application
B	Hammond et al., *Nature* 404:293-296 (2000)
C	Bernstein et al. *Nature* 409: 363-366 (2001)
D	Draft grant application to SBIR (Small Business Innovation Research)
E	Email of Primer Order to Invitrogen
F	Luciferase Simple Hairpin Slide
G	Short Hairpin RNA Experiment in Human 293 T Cells Slide
H	Short Hairpin RNA Experiment in Human HeLa Cells Slide
I	Short Hairpin RNA Experiment in *Drosophila* S2 Cells Slide
J	Short Hairpins Specifically Suppress Gene Expression Slide
K	Paddison et al., *Genes Dev.* 2002, 16:948-958
L	Manuscript of Paddison et al.
M	ScienceWatch Biology's Hottest 2002-2006
N	Paddison et al. Citations
O	2005 Award for Outstanding Achievement in Cancer Research from AACR
P	2007 Award in Molecular Biology from the National Academy of Sciences
Q	2007 Paul Marks Prize from Memorial Sloan-Kettering Cancer Center

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Hannon et al.	Confirmation No.:	8161
Application No:	11/894,676	Art Unit:	1635
Filed:	August 20, 2007	Examiner:	K. CHONG
Title:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Docket No.: 287000-130-US3

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Declaration Under 37 C.F.R. §1.131

We, Gregory J. Hannon, Patrick J. Paddison, Scott Hammond, Amy Caudy and Emily Bernstein, Douglas Conklin hereby declare as follows:

1. We are the inventors of the above-referenced patent application.

2. All the work described within this declaration was performed in the United States.

3. All of the work described within this declaration was performed by us, or on our behalf and under our direction.

4. We have reviewed our records, including the slides documents submitted herewith, and declare that the claimed invention, which is

> a method for attenuating expression of a target gene in a mammalian cell, the method comprising introducing into a mammalian cell a library of RNA expression constructs, each expression construct comprising:
>
> (i) an RNA polymerase promoter, and

(ii) a sequence encoding a short hairpin RNA molecule comprising a double-stranded region wherein the double-stranded region consists of at least 20 nucleotides but not more than 29 nucleotides,

wherein the short hairpin RNA molecule is a substrate for Dicer-dependent cleavage and does not trigger a protein kinase RNA-activated (PKR) response in the mammalian cells,

wherein the double-stranded region of the short hairpin RNA molecule comprises a sequence that is complementary to a portion of the target gene, and

wherein the short hairpin RNA molecule is stably expressed in the mammalian cell in an amount sufficient to attenuate expression of the target gene in a sequence specific manner, and is expressed in the cell without use of a PK inhibitor,

whereby expression of the target gene is inhibited

including original (and amended) claims 50, 52, 54-63 was conceived and reduced to practice at least prior to August 14, 2001, the publication date of Caplen et al., *PNAS*, Vol. 98, No. 17, pp. 9742-9747, which is also prior to December 28, 2001, *i.e.*, the filing date of U.S. Publication No. US 2002/0160393, Symonds et al., U.S. Serial No. 10/035,098 and which is also prior to the date of filing of the parent application of Kreutzer et al. (U.S. Serial No. 09/889,802, filed September 17, 2001).

A. Hannon Draft Grant Application

5. We attach a copy of a draft grant application (**Exhibit A**) which was prepared prior to August 14, 2001. A review of email indicates that this draft grant application was prepared at least by sometime in January 2000. The specific aims, as indicated on the first page of the draft grant application (**Exhibit A**, page 12), were directed to identifying and characterizing the critical components of the RNA interference (RNAi) machinery. The "Preliminary Results" this page refers to (see 4th paragraph on page 12) were reported in Hammond et al., *Nature* 404:293-

296 (2000) (**Exhibit B**) in a paper entitled "An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophia* cells."

6. In particular, one aspect of the proposed work was directed to isolating and cloning the protein and RNA components of the RISC complex (RNA-induced silencing complex), the nuclease complex responsible for degradation of target mRNAs, and characterizing it's function, both *in vitro* and *in vivo*. To allow us to carry out such studies, we established a model system using cultured *Drosophila* cells that provided a readily available source of material in sufficient quantities for the necessary biochemical studies.

7. The Summary on page 15 provides the rationale for the proposed work:

> My laboratory has devoted a number of years to creating improved tools for probing gene function in cultured mammalian cells; however, our experience indicates that a facile loss-of-function tool is lacking. Unfortunately, dsRNA induces somewhat generic responses in mammalian cells. It is our hope that by understanding the mechanistic basis of dsRNA-induced silencing, we may not only unravel a mysterious and important piece of biology but also provide the means to create improved tools for analyzing gene function in diverse organisms in which traditional genetic methods are either cumbersome or unavailable. This notion that has contributed to the decision to focus substantial effort in my laboratory toward elucidating the mechanism of RNA interference.

The final paragraph on page 36 further elaborates on this rationale:

> In this application, we propose a biochemical approach to deciphering the mechanisms that underlie dsRNA-induced gene silencing. RNA-interference allows an adaptive defense against both exogenous and endogenous dsRNAs, providing something akin to a dsRNA immune response. The primary goal of the work proposed in this application is to understand the mechanisms by which a cell can raise this response. We have presented evidence that RNA interference is accomplished, at least in part, through the action of a sequence-specific nuclease that is generated in response to dsRNA. Our data, and that of others (Hamilton and Baulcombe, 1999), is consistent with a model in which dsRNAs present in a cell are converted, in a manner analogous to antigen processing, into discrete, small RNAs that guide the nuclease in the choice of substrate.

> We propose to purify and characterize the nuclease and to clone the protein and RNA components of the enzyme. In addition, we propose to develop approaches that may allow the use of cultured *Drosophila* cells as a general tool for probing gene function. The combination of these studies may lead eventually to an ability to harness RNA interference as a genetic tool in other organisms, particularly mammals, in which analogous tools are presently lacking.

8. At that time, there was a lack of available practical loss-of-function tools for probing gene function in mammalian cells. The work proposed in this draft application to elucidate the mechanism of RNA interference was intended to develop such tools. In other words, by understanding the mechanistic basis of RNA interference, we could use that understanding to exploit the RNAi pathway and create new tools to study gene function and the lack of certain gene function in mammalian cells.

9. The work proposed in this application to identify and characterize components of the RNAi cellular machinery was carried out by us prior to August 14, 2001. Certain aspects of this work were reported in Bernstein et al. *Nature* 409: 363-366 (2001) (**Exhibit C**) in a paper entitled "Role for a bidentate ribonuclease in the initiation step of RNA interference." This paper describes the identification and cloning of the enzyme, which we named "Dicer." The paper describes how this enzyme is evolutionarily conserved in worms, flies, plants, fungi and mammals, and the paper reports the role of this newly discovered enzyme in the RNAi pathway in cells. In particular, these results indicated that the process of gene silencing through the RNAi pathway could be divided into at least two distinct steps. In the first step, long dsRNA (double-stranded RNA) is processed by Dicer into approximately 22 nt (nucleotide) "guide" sequences. In the second step, these guide RNAs are incorporated into a distinct nuclease complex we first called the "RNA-induced silencing complex" or RISC. The RISC complex uses the guide sequences to specifically identify and destroy homologous mRNAs. We named the RNAs that

were processed by Dicer "guide sequences" or "guide RNAs" based on their role in targeting RISC to specific mRNAs based on sequence. The results and work described in Bernstein et al. (2001) were included in this patent application, U.S. Serial No. 11/894,676, and also in the related application U.S. Serial No. 10/055,797, such as in Example 2.

B. Draft SBIR Grant Application

10. We attach as **Exhibit D** a copy of a draft grant application to SBIR (Small Business Innovation Research) which was prepared prior to August 14, 2001.[1]

11. The first page of this draft grant lists three Aims directed toward achieving stable gene silencing in mammalian cells. Aim 1 is the "creation of stable, loss-of-function mutations in embryonic cells using RNAi." Aim 2 is the "creation of stable loss-of-function mutations in non-embryonic cell types," which proposes "numerous strategies for bypassing [the] problem" that "long dsRNAs provoke a PKR response in differentiated cell types."

12. Attached pages 13-25 of **Exhibit D** provide more detail regarding each of these Aims. Starting on page 13, the grant application describes the Experimental Procedures for Aim 1. Aim 1 is defined as "Creation of stable, loss-of-function mutations in embryonic cells using RNAi." On page 14, the grant states that "[w]e have chosen to approach this goal by encoding dsRNA in the form of an inverted repeat or hairpin that can be expressed from a promoter of choice." Regarding this objective, on page 14 the draft grant states that "[w]e have achieved the goal of simplified hairpin construction by dividing the process into two steps (Fig. 6)." Figure 6 is on page 15 and depicts a "strategy for the creation of hairpin RNAs for stable expression of dsRNA" and illustrates that "expression of a GFP hairpin RNA induced stable silencing of an

[1] For convenience, we have added page numbers to this document.

exogenous GFP reporter in [mouse embryonic] P19 cells." The use of the strategy and also the results described in Aim 1 are described in Example 3 (entitled "A Simplified Method for the Creation of Hairpin Constructs for RNA Interference") and Fig. 27, and in Example 4 (entitled "Long dsRNAs Suppress Gene Expression in Mammalian Cells") and Figs. 28-34 of the parent application, U.S. Serial No. 10/055,797. Aim 1 also describes silencing mammalian genes for which assays are available to allow "positive selection for loss-or function" in mammalian cells, *e.g.*, HPRT and TK. (See 2nd paragraph on page 16 of **Exhibit D**.)

13. The grant application states the goals for Aim 2 on the top of page 18 of **Exhibit D**: "our goal is to devise strategies for presentation of the dsRNA trigger that allow it to elude PKR surveillance." The "Expression Strategies" provided in the grant state that "PKR requires approximately 30 bp of contiguous double-stranded sequence to trigger dimerization and activation of the enzyme." (See first paragraph under "Expression Strategies" on page 18. The third paragraph in that section on page 18 describes expression of hairpin RNAs in various mammalian cells: "NIH 3T3, 293, HeLa, U2OS, Rat 1 and C2C12" and various expression vectors incorporating various promoters, including U1, U6 and CMV.

14. In the section entitled "Short RNA hairpins" on page 19 of **Exhibit D**, the grant application describes use of short RNA hairpins that are "below the cut-off for triggering RNA for investigating "whether the expression of short RNA hairpins can be used to induce efficient silencing." The research plan here also refers to "short synthetic RNAs that mimic our Dicer products." In other words, this refers to RNAs that have a double-stranded region of 20 to 22 base pairs. It further states that "short synthetic hairpins directed against GFP, TK and HPRT will be expressed from CMV, U1 and U6 promoter vectors in the cell types noted above." (See page 19.)

15. The grant application on page 19 describes two methods for modifying the approach described in Aim 1 to "create hairpins with significantly shorter loops." The first is "to simply clone short hairpin sequences [either] as single, synthetic DNA fragments, and the second is to clone "in two steps if hairpin formation in such synthetic oligonucleotides competes too vigorously with intermolecular hybridization to produce clonable fragments." Furthermore, Figure 7 on page 21 of **Exhibit D** depicts the use of libraries of expression vectors expressing an encoded "dsRNA cassette" to carry out functional screens in cultured cells.

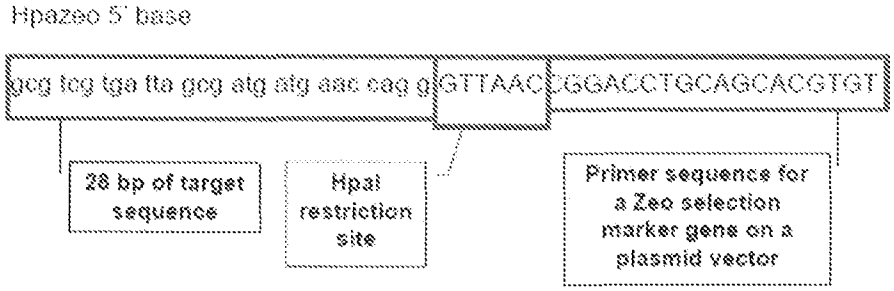
C. Primer Order to Invitrogen

16. Attached at **Exhibit E** is a copy of an email that was sent to Invitrogen to order oligonucleotide primers. The email was sent prior to August 14, 2001.

17. The email lists a number of pairs of oligonucleotide primers which were to be synthesized for use in cloning a sequence encoding a short hairpin RNA into a plasmid expression vector in order to obtain a short hairpin expression product as shown in Figure 37 of the parent application, U.S. Serial No. 10/055,797. These oligonucleotides requested through this e-mail order are examples of oligonucleotide primers designed for cloning such an expression vector using a two step cloning method, as referred to at paragraph 15 above and described in the grant application (**Exhibit D**) on the bottom of page 19. Note that the nucleic acids are synthesized in pairs (5' and 3') for use as 5' and 3' primers in a PCR amplification. For example, this is indicated by a "5" or a "3" at the end of each label, e.g., as in the first primer pair listed in the e-mail, "HPRTHpaZeo1 – 5" and "HPRThpazeo 1 – 3.".

18. Each primer consists of (a) a 28 nucleotide region of the target gene, followed by (b) a Hpa I restriction site (GTTAAC), followed by (c) a primer sequence for a Zeomycin selection

marker gene (Zeo) on a plasmid. Both a 5' nucleic acid and a 3' nucleic acid with these elements were to be synthesized as shown by the pairs of nucleic acids listed in **Exhibit E**. The elements of the first-listed nucleic acid in **Exhibit E** are labeled below:



19. The sequence of these nucleic acids reflects a two step cloning strategy for generating a DNA expression vector capable of expressing a short hairpin RNA having a double-stranded region of 28 base pairs. The nucleic acid pairs as indicated in **Exhibit E** are used as primers for a PCR reaction, using a Zeo selection marker gene as the PCR template. The amplified PCR product resulting from that PCR reaction is a double-stranded nucleic acid product that has a 28 nucleotide region of the target gene sequence, followed by a Hpa I restriction enzyme cleavage site, followed by the Zeomycin gene, followed by another Hpa I cleavage site, followed by the reverse complement of the 28 nucleotide region of the target gene.

20. In the first cloning step, the PCR product is cloned into an expression vector using Zeomycin selection. In the second cloning step, the vector is then digested using the HpaI restriction enzyme, resulting in a vector encoding a short hairpin consisting of (a) the target gene sequence, (b) a loop consisting of a HpaI restriction enzyme cleavage site and (c) the reverse complement of the target gene sequence. When transformed into bacterial cells, the HpaI site

facilitates selection of positive bacterial clones, i.e., those transformed with the expression vector. The draft SBIR Grant Application (**Exhibit D**) refers to such a two step cloning strategy at the bottom of page 19. s

21. The resulting expression vector constructed through this two step strategy encodes a short hairpin having a 28 base pair double-stranded region and an intervening loop consisting of an HpaI site. The short RNA hairpin encoded by an expression vector constructed using the primers listed in the Primer Order to Invitrogen (**Exhibit E**) has the same hairpin structure as shown in Figure 37 of the '797 application (see also **Exhibit F**).

22. The target genes referred to in **Exhibit E** and in **Exhibit D** include: human hypoxanthine—guanine phosphoribosyl transferase (HGPRT1 and HGPRT2 primers) and the mouse tyrosinase gene (tyro1 and tyro2 primers). The indicated target genes therefore indicate the resulting encoded short RNA hairpins (and expression constructs) are directed to silencing their corresponding target gene in mammalian cells, in particular, human cells and mouse cells. Additionally, as indicated in the Draft SBIR Grant Application (**Exhibit D**) on page 16 (second paragraph), HGRPT gene is directed to a gene target "for which exists a positive selection for loss-of-function" upon stable expression of the hairpin RNA in the cell.

D. Luciferase Simple Hairpin

23. Attached at **Exhibit F** is a copy of a slide dated at least by December 28, 2001. Information in this slide is also shown in Figure 37 in the parent application U.S. Serial No. 10/055,797. The slide illustrates two short hairpin RNA molecules. The second hairpin, the "Luciferase simple hairpin" has a double-stranded region consisting of 28 base pairs in length. .

The double-stranded region is highlighted. The double-stranded region of the short hairpin RNA molecule has a sequence that is complementary to a portion of the target gene, firefly luciferase.

24. The loop region of the hairpin on **Exhibit F** contains the sequence GUUAAC which is a HpaI restriction site. This is an example of a cloned simple hairpin that would be obtained using the methods described above in **Exhibit D** (specifically, the two-step method of hairpin cloning referred to here at paragraph 15) and using the PCR primers listed in **Exhibit E**.

E. Short Hairpin RNA Experiment in Human 293 T Cells

25. Attached at **Exhibit G** is a copy of a slide dated at least as early as October 2001. The title of the slide is "SHP 293T" indicating that this data is from an experiment using short hairpin RNA in 293T cells, a line of human embryonic kidney cells. This experiment assessed the ability of various short hairpin RNAs to specifically suppress gene expression in these cells, without provoking a PKR response. The 293T cells were co-transfected with a plasmid expressing the target gene, firefly luciferase, a plasmid expressing Renilla luciferase and one of various test hairpin RNAs. Subsequent to transfection, the level of expression of both luciferase proteins was measured. In the slide, the different test hairpin RNAs are indicated on the X axis of the slide underneath each of the bars. The respective bars indicate the degree to which the various introduced RNAs, including short hairpin RNAs, suppressed expression of the target firefly luciferase gene, as assayed by the ratio of firely luciferase to Renilla luciferase expression. As indicated in the slide, these results demonstrated that short RNA hairpins specifically suppressed expression of their target gene without provoking a PKR response in the cells.

26. For example, the nomenclature "SHP 25 luc hp" indicates a short hairpin RNA that has a double stranded region of 25 nucleotides in length. As the nomenclature indicates, the double-

stranded region of this short hairpin RNA molecule has a sequence that is complementary to a portion of the target gene, firefly luciferase. The bar graph shows, as a result of the experiment, a specific suppression of firefly luciferase gene expression in the 293T cells. See the bar labeled "SHP 25 luc hp" on the graph.

27. In the slide, the nomenclature "SHP 33 luc hp mism ngl3" indicates a short hairpin RNA that has a double stranded region of 33 nucleotides in length and has a mismatch in the sequence so that the sequence is not fully complementary to the sequence of the luciferase target gene. This bar of the bar graph shows, as a result of the experiment using a mismatched hairpin sequence, no specific suppression of firefly luciferase gene expression. The slide shows that short hairpin constructs with double-stranded regions of 32 nucleotides, 33 nucleotides, 34 nucleotides and 35 nucleotides did not exhibit attenuation of luciferase gene expression.

28. This slide shows an example of a short hairpin with a double-stranded region of 25 nucleotides in length, which did not trigger a protein kinase RNA-activated (PKR) response in the mammalian cell, and which did attenuate expression of the target gene, luciferase, in a sequence specific manner in the mammalian cells, 293T. The information in this slide was also included as Figure 39 of the parent application U.S. Serial No. 10/055,797.

F. Short Hairpin RNA Experiment in Human HeLa Cells

29. Attached at **Exhibit H** is a copy of a slide dated at least as early as October 2001. The slide shows data from an experiment using human HeLa cells (a cell line derived from human cervical cancer cells). We knew at the time of this experiment that long dsRNA initiates a PKR response in these cells. Using the same protocol as the experiment discussed above (**E**), this

experiment similarly assessed the ability of various short hairpin RNAs to specifically suppress gene expression in HeLa cells, without provoking a PKR response.

30. As indicated in the slide, these results demonstrated that short RNA hairpins specifically suppressed expression of their target gene without provoking a PKR response in the cells. For example, introducing a short hairpin RNA having a double-stranded region of 25 base pairs ("SHP 25 Luc hp") into the cells specifically suppressed expression of the firefly luciferase target gene. Longer double-stranded regions or mismatched target sequences did not result in suppression of target gene expression. The information in this slide was also included as Figure 40 of the parent application U.S. Serial No. 10/055,797.

G. Short Hairpin RNA Experiment in *Drosophila* S2 Cells

31. Attached at **Exhibit I** is a copy of a slide dated at least as early as October 2001. The data in this slide was generated using the same type of experimental procedure as discussed above in **Exhibits G and H**. The data in this slide indicates that short hairpin with a double-stranded region of 25 nucleotides ("SHP 25 luc hp") functioned to specifically inhibit expression of the target gene in the cells. The information in this slide was also included as Figure 38 of the parent application U.S. Serial No. 10/055,797.

H. Expression of Encoded Short Hairpins Specifically Suppress Gene Expression in Mammalian Cells

32. Attached at **Exhibit J** is a copy of a slide dated as least by January 2002 which shows results from an experiment which was included as Figure 42 (bottom) of the parent application U.S. Serial No. 10/055,797. The description of this experiment and the data can be found on page 17 of the '797 application. The results of this experiment demonstrate that expression of

encoded short hairpin RNAs effectively and specifically suppressed expression of a target gene in 293T cells, without provoking a PKR response. .

I. Paddison et al., *Genes Dev.* 2002, 16:948-958

33. The work described above culminated in several publications. One paper was published in *Genes and Development* in March 2002 entitled "Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells." (See **Exhibit K**.) This paper reports that "short hairpin RNAs (shRNAs) can be engineered to suppress the expression of desired genes in culture *Drosophila* and mammalian cells. shRNA can be synthesized exogenously or can be transcribed from RNA polymerase III promoters *in vivo*, thus permitting the construction of continuous cell lines or transgenic animals in which RNAi enforces stable and heritable gene silencing." (See Abstract of **Exhibit K**.)

34. A copy of a manuscript of the Paddison et al. paper (**Exhibit K**) that was prepared prior to publication and no later than January 31, 2002, as indicated by e-mails to which the manuscript was attached, is attached at **Exhibit L**.

35. Results of additional representative experiments, conducted similarly to the experiment referred to here in part **H**, "Expression of Encoded Short Hairpins Specifically Suppress Gene Expression in Mammalian Cells," are also reported in Paddison et al., among other places, at Fig. 4. (**Exhibit K**). Results of additional representative experiments conducted similarly to the Short Hairpin RNA Experiment in *Drosophila* S2 Cells (**G**), the Short Hairpin RNA Experiment in Human 293 T Cells (**E**) and the Short Hairpin RNA Experiment in Human HeLa Cells (**F**) are reported in Paddison et al., among other places, at Figs. 1 through 3. (**Exhibit K**). Figures 44A and 44B of the '676 application correspond to Figure 6A and 6B of Paddison et al. (**Exhibit K**).

J. Industry Awards

36. During 2002-2006, Paddison et al. (**Exhibit K**), having been cited by more than 500 subsequently published scientific papers, was therefore among the most highly cited "high impact" papers in the fields of molecular biology and genetics, as indicated by an analysis published by ScienceWatch.com (**Exhibit M**, see Table 2). A citation history summary for Paddison et al. (**Exhibit K**) is shown in **Exhibit N**.

37. Since we made the claimed invention and published Paddison et al., (**Exhibit K**), the invention of using stably expressed short hairpin RNAs to inhibit gene expression in mammalian cells has been recognized by industry organizations. For example, in 2005, Dr. Hannon received the Award for Outstanding Achievement in Cancer Research from the American Association for Cancer Research (AACR), which honored Dr. Hannon "...for his work uncovering the biochemical mechanism of RNA interference of gene expression (RNAi) and his contributions to the discovery and development of short hairpin RNAs as tools for genetic manipulation of mammalian cells." (**Exhibit O**).

38. In 2007, Dr. Hannon received two more prestigious awards, the Award in Molecular Biology from the National Academy of Sciences (**Exhibit P**), and the Paul Marks prize for the valuable contribution his RNAi work to cancer research from Memorial Sloan-Kettering Cancer Center (**Exhibit Q**). In granting that award, MSKCC noted how Dr. Hannon had applied his research in understanding the RNAi pathway to develop this valuable new technology, and his recognition as a leader in the field:

> Dr. Hannon is a leader in the relatively new field of RNA interference (RNAi). RNAi is a naturally occurring mechanism for regulating the expression of genes (controlling which genes are turned on and turned off in cells). In the laboratory, it is used as a

tool to study the function of specific genes, and it's being investigated as a therapeutic approach for treating many different diseases, including cancer.

Dr. Hannon's laboratory has elucidated key biochemical details of the components of the pathways involved in RNAi and is using these findings to develop molecular tools that can be used for gene discovery, the evaluation of gene function, and the generation of animal models. He has developed new techniques for using RNAi to study cancer development and is investigating possible cancer therapies that make use of small interfering RNAs (siRNAs).

Dr. Hannon discovered several proteins and enzymes that are an essential part of the RNAi mechanism, including Dicer, which cleaves double-stranded RNA into siRNAs; the RISC complex, which helps regulate protein translation and is involved in the body's defense against viral infections; and Argonaute2, which cleaves messenger RNA.

He also has been at the forefront of adapting RNAi techniques to study genes in mammals, and using these techniques to understand the variety of pathways that can lead to the formation of tumors.

K. Conclusion

39. The documents attached hereto as **Exhibits A - M** demonstrate that that the invention claimed, including claims 50, 52, 54-60, 62 and 63, was conceived at least as early as August 14, 2001, which is prior to the effective filing date of Caplen et al., Symonds et al., and Kreutzer et al. These documents and our declaration also show diligence and reduction(s) to practice.

40. We further declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that the making of willfully false statements and the like is punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United

States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed: _____
Gregory J. Hannon

Dated: _____

Signed: _____
Patrick J. Paddison

Dated: 1/19/11

Signed: _____
Scott Hammond

Dated: _____

Signed: _____
Amy Caudy

Dated: _____

Signed: _____
Emily Bernstein

Dated: _____

Signed: _____
Douglas Conklin

Dated: _____

Exhibits to Declaration Under 37 C.F.R. §1.131

Exhibit	Title
A	Hannon Draft Grant Application
B	Hammond et al., *Nature* 404:293-296 (2000)
C	Bernstein et al. *Nature* 409: 363-366 (2001)
D	Draft grant application to SBIR (Small Business Innovation Research)
E	Email of Primer Order to Invitrogen
F	Luciferase Simple Hairpin Slide
G	Short Hairpin RNA Experiment in Human 293 T Cells Slide
H	Short Hairpin RNA Experiment in Human HeLa Cells Slide
I	Short Hairpin RNA Experiment in *Drosophila* S2 Cells Slide
J	Short Hairpins Specifically Suppress Gene Expression Slide
K	Paddison et al., *Genes Dev.* 2002, 16:948-958
L	Manuscript of Paddison et al.
M	ScienceWatch Biology's Hottest 2002-2006
N	Paddison et al. Citations
O	2005 Award for Outstanding Achievement in Cancer Research from AACR
P	2007 Award in Molecular Biology from the National Academy of Sciences
Q	2007 Paul Marks Prize from Memorial Sloan-Kettering Cancer Center

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Hannon et al.	Confirmation No.:	8161
Application No:	11/894,676	Art Unit:	1635
Filed:	August 20, 2007	Examiner:	K. CHONG
Title:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Docket No.: 287000-130-US3

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Second Declaration of Professor Nouria Hernandez, Ph.D. Under 37 C.F.R §1.132

I, Nouria Hernandez, Ph.D., hereby declare and state that:

1. I am a Professor of Biology , and the Director of the Centre intégratif de génomique at the Université de Lausanne.

2. I am informed that the '676 application was filed based on a parent application, U.S. Serial No. 10/055,797, and is entitled to a priority date of January 22, 2002, which is the filing date of the '797 application.

3. At the time of the filing of the '797 application, *i.e.,* around January 2002, I was an Investigator of the Howard Hughes Medical Institute and a Professor at Cold Spring Harbor Laboratory. My C.V. is attached at **Exhibit A**.

4. I understand that the pending claims of this U.S. patent application, U.S. Serial No. 11/894,676 are directed to methods for attenuating expression of a target gene. A listing of the pending claims are attached at **Exhibit B**.

5. I understand the claimed methods are all directed to using RNA interference (RNAi) to stably attenuate expression of the target gene in a sequence specific manner in a mammalian cell, without activating a non-sequence specific PK response. As discussed below, the claimed methods would not have been obvious to a person of ordinary skill in the art as of January 21, 2002.

6. This Second Declaration is submitted in addition to my First Declaration in order to address additional references cited by the Examiner and comments made by the Examiner with regard to the '676 application. I incorporate the statements from my First Declaration into this Second Declaration.

7. I have reviewed the Office Action dated August 30, 2010. In this Office Action, I reviewed the comments from the U.S. Patent Examiner regarding my First Declaration and note that she has relied upon Kreutzer et al. (U.S. Application No. 20040102408) and Caplen et al. in making those comments.

8. As of January 22, 2002, a person of ordinary skill in the art would have had no reasonable expectation of success in carrying out sequence specific gene silencing by using an expression vector encoding a short hairpin RNA molecule having a double-stranded region consisting of 20 to 29 base pairs (bp). As discussed below, the references cited by the Examiner (along with the leading literature in the field) would have taught away from using an expressed short hairpin molecule, which to have gene silencing activity, must first be processed in the cell. Specifically, Caplen et al. and/or Kreutzer et al., individually, or combined, would not have taught a person of ordinary skill in the art that the claimed invention would be obvious with a reasonable expectation of success.

Elbashir et al.

9. The Examiner makes some statements regarding Elbashir et al. (Elbashir et al. (2001) *Genes Dev.* 15:188-200) on page 4 of the Office Action with which I disagree. The Examiner states "Elbashir et al. does conclusively provide evidence that a dsRNA of 29 base pairs in length did not efficiently mediate RNAi in Drosophila cells in vitro. However, there is no factual evidence provided in Elbashir et al. or any of the other references that expressly teach that dsRNAs having a duplex of less than 29 base pairs were not capable of mediating RNAi. This conclusion by Professor Hernandez appears to be opinion evidence without any factual support." I disagree with the Examiner.

10. Elbashir et al. does indeed provide a factual basis for my conclusion as a person of ordinary skill in the art as of January 22, 2002. The Elbashir et al. reference taught persons of ordinary skill in the art at that time that use of dsRNA having a double-stranded region of less than 29 base pairs would not be effective in mediating RNAi. Elbashir et al. disclosed negative results that would have caused one to expect that a short hairpin RNA with a double-stranded region consisting of 20-29 bp in length (a) would not be processed to the 21- and 22-nt siRNA structures necessary to mediate RNAi and (b) would consequently be ineffective in mediating RNAi.

11. In particular, among these results, the data in Elbashir et al. demonstrate a distinct negative linear correlation between the length of a dsRNA (from 500 bp to 29 bp) and its ability to act as an RNAi trigger (see Elbashir et al., Fig. 1). These data demonstrate that the longest dsRNAs (100-500 bp) were by far the most effective in acting as an RNAi triggers. Decreasing the length of the dsRNA to below 100 bp resulted in a marked, approximately linear decrease in the effectiveness of the dsRNA as

an RNAi trigger. In particular, as referred to in my first Declaration, shortening the length of the dsRNA to 30 or 29 bp completely eliminated the ability of the dsRNA to serve as an RNAi trigger. (Elbashir et al., Fig. 1). Notably, this lack of any RNAi activity for 29 and 30 bp dsRNA was observed even under optimized conditions, using a 100:1 molar ratio of dsRNA to the mRNA target. (Elbashir et al., Fig. 1 and page 189, first column). In this regard, dsRNA of 39-bp in length or longer all appeared to be efficiently processed into the 21 and 22-nt (guide) siRNAs ultimately responsible for mediating cleavage of the target RNA (see Elbashir et al., Figs. 2 and 7). In contrast, 29 bp RNA was only slowly processed to such guide fragments, strongly suggesting that without efficient processing to yield sufficient siRNA product, the dsRNA would fail to act as an RNAi trigger.

12. One of skill at the time would have understood these data to indicate there was a critical minimal length requirement for dsRNA to be able to serve as RNAi triggers. The dsRNA would have to be long enough, *i.e*, over 30 bp in length to provide for enough production of guide RNAs to result in degradation of the target mRNA. In other words, the data in Elbashir et al., as a whole, expressly teach that dsRNA below 29 bp in length (unless those RNAs were in the form of 21-23nt siRNAs) would fail to serve as RNAi triggers. The Examiner's suggestion otherwise takes the statement out of context. Specifically, dsRNAs of decreasing length were tested, and the results showed that the shortest length exhibiting activity was 38 base pairs and shorter lengths had no activity within experimental error. The Examiner's conclusion on page 4 of the Office Action that "there is no factual evidence provided in Elbashir or any of the other references that expressly teach that dsRNAs having a duplex of less than 29 base pairs were not capable

of mediating RNAi" has no scientific or logical basis, and especially in terms of how these data would have been understood and interpreted at the time by one of ordinary skill.

13. In short, in view of my understanding of the state of the art as of January 22, 2002 and based on how a person of ordinary skill in the art as of January 22, 2002 would have understood Elbashir et al., and in view of the other evidence I relied upon in my First Declaration, it is my conclusion that the methods for attenuating target gene expression as recited in the claims of Hannon et al. would not have been obvious in view of the art. It would have been backwards and contrary to the Elbashir paper's text for a person of ordinary skill in the art to interpret the negative results in Elbashir, as somehow indicating the complete opposite, that is, as providing any reasonable expectation that a dsRNA shorter than 29 bp could serve as an RNAi trigger.

14. The Examiner also states that "…the evidence provided by Elbashir et al. does not teach away from using a dsRNA 29 bp to mediate RNAi in mammalian cells and more importantly does not teach away from using a dsRNA of less than 29 bp to mediate RNAi in any cell type." See Office Action page 4.

15. I disagree with the Examiner and it is my conclusion that the results in Elbashir et al. would have taught one of ordinary skill in the art at the time *away* from using dsRNA of less than 29 bp in insect cells, and also in mammalian cells. The expectation of one of ordinary skill in the art at the time, for example, in view of the conservation across species of the RNAi machinery (see Bernstein et al, *Nature* 409, 363-366 (2001)), was that the negative results provided by Elbashir et al. in insect cells would also apply to the

use of short hairpin RNAs in mammalian cells. It would have been backwards and contrary to the Elbashir paper's text for a person of ordinary skill in the art to interpret the negative results in Elbashir as providing any reasonable expectation that one could have achieved gene silencing by stably expressing a short hairpin RNA in mammalian cells. Therefore, one of ordinary skill in the art would have been taught away from using short hairpin RNAs in mammalian cell types based on the Elbashir et al. paper and the state of the art at the time.

Kreutzer et al.

16. The Examiner stated that "the previous Office Action did in fact provide evidence that a dsRNA having a double stranded region of at least 21 bp was capable of mediating RNAi in cells which is direct evidence against the data provided by Elbashir. It is clearly shown in Kreutzer et al. (of record) that a dsRNA 21 nucleotide base paired molecule was capable of efficiently reducing gene expression in mammalian cells (see Examples). Applicant did not comment on this reference in this regard however this is direct evidence that one of ordinary skill in the art would have expected a dsRNA of less than 29 bp or having a double stranded region of at least 20 base pairs to be capable of mediating RNAi in mammalian cells." See page 3 of the August 30, 2010 Office Action.

17. I have reviewed Kreutzer et al. (US Publication No. 2004/0102408). As a person who was of ordinary skill in the art as of about January 22, 2002, and working in the field of RNA, I disagree with the Examiner's position. Kreutzer et al. would not have given a person of ordinary skill in the art a reasonable expectation of success that stably expressing a short hairpin RNA having a double-stranded region consisting of at least 20

nucleotides but not more than 29 nucleotides would attenuate gene expression in mammalian cells.

18. With regard to the Examiner's statement, a few facts are evident. The Examiner asserts that Kreutzer demonstrates " that a dsRNA 21 nucleotide base paired molecule was capable of efficiently reducing gene expression in mammalian cells (see Examples)." The "dsRNA" that Kreutzer describes, however, (see [0069]) is a synthetic and chemically altered RNA molecule (synthons modified by disulfide bridges) comprised of single strands linked by a disulfide bridge. Such a chemically altered species would not have provided any reasonable expectation of success with regard to how an unmodified dsRNA, or a hairpin RNA molecule that is expressed within a cell, would have affected gene expression.

19. The Examiner then alleges that "this is direct evidence that one of ordinary skill in the art would have expected a dsRNA of less than 29 bp or having a double stranded region of at least 20 base pairs to be capable of mediating RNAi in mammalian cells." With regard to mediating RNAi, it is noted that Kreutzer provides no evidence that the chemically modified RNA structures Kreutzer describes are even processed through the RNAi pathway. The Examiner's statement also appears to reflect a misunderstanding of what Elbashir taught. In particular, Elbashir taught that to overcome the inability of the cellular RNAi machinery to process short dsRNA molecules into the 21-23nt (guide) siRNA mediating target gene suppression, one instead could directly introduce a dsRNA mimicking an siRNAs into the cell. (See Figure 5 of Elbashir.) In view of Elbashir, one of skill would have expected that a 21 bp dsRNA could therefore serve as an RNAi trigger without the need for processing. To one of skill, such a result, however, would

have provided no evidence or expectation that a hairpin RNA molecule with a 21 bp double-stranded region could mediate RNAi, in particular because to mediate RNAi, the hairpin RNA would first have to be processed into a dsRNA. As explained in my first Declaration, in view of Elbashir, that a short hairpin RNA (having a double-stranded region of less than 29 bp or at least 20 base pairs) was capable of acting in such a way was in fact surprising and unexpected.

20. The Examiner is therefore mistaken in believing that a person of ordinary skill as of January 22, 2002 would view Kreutzer et al. "as direct evidence against the data provided in Elbashir et al." At that time, a person of ordinary skill in the art would have viewed Elbashir et al. described in my first Declaration – that is, Elbashir et al. expressly taught away from using short hairpin RNAs having double-stranded regions of less than 30 base pairs. In fact, nowhere in Kreutzer is there any teaching that an expressed short hairpin RNA as presently claimed could be used to suppress gene expression. The sole references Kreutzer makes to RNA hairpin structures are made in an entirely different context, that is, the problem of degradation of the dsRNA in the cell. To afford protection from degradation, Kreutzer et al. suggested use of chemically altered dsRNAs, generated through "chemical modification" of the dsRNA or by chemically modifying the nucleotides in the loop region of an RNA hairpin loop. See, for example, paragraph 19 of Kreutzer et al. Here, Kreutzer states "an RNA hairpin loop, in particular when using a vector according to the invention. To afford protection from degradation, it is expedient for the nucleotides to be chemically modified in the loop region between the double-stranded structure." The vector referred to and the fact that chemical modifications are proposed both indicate that this statement refers to an RNA produced in

vitro which would then need to be delivered into cells. Such a synthetic structure could not be expressed in a mammalian cell from the proposed vector.

21. Further, Kreutzer et al. would not have provided any reasonable expectation that one could have used the presently claimed methods to successfully suppress gene expression in a mammalian cell. The Examples in Kreutzer et al. do not show expression of a short hairpin RNA in mammalian cells. Instead, the Example 1 shows *in vitro* transcription (*e.g.*, starting at paragraph 44); generation of double-stranded RNA by in vitro hybridization (e.g., starting at paragraph 46). Similarly, Example 2 shows transfection (not stable expression) of dsRNA having a length of 315 bp (see Seq. I.D. No. 5 and paragraph 66) and microinjection of a chemically modified, synthetic, dsRNA of 21 bp (see Seq I.D. No. 8 and paragraph 69) into a murine cell line. The 21 bp dsRNA was not a hairpin, and was chemically modified and synthesized using solid state chemistry. In paragraph 69, Kreutzer et al. state: "A dsRNA linked chemically at the 3' end of the RNA as shown in sequence listing No. 8 to the 5' end of the complementary RNA via a C18 linker group was prepared (L-dsRNA). To this end, synthons modified by disulfide bridges were used." The paragraph goes on to describe solid support chemical methods used to carry out the chemical reactions needed to obtain the L-dsRNA. The disclosure of Kreutzer et al. would not have taught or made obvious to a person of ordinary skill in the art at the time the claimed methods of Hannon et al. because the Hannon methods require *in vivo* stable expression of a construct to express a short hairpin RNA having a double-stranded region consisting of at least 20 nucleotides but not more than 29 nucleotides.

22. The final sentence of Kreutzer et al. clarifies the meaning of the results presented in Example 2. The authors state in paragraph 76 "[t]his result demonstrates that even shorter dsRNAs can be used for specifically inhibiting gene expression in mammals when the double strands are stabilized by chemically linking the single strands." Therefore, in this statement, a person of ordinary skill in the art at the time would have understood that: (1) *in vitro* transcription of single stranded RNAs was required by the method of Kreutzer et al.; (2) that solid state chemical modification of those single strands was required by the method of Kreutzer et al. (also a set of *in vitro* chemical steps); and (3) microinjection of chemically modified dsRNAs into mammalian cells was required. None of these teachings would have made obvious the methods of Hannon et al.

Caplen et al.

23. The Examiner relies on Caplen et al. (PNAS Vol. 98, No. 17, August 14, 2001) to state that "based on Caplen et al. one of ordinary skill in the art would clearly have a reasonable expectation of success in using dsRNA of less than 29 bp to mediate RNAi in mammalian cells, and further provides factual evidence that even in cells, such as C. elegans or Drosophila as taught by Elbashir, ds RNA of less than 29 bp are capable of efficiently mediating RNAi." See Office Action on page 5.

24. I have reviewed the Caplen et al. reference and disagree with the Examiner's position. First, the Caplen paper reports results with regard to small inhibitory RNAs (siRNAs) and does not address short hairpin RNA structures at all. Caplen et al. report on experiments using siRNAs that are double-stranded RNAs having specific overhang structures that are designed to mimic the processed structure of siRNAs. In fact the

approach described by Caplen is the same approach that Elbashir describes in Figure 5 (see paragraph 19 above).

25. The last sentence of the Introduction of the Caplen paper states that "[g]iven the observations that (*i*) 21-25-nt dsRNAs with a characteristic structure can mediate RNAi in cell extracts…." The "characteristic structure" referred to by the authors is a double-stranded, non-hairpin, structure with the specific overhang structure shown in the paper which Caplen specifically designed to mimic the processed structure of siRNAs. The overhang structure of the dsRNAs used in the experiments is specified on page 9744 as "(20 and 21 nucleotides base-paired with 2-nt 3' overhangs)" and in the text below Table 1: "dsRNA molecules were formed with each strand carrying a 5'-OP_4, 3"-OH, and 2-base 3' overhangs."

26. As discussed above in regard to Elbashir, that such specific structures were found to mediate RNAi would have provided no insight or expectation that the different short hairpin RNA structure would have mediated RNAi in a mammalian cell. In fact, such results would have taught away from the use of short hairpin RNAs. A person of ordinary skill in the art at the time, reading Caplen et al. would have been taught to use dsRNAs with specific overhang structures mimicking siRNAs, not short hairpins.

27. In view of the August 30, 2010 Office Action, the Examiner's comments therein, Kreutzer et al., and Caplen et al., I am still of the opinion, as a person of ordinary skill in the art as of January 22, 2002, and in view of the state of the art at that time, that such a person of ordinary skill in the art would not have believed the claimed methods of

Hannon et al. in this application to be obvious and would not have had a reasonable expectation of success in carrying them out.

Symonds et al. (US 2002/0160393)

28. On page 10 of the August 30, 2010 Office Action, the Examiner rejected the pending Hannon et al. claims as obvious over Symonds et al., Leiber et al., Fire et al., Good et al. and Noonberg et al. I have reviewed each of these references and the Examiner's comments in the Office Action. It is my opinion that this combination of references does not make obvious the methods claimed by Hannon et al. in this application.

29. I have been informed that the Symonds et al. publication is a U.S. patent application that claims priority to two U.S. Provisional patent applications that are listed on the face page of the Symonds et al. publication as Provisional application No. 60/258,733, filed on December 28, 2000 and Provisional application No. 60/258,731, filed on December 28, 2000. I have reviewed both of these provisional applications.

30. The '733 provisional application describes "ribozyme-containing RNA molecules." The claims of the Hannon application require that the "short hairpin RNA molecule is a substrate for Dicer-dependent cleavage" which is not described in the '733 application. The linear molecules "for forming a double-stranded RNA complex" described in the '733 application include a sequence corresponding to a ribozyme and would not be substrates for Dicer-dependent cleavage. They would not make obvious a short hairpin molecule as claimed by Hannon. By describing an entirely different

approach for generating dsRNA within the cell in the '733 application, Symonds teaches away from the short hairpin approach described in the Hannon application.

31. The '731 provisional application notes that "the application of dsRNA for gene suppression in human cells has not been successful and may be due to the anti-viral pathways (unrelated to dsRNA-mediated gene suppression) in place that respond to the presence of viral dsRNA or intermediates of transposition." As an alleged solution, the provisional application describes using the Tat protein of HIV to suppress or inhibit these pathways, including suppressing or inhibiting the PKR pathway. This alleged solution is entirely different from and teaches away from the solution described in Dr. Hannon's application in expressing short hairpin RNAs that do not elicit a PK response.

32. In particular, the '731 application describes "linear RNA molecules" that require "a portion encoding HIV Tat protein." The claims of the Hannon application require that the short hairpin RNA be "expressed in the cell without the use of a PKR inhibitor." The disclosure of the '731 Symonds provisional application does not teach this requirement since the alleged solution provided includes the Tat protein for the purpose of inhibiting the PKR pathway.

33. The Examiner relies on pages 5 and 11 and Figure 2A of the '731 application. These portions of the '731 application do not make obvious the claimed invention of Hannon. This disclosure only describes a linear RNA molecule when it is in association with an RNA encoding HIV Tat protein. Specifically, on page 5 and 11 there is no disclosure of the "linear RNA molecule" without an RNA encoding HIV Tat protein, either on the same molecule or in association together with a linear RNA that can form a

dsRNA. Even when the RNA encoding HIV Tat is on a different molecule than the other linear RNA, the '731 application require that both be together in the same composition. There is no teaching or suggestion in the '731 application to obtain or use a short hairpin RNA as is recited in the Hannon claims.

34. The Examiner points to page 11 of the '731 application possibly to rely upon the last paragraph on that page regarding "the length of the instant linear RNA molecule…." (See first sentence of the last paragraph on page 11.) Here, the "instant" linear RNA molecule refers back to the preceding paragraph that indicates that the "linear RNA molecule" has two portions: (a) a portion encoding HIV Tat protein, and (b) a portion for forming a double-stranded RNA complex. The lengths have no upper limit and there are examples of wide ranges of lengths recited, such as 20-3,000 nucleotides, between 200 and 500 nucleotides, between 100 and 1000 nucleotides, and between 20 and 25 nucleotides in length. In the '731 application, there is no qualification about whether any one length would work better than any other length, so it would not have been obvious to a person of ordinary skill at that time which length to choose.

35. Another part of the '731 application that expands the number of possible choices of lengths for the first and second sequences is the definition of "hybridizing conditions" spanning pages 9-10. According to the '731 application, the first and second sequences hybridize with each other under hybridizing conditions. However, "hybridizing conditions" means that "two complementary strands having a length of at least seven nucleotides" are to hybridize. Therefore, the first and second strands could have some length longer that 7 nucleotides, such as 20 nucleotides or 100 nucleotides, but only seven nucleotides need to be hybridized to meet this definition. This adds to the vast

number of choices for length of the first and second strands provided for in the '731 application. This disclosure does not teach nor make obvious to a person of ordinary skill in the art the invention claimed by Hannon et al.

36. Figure 2A of the '731 application does not make any clearer the structure of the linear RNA molecules described by Symonds et al. Figure 2A shows a cartoon of a hairpin with apparently on 12 nucleotides hybridized in the double-stranded region and a much longer loop region. The description of Figure 2A on page 6 of the '731 application merely states that this cartoon illustrates a "mechanism" for forming dsRNA which generally "involves the cloning of an intervening sequence that, upon transcription, forms a loop as the complementary sequences bind." This gives a person of ordinary skill in the art no further information regarding the dsRNA complex or the linear RNA molecule referred to in the '731 application.

37. The Symonds '393 publication, read by a person of ordinary skill in the art as of January 22, 2002, would not have taught or made obvious the claimed invention by Hannon et al. The Examiner relies on paragraphs 108-114, 136, Figures 8A and 9. The disclosure in the Symonds '393 publication at paragraphs 108-114 does not appear to have support in either the '733 application or the '731 application.

38. Figure 8A is the same as Figure 2A in the '731 application which I discussed above. For the same reasons, this figure fails to describe or teach the short hairpin approach described in the Hannon application. Figure 9 is referred to in Example 6, which indicates the encoded RNA hairpin depicted in Figure 9 has a double-stranded region of approximately 500 bp. Figure 9, which describes a dsRNA construct for use in

mammalian cells, therefore not only fails to describe the short hairpin approach described in the Hannon application, it teaches away from that approach.

39. Paragraph 136 of the '393 publication appears similar to the paragraph in the '731 application on page 11 which I discussed above, except that here "instant linear RNA molecule" appears to refer to "a linear RNA molecule" described in paragraphs 124 and 134. This description indicates the linear RNA molecule always has a third sequence corresponding to a ribozyme. As discussed in paragraph 30, such a molecule would not be a substrate for Dicer-dependent cleavage, and would refer to an entirely different approach for generating dsRNA inside the cell that would neither teach nor make obvious the short hairpin approach described in the Hannon application. Moreover, as discussed in paragraph 34, the references here to various lengths provide no guidance as to the length one would use in the different approach of Hannon. Such references would therefore not have taught nor made obvious the use of short hairpins having a double stranded region of at least 20 nucleotides but not more than 29 nucleotides as claimed in the Hannon application.

40. Further, both the '731 application and the '393 publication include essentially the same definition of "hybridizing conditions," which expands the number of possible choices of lengths for the first and second sequences. As similarly discussed in paragraphs 34-36, this adds to the vast number of choices for length of the first and second strands provided for in the '393 publication. For this reason, the mere disclosure of various ranges, especially in the context of the statement that "there is no upper limit to the length of the linear RNA molecule or the first and second sequences thereof"

would not have taught nor made obvious to a person of ordinary skill in the art the use of short hairpin molecules as claimed by Hannon et al.

41. Regarding paragraphs 108-114, the only specific reference to length indicates that the RNA molecule referred to here has "at least 20 nucleotides identical with at least part of the nucleotide sequence of the nucleic acid of interest." There is no description here as to the length of a double-stranded region, or to the use of a short hairpin RNA in a mammalian cell to avoid the PK response. Instead, the '393 publication teaches away from that approach. The Background section of the '393 publication states that the problem was activation of the PK response (see paragraph 5 of the '393 publication). To avoid that problem, a person of ordinary skill in the art, reading the '393 publication would understand the disclosure to instead teach use of some PK inhibitor, such as HIV Tat. In contrast, the shRNA approach claimed in the Hannon application does not use a PK inhibitor. In particular, the claimed method states that the shRNA is expressed in the mammalian cell without the use of a PK inhibitor. The '393 publication therefore not only fails to teach or make obvious such an approach, it directs one to use an entirely different approach from the methods claimed in the Hannon application.

42. I previously reviewed and commented upon Leiber et al., Fire et al., Good et al., and Noonberg et al. in my First Declaration and I continue to be of the opinion that the combination of all of these references with Symonds et al. would not have made the methods claimed by Hannon et al. in this application obvious to a person of ordinary skill in the art at the time with a reasonable expectation of success.

43. Further evidence of the non-obviousness of the Hannon et al. invention is evidenced in the complicated nature of the methods provided in the cited references, such as chemically modifying RNAs and using microinjection (Kreutzer), making double-stranded RNAs with specific overhang structures (Caplen), and using Tat-type or ribozyme-type structures (Symonds). It is notable that none of these complicated approaches became commonly used methods to stably silence genes in mammalian cells. In contrast, the methods claimed by Hannon et al. have become widely used in research for stably silencing gene expression in mammalian cells.

I hereby declare that all statements are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 6th January 2011 By: Nouria Hernandez

Nouria Hernandez, Ph.D.

Exhibit B – Second Declaration of Dr. Hernandez Under 37 C.F.R. §1.132

U.S. Serial No. 11/894,676

Attorney Docket No. 0287000.130.US3

Claim Listing

1-49. (Cancelled)

50. (Currently Amended) A method for attenuating expression of a target gene in a mammalian cell, the method comprising

introducing into [[a]] mammalian cells a library of RNA expression constructs, each expression construct comprising:

(i) an RNA polymerase promoter, and

(ii) a sequence encoding a short hairpin RNA molecule comprising a double-stranded region wherein the double-stranded region consists of at least 20 nucleotides but not more than 29 nucleotides, [~~such that the short hairpin RNA does not trigger a protein kinase RNA-activated (PKR) response in the mammalian cells~~],

wherein the short hairpin RNA molecule is a substrate for Dicer-dependent cleavage and does not trigger a protein kinase RNA-activated (PKR) response in the mammalian cell,

wherein the double-stranded region of the short hairpin RNA molecule comprises a sequence that is complementary to a portion of the target gene, and

wherein the short hairpin RNA molecule is stably expressed in the mammalian cell in an amount sufficient to attenuate expression of the target gene in a sequence specific manner, and is expressed in the cell without use of a PK inhibitor, whereby expression of the target gene is inhibited.

51. (Cancelled)

52. (Previously presented) The method of claim 50, wherein the expression construct further comprises LTR sequences located 5' and 3' of the sequence encoding the short hairpin RNA molecule.

53. (Cancelled)

54. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of at least 21 nucleotides.

55. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of at least 22 nucleotides.

56. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of at least 25 nucleotides.

57. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of 29 nucleotides.
58. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule has a total length of about 70 nucleotides.
59. (Previously presented) The method of claim 50, wherein the RNA polymerase promoter comprises a pol II promoter or a pol III promoter.
60. (Previously presented) The method of claim 59, wherein the pol III promoter comprises a U6, an H1, or an SRP promoter.
61. (Withdrawn) The method of claim 59, wherein the pol II promoter comprises a U1 or a CMV promoter.
62. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule attenuates expression of the target gene in the mammalian cell by at least about 60%.
63. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule attenuates expression of the target gene in the mammalian cell by about 60% to about 90%.
64. (Cancelled)

PTO/SB/22 (07-09)
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PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a) FY 2009 *(Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).)*	Docket Number (Optional) 0287000.00130US3
Application Number 11/894,676-Conf. #8161	Filed August 20, 2007
For METHODS AND COMPOSITIONS FOR RNA INTERFERENCE	
Art Unit 1635	Examiner K. Chong

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

		Fee	Small Entity Fee	
☐	One month (37 CFR 1.17(a)(1))	$130	$65	$
X	Two months (37 CFR 1.17(a)(2))	$490	$245	$ 245.00
☐	Three months (37 CFR 1.17(a)(3))	$1110	$555	$
☐	Four months (37 CFR 1.17(a)(4))	$1730	$865	$
☐	Five months (37 CFR 1.17(a)(5))	$2350	$1175	$

X Applicant claims small entity status. See 37 CFR 1.27.

☐ A check in the amount of the fee is enclosed.

X Payment by credit card. ~~Form PTO-2038 is attached.~~

☐ The Director has already been authorized to charge fees in this application to a Deposit Account.

X The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 08-0219 .

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

☐ applicant/inventor.

☐ assignee of record of the entire interest. See 37 CFR 3.71.
Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96).

X attorney or agent of record. Registration Number 42,812

☐ attorney or agent under 37 CFR 1.34.
Registration number if acting under 37 CFR 1.34 _____

/Jane M. Love, Ph.D./ — Signature
January 31, 2011 — Date

Jane M. Love, Ph.D. — Typed or printed name
(212) 230-8800 — Telephone Number

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below.

☐ Total of 1 forms are submitted.

Specific Aims

In an evolutionarily diverse group of organisms that includes C. elegans, Drosophila, trypanosomes, planaria, hydra, zebrafish, plants and fungi, introduction of double-stranded RNA induces gene silencing in a sequence-specific fashion (Sharp, 1999; Sanchez Alvarado and Newmark, 1999; Lohmann et al., 1999; Cogoni and Macino, 1999; Waterhouse et al., 1998; Montgomery and Fire, 1998; Ngo et al., 1998). These processes have been termed variously RNA interference (RNAi), PTGS (post-transcriptional gene silencing) and quelling. dsRNA-induced gene silencing has been proposed to provide an antiviral defense, to modulate gene expression, and to play a critical role in maintaining genome stability by regulating the activity of transposable elements (Sharp, 1999; Montgomery and Fire, 1998; Tabara et al., 1998; Tabara et al., 1999; Ketting et al., 1999; Ratcliff et al., 1997).

Although dsRNA-induced gene silencing may play important biological roles, general interest in this phenomenon has been fueled by its utility as a research tool. RNA interference has revolutionized reverse genetics in C. elegans and may prove to be similarly useful in Drosophila (Tabara et al., 1998; Misquitta and Paterson, 1999; Kennerdell and Carthew, 1998). In addition, dsRNA-induced silencing has provided methods to analyze gene function in organisms for which genetic tools had previously been either cumbersome or non-existent (e.g., trypanosomes, Ngo et al., 1998).

Despite the obvious importance of dsRNA-induced gene silencing, the mechanisms underlying this phenomenon have remained obscure. In a number of organisms, including C. elegans, evidence indicates that dsRNA provokes gene silencing at a post-transcriptional level (Montgomery et al., 1998). However, particularly in plants, there are also indications of a parallel, if not related, process that accomplishes gene silencing by modification of chromatin structure (see for example, Jones et al., 1998; Jones et al., 1999).

We have taken a biochemical approach toward deciphering the mechanisms by which dsRNA suppresses gene expression. We have shown (see **Preliminary Results**) that, upon transfection into cultured, Drosophila S2 cells, dsRNA inhibits gene expression by reducing the levels of mRNAs that are homologous to the dsRNA. Extracts of transfected cells contain a nuclease that degrades cognate, synthetic mRNAs but that is inactive against heterologous RNAs. We have demonstrated that this nuclease is an RNP and have identified an RNA component that may guide substrate selection. We have designated this enzyme **RISC** (RNA-induced silencing complex). In this application, we outline our approach toward elucidating the mechanisms underlying dsRNA-induced gene silencing. Specifically, we propose the following aims:

Aim 1. RNA interference in Drosophila S2 cells. We will begin by characterizing the response to dsRNA in S2 cells. This will serve two purposes. The first will be to optimize the source of material for purification and biochemical characterization of the nuclease. The second will be to develop the S2 system as a general tool for probing gene function.

Aim 2. RNA interference in vitro. The RISC is composed of protein and RNA components that form a sequence-specific nuclease in response to dsRNA. We will examine the process that leads to the production of the nuclease, with particular attention to the generation of the putative guide RNA. A number of genes have been linked to RNA interference in C. elegans. We will also assess whether any of these are components of the RISC. If no known gene provides a biochemical handle on the activity, we will purify the nuclease by conventional methods.

Aim 3. Components of the RNAi nuclease. The purified nuclease complex will be used for cloning of both the protein and RNA components of this RNP enzyme. Once this is achieved, we will test the dependence of dsRNA-induced gene silencing on each of these components *in vitro* and *in vivo.*

Background and Significance

In most organisms, the presence of double-stranded RNA signals trouble. For example, dsRNAs could indicate invasion by viral pathogens or could reflect the activity of mutagenic and potentially deleterious mobile genetic elements. Therefore, cells have evolved mechanisms for detecting and responding to these threats. In mammals, responses are rather general and include suppression of translation and non-selective RNA degradation (reviewed in Williams, 1999; Clemens and Elia, 1997). However, in a variety of other systems, the response is specific, interfering only with the expression of sequences that are homologous to the dsRNA (Sharp, 1999; Sanchez Alvarado and Newmark, 1999; Lohmann et al., 1999; Cogoni and Macino, 1999; Waterhouse et al., 1998; Montgomery and Fire, 1998; Ngo et al., 1998). The subject of dsRNA-induced gene silencing has recently achieved a more prominent place in the general consciousness because of its application as a reverse genetic tool (see for example Tabara et al., 1998), and this has highlighted the almost complete mystery surrounding the mechanisms by which dsRNAs can suppress the expression of specific target genes.

RNA interference

The discovery of RNAi grew out of experiments from Guo and Kemphues (Guo and Kemphues, 1995) which attempted to use antisense RNA to probe the function of PAR-1, a kinase that is involved in specifying asymmetric division during C. elegans development. These investigators noted that introduction of antisense RNA into the maternal germline gave a precise phenocopy of a par-1 mutant allele. Surprisingly, a sense-oriented transcript was equally effective. A resolution to this apparent paradox came from the discovery by Fire and colleagues that dsRNAs were much more potent than were ssRNA of either polarity (Fire et al., 1998). In retrospect, it seems likely that early success with antisense RNAs derived from low-level contamination of ssRNA preparations with dsRNA. These are routinely generated in *in vitro* transcription reactions by non-specific initiation at the free ends of the template.

The success of RNAi as a genetic tool is based, in part, on the unusual properties of this biological phenomenon. The first is that RNA interference is non-cell autonomous. Injection of RNA into, for example, the gut of the worm can cause suppression of the targeted gene in all tissues of the animal (Fire et al., 1998). This indicates that either the signals for or the effectors of RNAi can travel across cellular boundaries. The dsRNA itself need not be introduced by injection. In fact, soaking larval worms in a solution containing liposome-encapsulated dsRNAs can ablate gene expression (Tabara et al., 1998). RNA-interference can even be provoked by feeding worms a diet of E. coli that express dsRNAs (Timmons and Fire, 1998). In addition, the gene silencing that is provoked by RNAi is heritable. Suppression is often transmitted in a dominant fashion to the F1 progeny and can persist into the F2 generation (Fire et al., 1998). However, the response ultimately decays with consequent restoration of expression of the targeted gene.

Although, the mechanisms that underlie double-stranded RNA-dependent gene silencing remain a mystery, substantial evidence points to RNAi acting at the post-transcriptional level (Montgomery et al., 1998). The first indication of this came from the observation that dsRNAs directed against promoters and intronic sequences were ineffective at silencing gene expression (reviewed in Sharp, 1999). Furthermore, exonic dsRNAs did not affect the abundance of pre-mRNAs nor were any physical changes in the DNA sequence of targeted genes apparent (Montgomery et al.,

1998). However, the most convincing evidence for a post-transcriptional mechanism arises from the unusual nature of mRNA synthesis in nematodes. In C. elegans, many pre-mRNAs are transcribed as components of multigene operons. Individual mRNAs are separated from the larger precursor by addition of a small leader sequence (the SL-RNA) that is donated from an snRNP via trans RNA splicing (reviewed in Nilsen, 1995). Thus, if RNAi acted at the transcriptional level, a coordinate effect on operatively linked genes would be expected. For the most part, such effects are absent (Montgomery et al., 1998). However, a recent report challenges some of these findings (Bosher et al., 1999). Double-stranded RNA directed against the lir-1 gene caused an embryonic lethality that was unexpected considering the phenotype of a lir-1 null animal. More detailed analysis revealed that lir-1 RNAi also caused a severe reduction in lin-26, an essential gene that is linked to lir-1 in a multigene operon. Also in contrast to previous reports, intronic sequences were effective in suppressing expression of the locus. At a minimum, these data demonstrates that RNA interference can target pre-mRNAs and raises the possibility that both transcriptional and post-transcriptional mechanisms can contribute to dsRNA-induced gene silencing in C. elegans.

Genetic approaches to mechanism

Several groups have taken genetic approaches toward illuminating the mechanisms underlying RNA interference (Ketting et al., 1999; Tabara et al., 1999). These have relied on the identification of animals that show resistance to dsRNA-induced gene silencing. By a combination of genetic selection and analysis of pre-existing mutant strains, six genes have been linked to RNAi so far. Mello and colleagues have identified four loci, rde1-4, mutation of which can provide resistance to dsRNA homologous to an essential gene (Tabara et al., 1999). Two mutator strains, mut-2 and mut-7, also proved to be insensitive (Tabara et al., 1999; Ketting et al., 1999). All of these mutants provide resistance to RNA interference in the germline, however, only rde1, 3, 4 and mut-2 gave complete resistance in the soma. Thus far, only rde-1 and mut-7 have been characterized at the molecular level.

The rde-1 gene encodes a member of a multi-gene family that is represented in evolutionarily diverse organisms (Tabara et al., 1999; Benfey, 1999). However, members of this family contain no discernable structural motifs that provide functional clues. In Drosophila, two homologs of rde-1 have been characterized. The Sting gene plays a role in silencing of the X-linked, repetitive stellate locus and in meiotic drive while Piwi has been implicated in the maintenance of the stem cell phenotype (Schmidt et al., 1999; Cox et al., 1998). Similarly, Arabidopsis homologs, Zwille and Argonaute, function in the maintenance of the undifferentiated state of stem cells (Moussian et al., 1998; Bohmert et al., 1998). These possible links to gene silencing are consistent with the derepression of transgenes in some RNAi-resistant mutants in C. elegans (although this is not seen in rde-1 animals, (Tabara et al., 1999; Ketting et al., 1999). A potentially informative homology comes from the rabbit family member, eIF2C, which was isolated as a major component of a fraction that promoted ternary complex formation between Met-tRNA, GTP and eIF2 (Zou et al., 1998). This might indicate that RNAi acts as a translational surveillance mechanism; however, the link between so-called eIF2C and the translational machinery has not been rigorously proven.

The mut-7 gene encodes a member of the reqQ/Werner/Bloom helicase family (Ketting et al., 1999). The authors also identified a weak homology to RNaseD, a bacterial RNA processing enzyme. This led to the suggestion that mut-7 protein might provide a nuclease activity that could degrade targeted mRNAs. The link between mut-7 and gene silencing has been strengthened by the finding that another member of this helicase family, qde-3, is required for homology-dependent gene-silencing (quelling) in Neurospora (Cogoni and Macino, 1999). Quelling refers to suppression of endogenous genes that is provoked by the introduction of transgenes. Although definitive evidence is

lacking, existing data are consistent with quelling operating at the post-transcriptional level (Cogoni et al., 1996).

An interesting feature of mut-7 and mut-2 strains is that both show increased activity of a variety of transposons (Ketting et al., 1999). This suggests that the mechanisms that protect the genome from an undesirable level of transposon activity may be related to RNAi. In fact, most transposons possess inverted repeat sequences at their termini that would be expected to form dsRNA. However, not all RNAi-deficient strains show elevated levels of transposition. For example, neither rde-1 nor rde-4 mutant animals have any evidence of this phenotype (Tabara et al., 1999).

Although genetics studies have begun to yield components of the dsRNA-induced silencing process, mechanistic insights have not been forthcoming. This argues strongly for the development of parallel, biochemical approaches that can synergize with genetics to unravel this complex phenomenon.

dsRNA-induced gene silencing in other systems

Investigators attempting to construct transgenic plants have long been plagued by the phenomenon of co-suppression (reviewed in Jorgensen et al., 1998). This refers to a copy-number-dependent silencing of transgenes and of endogenous sequences that are homologous to the transgenes. One component of co-suppression is post-transcriptional gene silencing (PTGS) which is characterized by decreased stability of target mRNAs (reviewed in Wassenegger and Pelissier, 1998; Baulcombe, 1996). A close relationship between PTGS in plants and RNAi is suggested by several observations. First, PTGS can be provoked by double-stranded RNA viruses that carry fragments homologous to endogenous genes (Waterhouse et al., 1998; Angell and Baulcombe, 1999; Angell and Baulcombe, 1997). This response suppresses not only the endogenous sequences but also provides virus resistance. As in C. elegans, silencing is non-cell autonomous and can be propagated throughout the plant (Voinnet and Baulcombe, 1997; Voinnet et al., 1998; Smyth, 1997). In fact, in cases of transgene co-suppression, systemic PTGS can be transferred from plant to plant by engraftment (Palauqui and Vaucheret, 1998; Palauqui and Balzergue, 1999). Finally, co-suppression is stable and heritable. However, in contrast to C. elegans, as the plants are propagated, a transition from post-transcriptional gene silencing to an epigenetic silencing that is maintained by alterations in chromatin structure may occur (Jones et al., 1999). In fact, dsRNA viruses can induce *de novo* methylation of genes that are homologous to sequences carried by the virus (Jones et al., 1998; Jones et al., 1999).

The successful use of dsRNA-induced gene silencing to probe gene function in C. elegans has encouraged investigators to attempt this approach in other systems. Virus-induced gene silencing is being deployed on a large scale in plants, and suppression of gene function by dsRNA has been successfully used in trypanosomes, hydra, planaria, zebrafish and Drosophila. As the availability of complete genome sequences becomes more common, the need for tools that enable investigators to link sequence to function will become acute. This is felt urgently in mammalian systems wherein procedures for creating mutant animals are time-consuming and costly.

Summary

My laboratory has devoted a number of years to creating improved tools for probing gene function in cultured mammalian cells; however, our experience indicates that a facile loss-of-function tool is lacking. Unfortunately, dsRNA induces somewhat generic responses in mammalian cells. It is our hope that by understanding the mechanistic basis of dsRNA-induced silencing, we may not only unravel a mysterious and important piece of biology but also provide the means to create improved tools for analyzing gene function in diverse organisms in which traditional genetic methods are either

cumbersome or unavailable. This notion that has contributed to the decision to focus substantial effort in my laboratory toward elucidating the mechanism of RNA interference.

Preliminary Results

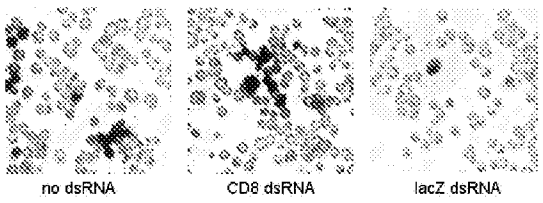
We have sought to complement genetic approaches to RNAi by establishing a model system in which double-stranded RNA-induced gene silencing could be approached biochemically. Of the organisms in which RNA interference has been shown to occur, only Drosophila offers the possibility of using easily cultured cells as a source of material for study.

To us, there seemed a number of advantages to using cultured Drosophila cells for investigating the mechanisms of double-stranded RNA-induced gene silencing. First, my laboratory has a great deal of experience growing and manipulating cultured mammalian cells. Thus, it was much easier to make the transition to cultured Drosophila cells than to a whole-organism system such as C. elegans or Drosophila embryos. Second, the source material for biochemical study is homogeneous and easily converted into cell-free extracts by established protocols. In fairness, this may also be said of Drosophila embryos (Tuschl et al., 1999). Third, source material is easy to prepare and is available in almost unlimited amounts. My prior experience in RNA processing was in the use of Ascaris embryo extracts for studies of trans-RNA splicing (see for example, Hannon et al., 1992; Hannon et al., 1990; Hannon et al., 1991). While these studies were fruitful, the preparation of source material was always a time-consuming and expensive process. In contrast, 100's of liters of cultured suspension cells can be easily produced. In fact, CSHL has a facility that for a nominal charge will generate large quantities of any suspension cell line. Finally, development of a cell culture system in which the expression of specific genes could be specifically and acutely suppressed would open the door to a tremendous amount of interesting biology.

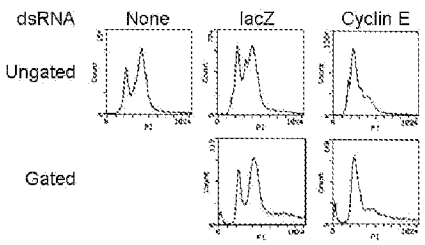
Recognizing the potential of cultured Drosophila cells for mechanistic studies of RNA interference, we tested whether introduction of dsRNA into S2 or Kc cells affected gene expression in a sequence-specific fashion. We began by probing effects on an ectopically expressed gene. Transient transfection of cultured, *Drosophila* S2 cells with a vector that directs lacZ expression from the copia promter resulted in β-galactosidase activity that was easily detectable by an *in situ* assay (Fig. 1A).

To determine whether dsRNA could suppress lacZ expression, we prepared a dsRNA corresponding to the first 300 nt. of lacZ. A transcription template containing T7 RNA polymerase promoters at each end was prepared by PCR. Routinely, ~100 μg of dsRNA can be prepared in a single *in vitro* transcription reaction that includes ~5 μg of transcription template. As a quality control, all of our dsRNA preparations are tested for sensitivity to RNAse III, an endoribonuclease that specifically digests dsRNA (Zhang and Nicholson, 1997; Nicholson, 1999), a kind gift of A. Nicholson, Wayne State Univ.).

A.



B.



C.

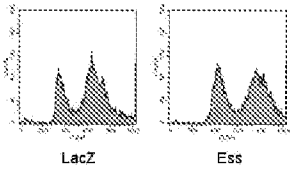


Figure 1. **A.** Drosophila S2 cells were transfected using a calcium phosphate protocol with a plasmid that directs lacZ expression from the copia promoter in combination with either no dsRNA or the indicated dsRNAs. Following optimization of transfection conditions, we achieve up to 50% (usually 20%) transfection rates using a phenotypic marker (e.g. LacZ or GFP expression) as a measure of efficiency. Identical results were obtained by transfection using lipid reagents (e.g. Superfect, Qiagen) and using Kc rather than S2 cells. **B**. Cells were transfected with dsRNAs corresponding to cyclin E or with a control dsRNA (lacZ), as indicated. Cells that successfully incorporated co-transfected DNA (lower panels, Gated) were marked using a plasmid that directs expression of a membrane linked GFP from the Drosophila actin promoter. This marker was chosen since the fluorescence of this fusion had been previously shown to survive fixation with ethanol (Kalejta et al., 1999). **C.** S2 cells were transfected either with a control dsRNA or with a single-stranded antisense RNA corresponding to the first 540 nucleotides of the cyclin E cDNA.

Transfection of S2 cells with lacZ dsRNA almost completely suppressed β-galactosidase activity, whereas transfection with a control dsRNA (CD8) had no effect (Fig. 1A). This result was obtained irrespective of whether the plasmid DNA and the dsRNA were co-transfected or whether transfection with the dsRNA preceded introduction of the plasmid by 1-2 days (not shown). Suppression of β-galactosidase activity was less effective if dsRNA was introduced subsequent to the plasmid; however, this observation may result from the extremely long half-life of the lacZ protein.

To determine whether RNAi could also target endogenous genes, S2 cells were transfected with a dsRNA corresponding to the first 540 nucleotides of *Drosophila* cyclin E, a gene essential for progression into S phase (Richardson et al., 1993; Knoblich et al., 1994). During log-phase growth, untreated S2 cells reside primarily in G2/M (Fig. 1B). While transfection with lacZ dsRNA had no effect on the cell-cycle distribution, transfection with the cyclin E dsRNA caused a G1 phase cell-cycle arrest (Fig. 1B).

One remarkable feature of our results is the apparent efficiency with which dsRNA provokes the response. While we achieve a maximal transfection efficiency of~50% with DNA plasmids (and more routinely 20%), essentially all of the cells in a dsRNA-transfected culture seem to be affected (e.g. Fig. 1). This could be explained in several ways. It is possible that the interfering activity could migrate from cell to cell. This is a feature of RNA interference in intact organisms (see **Background**) wherein injection of dsRNA at one site can suppress gene expression in remote tissues. Alternatively, dsRNA could be more effective than dsDNA at either entering cells or creating a phenotype. Based upon the characteristics of RNA interference *in vivo* (e.g. the ability of only a few molecules of dsRNA to ablate expression in an entire animal), it is possible that a small amount of dsRNA could provoke a disproportionate response in a cultured cell.

In the cases that have been examined to date, RNA interference has several distinguishing features. One is the requirement for dsRNA. To insure that the effects observed in S2 cells were due to dsRNA-induced gene silencing, we tested the ability of single-stranded RNAs of either sense or antisense orientation to provoke the phenotypes that we had observed with dsRNA. In all cases, ssRNA had small but reproducible effects. This was indicated, for example, by a small increase in the G1 population in cyclin E dsRNA-transfected cells (Fig. 1C). In all experiments, identical effects were observed with sense and antisense-oriented transcripts. These results coincide precisely with the original reports from C. elegans that described small but equal effects of sense or antisense transcripts and much more pronounced effects of dsRNA (Fire et al., 1998).

A second feature of RNA interference is a dependence on the length of the dsRNA. In C. elegans, drosophila and trypanosomes, only dsRNAs longer than ~150 nucleotides could efficiently interfere with gene expression (reviewed in Sharp, 1999). Similarly, in our assay, the ability of cyclin E dsRNA to provoke G1 arrest was length-dependent (Fig. 2A). Double-stranded RNAs of 540 and 400 nucleotides were quite effective, whereas dsRNAs of 200 and 300 nucleotides were less potent. Cyclin E dsRNAs of 50 or 100 nucleotides were inert in our assay.

Double-stranded RNA-dependent gene silencing in diverse organisms is associated with a reduction in the level of mRNA that is homologous to the dsRNA. Similarly, transfection of Drosophila S2 cells with cyclin E dsRNA (bulk population) caused a dramatic reduction in level of endogenous cyclin E mRNA as compared with control cells (Fig. 2B). This effect was specific since the levels of a number of other transcripts were unaffected. However, these could be suppressed by transfection with cognate dsRNAs (Fig 2B). For example, transfection of cells with dsRNAs homologous to *fizzy*, a component of the anaphase promoting complex (APC) or *cyclin A*, a cyclin that acts in S, G2 and M, also caused specific reduction of their mRNAs. The modest reduction in *fizzy* mRNA levels in cells transfected with *cyclin A* dsRNA probably resulted from arrest at a point in the division cycle at which *fizzy* transcription is low (Wolf and Jackson, 1998; Kramer et al., 1998). These results suggest that RNA interference may be a generally applicable tool for probing gene function in cultured, Drosophila cells. As the Drosophila genomic sequence nears completion, the coupling of RNAi with microarray analysis will allow the use of S2 cells as a tool for analyzing the functional role of individual gene products in complex biological pathways (see Aim 1).

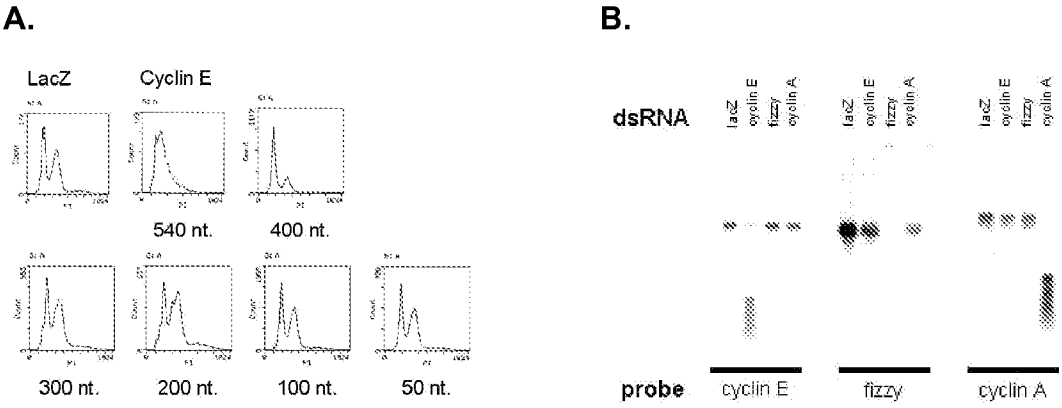


Figure 2. **A.** Templates for the generation of dsRNAs of the indicated lengths were prepared by PCR. FACS analysis of the bulk population of transfected cells (not gated on a co-transfected marker) is shown. **B.** Drosophila S2 cells were transfected with the indicated dsRNAs. After three days, cells were lysed and total RNA was prepared. This was analyzed by northern blotting with the indicated probes. Equal loading was insured by over-probing the blot with the RP49 cDNA (encoding a ribosomal protein). The smears of hybridization seen in some of the targeted samples may represent degradation products or could arise from cross hybridization of the probe to the transfected dsRNA. Although probes were designed to exclude sequences included in the dsRNA, minor contamination of the probe could potentially produce the observed signal.

The forgoing data indicate that gene silencing can be provoked by transfection of S2 cells with dsRNA. To probe the mechanisms underlying RNAi, we worked to create a cell-free assay that could reflect this process, at least in part. The decrease in mRNA levels that are observed upon transfection of specific dsRNAs into Drosophila cells could be explained by effects at transcriptional or post-transcriptional levels. However, data from other systems have strongly suggested that some elements of RNA interference affect mRNA directly (see **Background**).

A simple model for the observed properties of RNA interference would be the induction by dsRNA of a nuclease activity that could specifically target cognate mRNAs. We therefore designed an assay to search for such an activity. S2 cells were transfected with dsRNAs corresponding to either cyclin E or lacZ, and whole-cell extracts were prepared by a simple, hypotonic lysis procedure. To test for the presence of nuclease activity, these extracts were incubated with ^{32}P-labelled, synthetic transcripts derived from either the cyclin E or the lacZ cDNAs.

Extracts prepared from cells transfected with cyclin E dsRNA efficiently degraded the cyclin E transcript; however, the lacZ transcript was stable in these lysates (Fig. 3). Conversely, lysates from

cells transfected with the lacZ dsRNA degraded the lacZ transcript but left the cyclin E mRNA intact. Optimization of salt concentrations indicated maximal activity in the physiological range (not shown). The nuclease was inhibited by EDTA but not EGTA, suggesting a requirement for a divalent cation, probably Mg^{2+}. Degradation was not stimulated by the addition of exogenous nucleotides or by inclusion of an ATP regeneration system. Treatment of extracts with apyrase had no effect, strongly suggesting the lack of an NTP requirement.

These results suggest that RNA interference reduces the level of target mRNAs, at least in part, through the generation of a sequence-specific nuclease activity. Although we occasionally observed possible intermediates in the degradation process (see Fig 3), the absence of stable cleavage end-products indicates an exonuclease (perhaps coupled to an endonuclease). However, it is possible that the RNAi nuclease makes an initial endonucleolytic cut and that non-specific exonucleases in the extract complete the degradation process (see Shuttleworth and Colman, 1988). In addition, our ability to create an extract that targets lacZ *in vitro* indicates that the presence of an endogenous gene is not required for the RNAi response.

Recently, Sharp and colleagues have reported the preparation of extracts from Drosophila embryos in which the addition of specific dsRNAs can cause inhibition of translation and degradation of a luciferase RNA (Tuschl et al., 1999). Although our results are similar to those reported, we feel that the S2 cell system may have a number of advantages over the embryo system for the characterization of the dsRNA-induced nuclease activity. Chief among these is the relative ease with which the material can be prepared. However, continuing comparison of these systems may reveal differences which make each uniquely suited to the analysis of specific aspects of RNA interference.

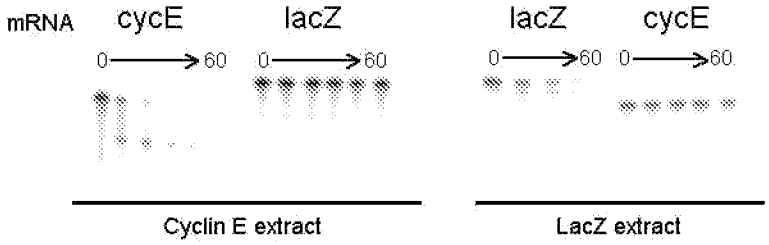


Figure 3. S2 cells were transfected with dsRNAs derived from either the cyclin E or lacZ cDNAs, as indicated. After 3 days, cells were tested for a successful dsRNA response by monitoring the cell cycle arrest induced by ablation of cyclin E. Extracts were prepared by havesting cells in 5mM EGTA/5mM EDTA. Cells were washed in PBS three times and in hypotonic lysis buffer (10 mM Hepes pH 7.3, 6 mM β-mercaptoethanol) once. Cells were disrupted by 20 strokes in a dounce homogenizer (type B pestle). The resulting lysates were centrifuged for 20 min. at 30,000xg, and supernatants were used in the degradation assay. Assays were carried out for the indicated times in a reaction buffer (20 mM hepes pH 7.3, 110 mM KOAc, 1 mM $Mg(OAc)_2$, 3 mM EGTA, 2 mM $CaCl_2$, 1 mM DTT). Samples were analyzed by electrophoresis on 8% denaturing polyacrylamide gels.

To examine the substrate requirements for the dsRNA-induced, sequence-specific nuclease, we incubated a variety of cyclin E-derived transcripts with an extract derived from cells that had been transfected with cyclin E dsRNA (Fig. 4A). Just as a length-requirement was observed for the transfected dsRNA, the RNAi nuclease activity showed a dependence on the size of the RNA substrate. Either a 600 nt. transcript that extends slightly beyond the targeted region (Fig. 4A) or an ~1 kb. transcript that contains the entire coding sequence (not shown) were completely destroyed by the extract. Surprisingly, shorter substrates were not degraded as efficiently. Reduced activity was observed against either a 300 or a 220 nt. transcript, and a 100 nt. transcript was nuclease-resistant in our assay. This was not due solely to position effects since ~100 nt. transcripts derived from other portions of the transfected dsRNA behaved similarly (not shown). In the *in vitro* system, neither a 5'

cap nor a polyA tail was required since such transcripts were degraded as efficiently as uncapped and non-polyadenylated RNAs.

Since RNAi is provoked by transfection with dsRNA, the nuclease activity present in the extract was expected to recognize both sense and antisense strands of the cyclin E mRNA. In accord with this prediction, antisense substrates that contained a substantial portion of the targeted region were degraded efficiently (Fig. 4B). As was observed for the sense-oriented transcripts, degradation depended on the size of the substrate. Short anitsense transcripts (~100 nt.) that fell within the targeted region were nuclease resistant (not shown). Interestingly, recognition by the nuclease did not depend solely on the size of the transcript but on the size of the region homologous to the dsRNA. Transcripts that are of sufficient length to be degraded efficiently but that contain only short stretches of homologous sequence (~130 nt.) largely resisted the activity of the RNAi nuclease (as600, Fig. 4B). For both the sense and antisense strands, transcripts that contained no homology to the transfected dsRNA (Eout, Fig. 4A and as300, Fig. 4B) were not degraded. Although we cannot rigorously exclude the possibility that nuclease specificity could have migrated beyond the targeted region, the resistance of transcripts that do not contain homology to the dsRNA is consistent with data from *C. elegans.* In most cases, double-stranded RNAs homologous to an upstream cistron have little or no effect on a linked downstream cistron, despite the fact that polycistronic mRNA precursors can be readily detected (see **Background**).

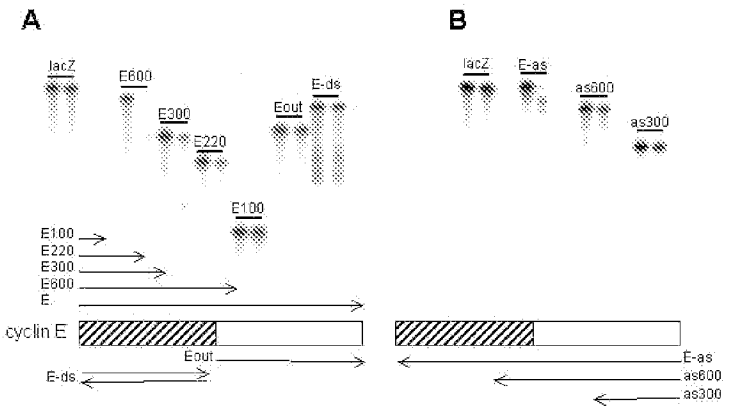


Figure 4. Transcription templates were prepared by PCR of the Drosophila cyclin E cDNA. Synthetic, ^{32}P-labelled transcripts were incubated in an extract of S2 cells that had been transfected with the cyclin E dsRNA for 0 or 30 minutes. The cyclin E cDNA is shown below each panel as a box. Cross-hatching indicates the portion of the cDNA that was present in the transfected dsRNA.

Somewhat surprisingly, the nuclease was incapable of digesting a dsRNA identical to that used to provoke the RNAi response *in vivo* (Fig. 4A). This seemed counterintuitive since dsRNA provokes the creation of the nuclease. Mechanistically, this suggests that either the recognition or degradation step is specific for ssRNA. This raises many questions concerning the ability of the nuclease to recognize and degrade highly structured RNAs such as rRNA or dsRNAs that act as replication intermediates for RNA viruses. One goal of the studies outlined in this application will be to address these issues.

Gene silencing provoked by dsRNA is sequence-specific. A plausible mechanism for determining specificity would be incorporation of nucleic acid guide sequences into the complexes that accomplish silencing (Hamilton and Baulcombe, 1999 and reviewed in Sharp, 1999). A well-established method for testing the dependency of a process on a nucleic acid component is through use of a conditionally active nuclease (see for example Furneaux et al., 1985; Krainer and Maniatis, 1985). The activity of micrococcal nuclease depends on Ca^{2+}. Thus, extracts can be treated with this

nuclease in the presence of calcium to destroy endogenous RNA and DNA. The micrococcal nuclease can be inactivated by addition of EGTA, and the reaction in question can be carried out. Inhibition is interpreted as evidence for a nucleic acid requirement. Pre-treatment of S2 extracts with micrococcal nuclease abolished the ability of these extracts to degrade cognate mRNAs (Fig. 5A), indicating the requirement for a nucleic acid cofactor. This is likely to be an RNA since treatment of the extract with DNAse I had no effect (Fig 5A). Sequence-specific nuclease activity, however, did require protein (not shown).

One potential artifact in this type of experiment is a false-positive result that arises from the release of RNA binding proteins that could coat the substrate molecule and block access by the RNAi nuclease. In addition, micrococcal nuclease itself can bind RNA non-specifically in the absence of calcium. We therefore tested whether addition of non-specific competitor RNA following nuclease treatment could rescue activity. Neither yeast tRNA nor total S2 RNA had any effect. Considered together, our results support the possibility that the RNAi nuclease is an RNP, requiring both RNA and protein components. Biochemical fractionation (see below) is consistent with these components being associated in extract rather than being assembled on the target mRNA following its addition.

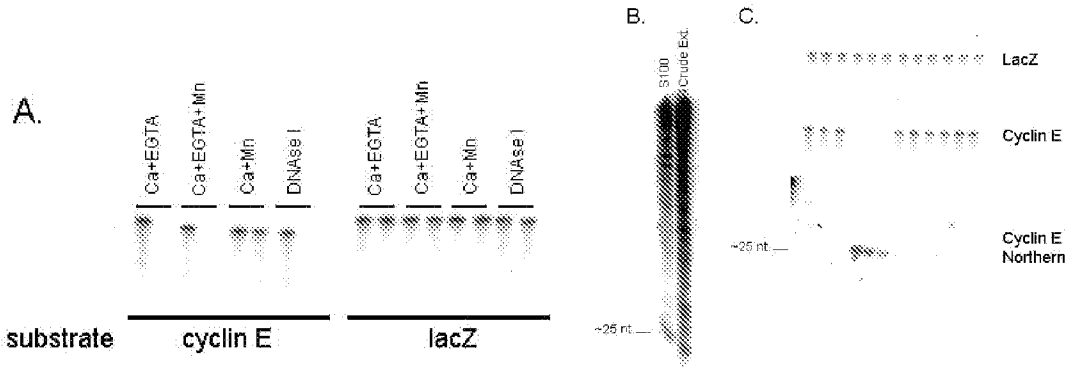


Figure 5. **A**. S2 cells were transfected with cyclin E dsRNA and extracts were prepared as described above. 30 μl aliquots of extract were treated with 60U of micrococcal nuclease (Mn, Worthington) for 30 min at 30°C in the presence of 1 mM Ca^{2+}. At the end of 30 minutes, EGTA was added to 5 mM. Controls included pretreatment with Ca^{2+} in the absence of nuclease (lanes Ca) and treatment with the nuclease in the absence of calcium (lanes Ca+EGTA+Mn). DnaseI (RQ1, Promega) was added to 2U per reaction. Following pretreatment, the aliquots of extract were incubated for 30 min with either the lacZ or cyclin E substrate as indicated. **B, C** S2 cells were transfected with cyclin E dsRNA and extracts were prepared as described above. **B**. RNA was prepared either from crude lysates or from S100 (ribosomal) pellets. This was electrophoresed on a 15% polyacrylamide/Urea gel and transferred to Hybond N+ by electroblotting. A probe was prepared by *in vitro* transcription and corresponded to the 540 nt cyclin E substrate (sense orientation). The blot was hybridized in a moderate stringency buffer (500 mM $NaPO_4$, pH 7.0, 15% Formamide, 7% SDS, 1% BSA) overnight at 45°C. Washing was at 37°C in 1X SSC. **C**. The activity was extracted from the S100 pellet with 300 mM KCl, diluted and chromatographed on a Q-sepharose column. Fractions surrounding the peak of activity are shown. The top panel demonstrates a lack of activity toward a control substrate, lacZ. The center panel shows activity toward the cognate substrate, cyclin E. The bottom panel shows a northern blot of RNAs contained in the fractions.

Data indicating the dependence of the RNAi nuclease on an essential RNA component prompted a search for the nature of the cofactor. In plants, the phenomenon of post-transcriptional gene silencing has been associated with the existence of small (~25nt) RNAs that correspond to the gene that is being silenced (Hamilton and Baulcombe, 1999). To address the possibility that a similar RNA might exist in *Drosophila* and guide the sequence-specific nuclease in the choice of substrate, we partially purified our activity and searched for co-fractionating RNAs that are homologous to the substrate.

We initially attempted to fractionate the activity by sedimentation through glycerol and sucrose density gradients. These indicated a very high molecular weight for the nuclease; however,

examination of active fractions indicated that the nuclease was associated with ribosomes. This was of interest for several reasons, principle among which was the previously articulated notion that RNAi might work as a translational surveillance mechanism (see **Background**). However, we have not established definitively whether association with ribosomes is biologically relevant or whether our observations reflect an artifact of extract preparation.

In any case, it was necessary to dissociate the nuclease from ribosomes before any serious attempt at purification could be made. Numerous proteins that associate with ribosomes can be released by incubation at high salt concentrations (for example, reviewed in Merrick, 1994). Similarly, the RNAi nuclease can be quantitatively released from the ribosome fraction by incubation with 300 mM KCl. Gel filtration of the soluble nuclease indicates a size of between 200 and 500 kDa. (although this estimate is still quite crude). Chromatography of soluble nuclease over an anion exchange column (Q-sepharose) resulted in a discrete peak of activity (Fig 5C, cyclin E) that retained specificity since it was inactive against a heterologous mRNA (Fig 5C, lacZ).

Crude extracts contained both sequence-specific nuclease activity and abundant, heterogeneous RNAs homologous to the transfected dsRNA (Fig. 2,5). Active fractions contained a discrete RNA species of 25 nt that is homologous to the cyclin E target (Fig 5C, northern). This band is likely to represent a family of distinct RNA species since it could be detected with probes specific for both the sense and antisense cyclin E sequences and with probes derived from completely independent segments of the cyclin E dsRNA (not shown).

RNA-interference allows an adaptive defense against both exogenous and endogenous dsRNAs, providing something akin to a dsRNA immune response. The results presented in this section provide a framework within which models of dsRNA-induced gene silencing can be created. According to our data, introduction of dsRNA into Drosophila cells provokes the assembly of a sequence-specific nuclease. This occurs through conversion of the dsRNA, either via processing or replication, into small RNAs that are homologous to the substrate. These are incorporated into a nuclease that is apparently a ribonucleoprotein particle, containing both essential protein and RNA subunits. Fractionation of the nuclease and northern analysis of the 25 nt RNA indicates that a family of nucleases is created, with each member having a 25mer that targets a specific portion of the substrate.

Our data draws a striking parallel between RNA interference in Drosophila and post-transcriptional gene silencing in plants. As stated above, plants in which PTGS is occurring contain 25 nt RNA species that are homologous to the gene that is being suppressed. The identical size of putative specificity determinants in plants (Hamilton and Baulcombe, 1999) and animals predicts a conservation of both the mechanisms and the components of dsRNA-induced, post-transcriptional gene silencing in diverse organisms.

As this proposal was nearing completion, a manuscript reporting the data in this section was accepted for publication (Hammond et al., Nature, in press).

Based upon the preliminary results presented herein, we feel that we have developed a system that can be used to decipher the mechanisms underlying RNA interference in Drosophila. We have not only demonstrated that elements of the RNAi response can be recapitulated *in vitro* but that the sequence-specific nuclease activity in these extracts can be fractionated through multiple purification steps. The data presented in the forgoing section also raise the possibility of using the S2 system to ask a broad range of biological questions. While the aims of this proposal are focused largely on the mechanisms of RNA interference, ancillary benefits may accrue in the form of improved

tools for probing the function of the many genes that are emerging from the Drosophila genome sequencing effort.

Experimental Procedures

Aim 1. RNA interference in cultured Drosophila cells.

The experiments outlined in this section of the proposal are aimed at characterizing the dsRNA response in cultured S2 cells. Such experiments will serve two purposes. The first is to act as a prelude to isolation and characterization of the sequence-specific nuclease. Optimization of the cell system should yield significant improvements in the preparation of starting material for the purification process. The second is to set the stage for the use of S2 and other Drosophila cell lines as generic tools for probing gene function. Data presented in the **Preliminary Results** strongly suggest that RNA interference can be used to create relevant loss-of-function phenotypes in culture. However, generalizing the approach requires not only a better understanding of the dsRNA response and the ways in which it can be provoked but also perhaps the design of improved tools for manipulation of Drosophila cells.

Time-course of the dsRNA response

In the experiments that were presented above, the phenotypes of cells transfected with dsRNA were determined at approximately 3-5 days post-transfection. Both for analysis of gene function and for maximizing the material available for biochemistry, it will be essential to know the time-course of the response to a single dose of dsRNA. For optimizing preparation of extracts, it is the duration which is critical because long-lived response may allow us to amplify transfected cells prior to lysis. For analysis of gene function, it is important to know at what point effective suppression of gene expression is achieved and at what point the response decays.

For these experiments, it is essential to have a read-out that is not counter-selected in the population, as is cell cycle arrest provoked by loss of cyclin E. So that changes in RNA levels are accurately reflected by the phenotype, a short half-life protein should be used (ruling out lacZ). Finally, the readout should be at least semi-quantitative. All of these criteria are fulfilled by GFP derivatives that have been destabilized by fusion to degrons (signals that specify degradation by the ubiquitin-proteosome pathway, (Li et al., 1998), and available commercially from Clontech).

As a preliminary test of the strategy, we have used dsRNA to target several GFP derivatives in S2 cells in transient assays. As mentioned above, a GFP-US9 fusion protein is easily detectable by FACS following transient transfection. This is also true of a destabilized GFP derivative that has a half-life in mammalian cells of ~30 min (we are presently determining the half-life in S2 cells). Transfection with a dsRNA comprising the first 450 nt. of the GFP coding sequence reduces expression of either GFP to undetectable levels (not shown).

We will begin by constructing a stable S2 cell line that expresses the destabilized GFP (see below). These cells will be transfected with the GFP dsRNA under conditions that quantitatively eliminate GFP positive cells. Although it is reasonable to assume that this is possible based upon our prior results, transfected cells could be isolated using a co-transfection marker (we have successfully used CD8 and magnetic affinity resins in S2), if necessary. Cell populations will be tested by FACS for changes in the pattern of GFP fluorescence. Assays will begin at 4 hours post transfection and will be carried out at four-hour intervals until the 24 hour time point. Cells will then be tested at each subsequent 12-hour mark. For reference, we will assay, in parallel, cell free extracts for the presence of sequence-specific nuclease activity. Of course, it will also be necessary to extend any results to several different genes.

These studies should give us a general idea of how the dsRNA response proceeds in cell culture. The rapidity of the response will be an important parameter in judging the utility of S2 cells for probing gene function (see below). Our preliminary experiments have demonstrated that cell cycle arrest in response to cyclin E dsRNA occurs within 24 hours of transfection (not shown). However, searching, for example, for downstream targets of signaling pathways or transcriptional activators would be best accomplished if the response could be provoked even more quickly. Analysis of the decay of the RNAi response may also provide important mechanistic clues. For example, if the *in vitro* and *in vivo* assays diverge, this could indicate that the dsRNA-induced gene silencing is a multifaceted response, involving more that just degradation of cognate mRNAs. There is indeed evidence for a transcriptional component to persistent dsRNA-induced silencing in plants (see **Background**), and experiment to address this possibility in S2 cells are outlined, below.

Can the dsRNA be encoded?

Our experiments, to date, have provoked gene silencing by introducing into cells dsRNA that had been synthesized *in vitro*. Data from other systems indicates that the duration of the response to an acute exposure to dsRNA is limited. In C. elegans, dsRNA-induced gene silencing can last for more than one generation; however, this comprises only a few days. Experiments detailed above should provide a measure of the duration of the response in S2 cells; however, it is reasonable to assume that this, as in other systems, will be finite. Therefore, it is unlikely that we will be able to create stable, knockout cell lines simply by delivering a single dose of dsRNA. The ability to provoke gene silencing by stably expressing dsRNAs will have tremendous impact on our efforts to analyze the mechanisms underlying RNA interference, allowing the preparation of almost unlimited amounts of starting material for biochemistry and purification. With regard to the use of S2 cells for probing gene function, the ability to create stable loss-of-function mutants will potentially allow monitoring of phenotypes over longer time periods and may also permit the use of dsRNA expression libraries for forward genetics in cell culture.

A number of strategies have proven successful in other systems for provoking gene silencing by expression of dsRNAs, and these will guide our efforts to achieve a similar goal in S2 cells. The simplest strategy involves the use of RNA polymerase II promoters to drive expression of both the sense and antisense strands in the target cells. Unfortunately, we have attempted this method without success. There are a number of possible explanations for the failure of this simple approach. First the two strands of cyclin E may have failed to associate *in vivo*. Second, if association did occur, insufficient dsRNA may have been produced. We, therefore, propose several alternative strategies.

In trypanosomes, the most efficient method for inducing RNAi is by transfection with plasmids that direct separately expression of each strand of the dsRNA using bacteriophage T7 RNA polymerase (Ngo et al., 1998, E. Ullu, personal communication). T7 RNA polymerase has been used to direct high-level expression of RNAs in diverse systems, including mammals, fungi and insect cells (Studier et al., 1990; Dunn et al., 1988; Benton et al., 1990; van Poelwijk et al., 1995). This system generally has two components. The first directs the synthesis of the polymerase, and the second contains the T7 promoter (and often terminator) and the RNA that is to be expressed. To avoid complications that can arise from transient transfection, we will begin by creating an S2 cell line that stably expresses the T7 polymerase. For this purpose, we have obtained from Bill Studier (Brookhaven National Labs) mammalian expression vectors that contain the T7 polymerase coding sequence, either with or without an appended nuclear localization signal (Dunn et al., 1988). To achieve expression in S2 cells, we will transfer the T7 polymerase gene to an expression vector (pIZ, Invitrogen) that contains the baculovirus IE2 promoter. This vector contains in addition a Zeocin

resistance gene as a selection marker (we have also constructed a version with the more manageable neomycin resistance gene). Clonal cell lines will be established and tested for polymerase expression using a variety of *in vitro* and *in vivo* assays. We have previously used T7 polymerase to direct expression of RNAs in mammalian cells and are well versed in assays for its function.

Once T7-expressing S2 (S2T7) cells have been generated we will examine the possibility that T7-driven transcripts can be used to provoke RNA interference. We will create an expression vector in which T7 promoter sequences flank a ~500 nt segment of the cyclin E cDNA. The expression cassette will be surrounded by strong T7 termination signals (Lyakhov et al., 1998). In this way, a single plasmid can give rise to both strands of the dsRNA. Transfection along with a GFP marker should allow us to assess the ability of this plasmid to provoke a response using cell cycle arrest as an assay.

It is conceivable that separately expressed transcripts (even from the same plasmid) may not form duplexes efficiently *in vivo*. Therefore, we will also take a second strategy that has proven effective in trypansomes (Ngo et al., 1998). Intramolecular duplexes may form much more efficiently than intermolecular duplexes (see for example Jones and Sullenger, 1997). Thus, we will attempt to elicit silencing by expression of an RNA hairpin. The 540 nt. fragment of the cyclin E RNA will be cloned in opposing directions around a spacer sequence. For convenience, the spacer will be the 400 nt. Zeocin resistance gene. This will allow not only sufficient spacing to permit hybridization but will provide selective pressure against deletion events that are common in the propagation of this type of construct in E. coli. Our previous experience in the construction of long hairpins suggests that the use of either Sure (Stratagene) or STBL2 (Gibco) cells produces the best results. Hairpin constructs will be produced in two formats, one driven by an RNA polymerase II promoter (initially actin, but HSP70 could eventually provide inducibility) and T7. Following transient transfection into S2 cells, G1 arrest will provide an assay for successful silencing.

If expression of the hairpin does silence cyclin E expression, it will be of interest to determine whether the response is restricted to the double-stranded portion of the RNA. This can be tested in one of several ways. First, using the *in vitro* assay described above, we can search for nuclease activity against a synthetic transcript that is homologous to the spacer region. Alternatively, we could replace the zeocin resistance gene that acts as a spacer with sequences from another cellular gene (e.g. cyclin A) and look for loss of endogenous cyclin A transcripts by northern blotting. Without knowing the mechanisms by which dsRNAs are converted into sequence directed silencing complexes, it is difficult to predict whether a response will occur to both the single and double stranded portions of the hairpin. If the dsRNA is simply processed to create the specificity determinant of the nuclease, it seems likely that the response will be restricted to the double-strand only. However, if a replication strategy is used to generate the 25mer, then the single stranded portion of the hairpin may also be targeted. It should be noted that similar experiments will also be carried out by transfection of S2 cells with hairpin and partial duplex RNAs that have been synthesized *in vitro*.

Probing gene function in cultured S2 cells

The ability to encode dsRNAs would open the door to the use of S2 cells as a “genetic organism” in cell culture. My laboratory has devoted significant effort to the creation of improved tools for genetic, complementation screening in cultured mammalian cells (Hudson et al., 1999; Maestro et al., 1999; Hannon et al., 1999; Sun et al., 1998, Fig 6). These are based upon a series of modified retroviral vectors that have been designed to allow efficient delivery of cDNAs and cDNA libraries to cells in culture. The inclusion of recombination signals in the retroviral LTRs and the placement of a

700 nt. bacterial replicon between the LTRs allows efficient recovery of cDNAs from cell populations that have been selected based upon phenotypic criteria. Retroviral constructs that have been recovered can be used directly for the production of infectious virions for subsequent rounds of screening or for confirmation that a particular cDNA can produce a given phenotype.

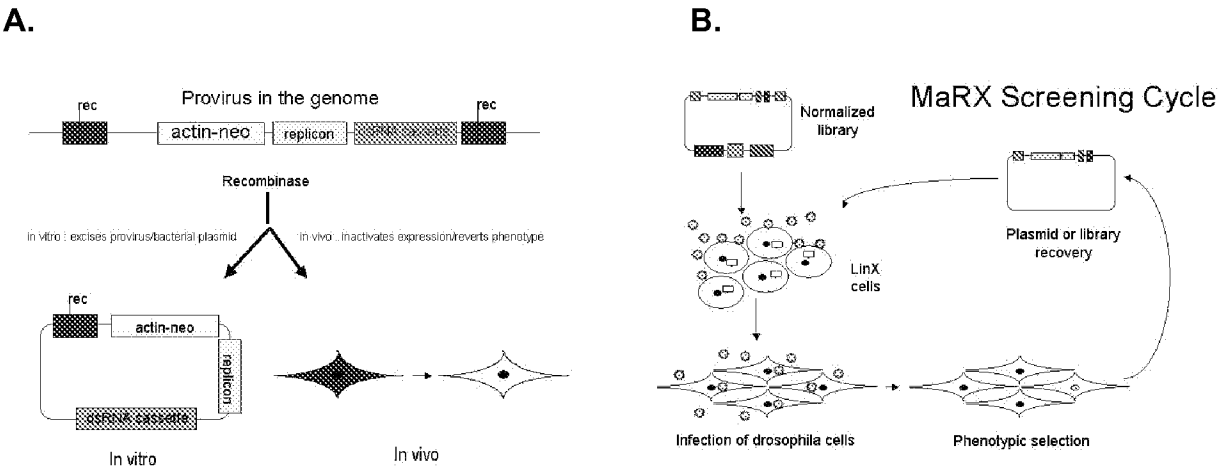


Figure 6. Adaptation of the MaRX system for use in Drosophila cells. We have created a retroviral gene transfer system that allows the isolation of genes from cDNA libraries based upon functional screens in cultured cells. A. Schematic diagram of a MaRX provirus. Inclusion of recombinase sites in the LTR allow excision and recovery of the provirus from the genome of infected cells. The excised plasmid can not only be propagated in bacteria but also be used to create infectious virus without intervening cloning steps. B. A schematic of the screening cycle. Plasmid libraries can be converted into high-titer retrovirus in LinX packaging cells. These are used to infect recipient (e.g. S2) cells. Infected cells are selected based upon the desired phenotype and the gene that presumably conferred the phenotype is rescued by in vitro excision. Recovered plasmids can be used in further rounds of screening or for confirmation of results.

Drosophila cells can also be efficiently infected by MuLV-based retroviruses. This can be accomplished either by use of the promiscuous envelope protein, VSV-G protein, derived from vesticular stomatitis virus or by use of the gypsy envelope protein (Teysset et al., 1998; Jordan et al., 1998). We have tested the use of retrovirus vectors in S2 cells and have achieved good infection efficiencies (~5-10% rates of stable gene transfer without optimization). Modification of our existing retrovirus vectors and retrovirus packaging cell lines for use in S2 cells will be a simple matter. The precise nature of the modifications will be guided by the experiments proposed above. At a minimum, we must include a marker gene (e.g. neomycin resistance) under the control of the Drosophila actin promoter. In addition, we may, for example, include a T7 transcription unit that drives the synthesis of a hairpin RNA or opposing T7 promoters to drive expression of each strand of the dsRNA separately (Fig 6).

In any case, the coupling of RNA interference with an efficient method for creating stable cell lines may allow S2 cells to be used to probe the broad range of biological questions that are accessible in cultured cells. One example would be screening loss-of-function cDNA libraries, however, there is a potential problem. One characteristic on RNAi *in vivo* is that is non-cell autonomous. *In vitro*, transfection with dsRNA is much more efficient at creating a defined phenotype than is transfection with a DNA expression construct. This raises the possibility that RNA interference can also be passed from cell to cell *in vitro*. If this is the case, screening of complex populations would be impossible since phenotypes would disperse throughout the culture. To address this possibility, we will use mixing experiments. Populations of S2 cells will be separately transfected with the GFP-US9 expression vector and with cyclin E dsRNA. These cell populations will then be mixed. The cyclin E-transfected cells should reside mainly in G1. The GFP-transfected cells should show a

normal cell cycle distribution, with the majority of cells in G2. If the RNAi activity can be transferred efficiently between cells, the GFP-labelled cells should shift into G1 upon mixing with a population of cells that have been transfected with the cyclin E dsRNA. To maximize our chances of observing the phenomenon, cyclin E transfected cells will initially be in excess. However, if we find evidence of migration, we can gauge its efficiency by varying the ratio of the two cell populations.

Although migration *in vitro* could be problematic for the use of RNAi as a library screening tool, it would raise a series of interesting biological questions. For example, we would, of necessity, investigate the nature of the transferred entity. Could activity be transferred through media independently of the transfected cells (as might be expected for a virus-like particle)? Could the entity be propagated indefinitely? However, since it is unknown whether such transfer occurs, it would be far too speculative to discuss precise experiments at this point.

A use of S2 cells that would not be affected by migration of the activity would be probing the function of individual genes. For example, one could create acutely loss-of-function mutations in a given endogenous gene (see Preliminary results). Targeting only 5' or 3' untranslated regions would allow wild-type copies to be replaced by mutant versions of the gene – e.g., by expression of altered mRNAs containing only the coding sequence. In this way, one might link individual protein domains with specific biological functions.

We are currently attempting such an analysis with the Drosophila Myc protein. Although the Myc oncogene has been known for more than 20 years, its biological functions remain obscure and hotly debated. The Drosophila Myc protein and other components of the pathway such as Max) is well conserved both structurally and functionally (Gallant et al., 1996; Johnston et al., 1999; Schreiber-Agus et al., 1997). By creating acute loss of function mutants of dMyc, we can examine both phenotypic consequences and changes in gene expression using DNA microarrays (a unigene set of 7000 genes is expected in April, and CSHL has a commitment to producing arrays of these clones). We can then move to linking specific changes in gene expression to specific regions of the protein by expression of mutants that are not targeted by the dsRNA. Furthermore, consequences of disrupting individual effector pathways (e.g. Max) can also be correlated. These pilot experiments could potentially form the basis for a much broader program which incorporates RNAi into other projects within the lab, not only in the study of the role of myc in cellular transformation (Wang et al., 1998) but also in ongoing studies of centrosome duplication and apoptosis (Maestro et al., 1999).

Is there a transcriptional component to dsRNA-induced silencing?

In C. elegans, the majority of the evidence is consistent with dsRNA-induced gene silencing operating at the post-transcriptional level (see **Background**). However, in plants, dsRNA clearly induce both post-transcriptional effects and silencing at the corresponding genomic locus by alterations in chromatin structure (Jones et al., 1998; Jones et al., 1999). The dsRNA-induced, sequence-specific nuclease is sufficient to account for the effects that we have observed, to date, in S2 cells. However, we will also probe the possibility that dsRNA affects expression at the transcriptional level.

The most straightforward way to assess the effects of dsRNA on the transcription of specific genes is through nuclear run-on assays. Nuclei are prepared rapidly at low temperature. Polymerases that are transcribing the gene are trapped on the template. Isolated nuclei are supplied with radioactive precursors and incubated for a short time (2-30 min) at physiological temperature to allow engaged polymerases to complete transcription. The polymerase density on a given gene is then reflected by the amount of radioactivity that has been incorporated into that transcript. This is measured by hybridization, usually to a single stranded probe comprised of the gene sequence.

Although the assay is simple, in principle, it is sometimes difficult in practice. Fortunately, a number of colleagues at CSHL have significant experience in this type of analysis (see attached letter from Nouria Hernandez).

S2 cells will be transfected with dsRNAs corresponding to lacZ, fizzy, cyclin E and cyclin A, and the transcription rates of each endogenous gene will be measured at 1, 2, 3, 4, and 5 days post-transfection. LacZ-transfected cells will serve as a control. The RP49 gene will serve to standardize the quality of the nuclear preparations.

Nuclear run-ons, although suggestive, will not distinguish between a transcriptional effect and a nuclease that acts quickly on newly synthesized RNA. Therefore, it will be necessary to correlate results with another method of probing transcriptional activity. The most straightforward is to measure the density of RNA polymerase II by chromatin immunoprecipitation (reviewed in Kuo and Allis, 1999). We have enlisted the aid of Nouria Hernandez in whose lab this approach is being used to help us with these experiments (see attached letter). If we note that transfection with dsRNA specifically reduces transcription of the targeted gene, then we will address the question of dsRNA-induced chromatin remodeling in more detail.

Lacking the primary result, a detailed description of these experiments is premature. Briefly, we will examine the state of chromatin on the silenced gene using a battery of well-established assays. Since Drosophila essentially lack endogenous DNA methylation, exogenously expressed methylases can be used to examine the accessibility of the targeted sequence (Wines et al., 1996; Boivin and Dura, 1998). A number of investigators have successfully use E. coli Dam methylase to probe chromatin structure both in S2 cells and in intact flies. We have obtained the necessary constructs, and can call upon a local colleague (Shiv Grewal) who works on gene silencing in S. pombe for help with these studies. In addition, we can monitor histone acetylation and association of polycomb group proteins (since these have been associated with cosuppression in Drosophila) with the targeted locus using CHIP assays (chromatin immunoprecipitation, reviewed in Kuo and Allis, 1999). Ultimately, our goal would be to establish *in vitro* assays that reflect any chromatin remodeling that we observe *in vivo*. These could allow eventual biochemical purification of sequence-specific chromatin remodeling complexes, should they exist.

Summary

Work described in this section is aimed at a characterization of RNA interference in cultured Drosophila cells. These studies will serve as a launch pad for the biochemical studies proposed below but may also pave the way for an expanded use of S2 and other cultured Drosophila cells for analysis of gene function in diverse biological pathways.

Aim 2. RNA interference *in vitro*.

The goal of the experiments outlined in this section is to examine dsRNA-dependent gene silencing using our *in vitro* system. We will first investigate the steps leading up to the generation of the sequence-specific nuclease activity and then focus our efforts on the nuclease itself. Purification of the nuclease activity will allow a biochemical characterization of the enzyme and ultimately lead, through procedures outlined in Aim 3, to the identification of the protein and RNA components that form the RISC.

dsRNA metabolism and the source of the putative guide RNA

RNA interference is initiated upon detection of dsRNA in the cell. This results ultimately in the production of a family of nuclease complexes that incorporate sequences from the dsRNA as possible

guides to substrate selection (See **Preliminary Results**). Despite their heterogeneous sequence composition, the putative guide RNAs are remarkably discrete. Incoming dsRNA could be converted into guide RNAs through processing, replication or a combination thereof. Analysis of RNA interference in C. elegans has provided compelling evidence for amplification of the response. Injection of as few as 30 molecules of dsRNA is sufficient to ablate gene expression throughout the worm (Fire et al., 1998). One plausible explanation for this observation is that the dsRNA or the guide RNAs could be replicated in a self-perpetuating fashion as the response is transmitted throughout the animal.

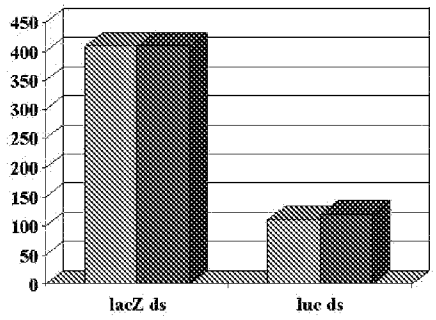


Fig. 7 Naïve S2 extracts were tested for dsRNA dependent gene silencing using translation of luciferase mRNA as an assay (this is similar to the RNAi assay described in Tuschl et al., 1999). Addition of cognate dsRNA but not heterologous dsRNA suppressed luciferase activity by ~4-fold. Duplicate reactions are shown.

In early experiments, we used extracts of naïve cells in which RNA interference was initiated by addition of dsRNA *in vitro* (see Fig. 7). Our focus on extracts of dsRNA-transfected cells was prompted by their high efficiency. However, lysates of naïve S2 cells are well suited to probing the process that converts input dsRNAs to 25mer guide sequences. To test the possibility that input dsRNA is converted directly into guide RNAs that are incorporated into the nuclease, we will initiate RNA interference *in vitro* using labeled dsRNAs. We will then partially purify the nuclease by chromatography on Q sepharose. For nuclease that is generated *in vivo*, this step effectively separates activity and consequently the guide RNA from the bulk of dsRNA that is present within the extract (see Fig 5). If we find that the 25mer that is present in active fractions is labeled, this would suggest that the dsRNA is directly processed to generate the specificity determinant of the enzyme. The relevancy of this result can also be confirmed through *in vivo* experiments. By transfection with biotin-labelled dsRNA, we may probe the origins of the specificity determinant that is incorporated into the enzyme *in vivo*. Of course, such experiments must be controlled for the possibility that salvage pathways could allow incorporation of biotinylated nucleotides into a replicated guide RNA giving a false-positive result.

We will also examine the possibility that the dsRNA is replicated to produce the putative guide RNAs. This can be tested by initiating the response with unlabelled dsRNA in the presence of ^{32}P-rNTPs. If the 25mer that is incorporated into the nuclease complex becomes labeled, this would indicate production of the guide RNAs through replication. Alternatively, a positive result could suggest the addition of a tag sequence that specifies association with the nuclease. Of course, we must control for the possibility that label could be incorporated through non-specific, end-addition activities that are common in cellular extracts. For example, if 25mer's are synthesized in the extract, then the RNAi response should be sensitive to chain terminating nucleotides such as cordycepin.

It is alternatively possible that the 25mer is produced by a combination of replication and processing. For example, separate activities could amplify the dsRNA and process it into discrete guide RNAs. In this event, both of the experimental approaches described above would yield positive

results. In any case, the experiments outlined in this section could provide the basis for the development of *in vitro* assays specific for the first step of RNA interference, recognition and metabolism of the dsRNA. If we achieve this goal, we will attempt, in the long term, to use such assays for the purification and characterization of the components involved in this process.

Purification of the sequence-specific nuclease complex

One of the many mysteries surrounding dsRNA-induced gene silencing is how a dsRNA can elicit the formation of an adaptive nuclease complex that can efficiently reduce the level of virtually any mRNA in a sequence-specific fashion. This question may be answered, at least in part, by understanding the nature of the nuclease complex itself. This section describes our strategies for the purification of the nuclease complex. This effort will not only allow a biochemical characterization of the enzyme but also serve as a prelude toward molecular cloning of the constituents of the activity.

Potential components from genetic analysis

As discussed in the **Background**, genetic approaches in C. elegans and in Neurospora have identified a number of proteins that play critical roles in dsRNA-induced gene silencing. However, these studies have failed to illuminate the specific functions of any of these components. Before undertaking a potentially arduous conventional purification, it seems prudent to determine whether any of these proteins forms an integral part of the sequence-specific nuclease complex since this would provide a molecular handle that could aid the isolation of the enzyme. Presently, genetic analyses have identified two candidates that could plausibly form components of the nuclease complex. These are rde1 and mut-7/qde3. However, it is almost certain that such candidates will continue to emerge in the literature. It is assumed that approaches described below will be generalized and extended as warranted by the maturation of genetic studies from other laboratories.

The rde1 gene was isolated in a genetic selection for mutations that caused resistance to dsRNA-induced gene silencing (Tabara et al., 1999). This gene is a member of a protein family that is represented in diverse organisms ranging from mammals to plants to yeast. Unfortunately, little or no functional information is available for any family member. In Drosophila, rde1 has two homologs that have been identified to date, sting and piwi (see **Background**).

We have obtained full-length cDNAs enconding sting and piwi. Each of these will be linked to a T7 epitope tag (N- and C- terminal, individually) and expressed in S2 cells. Expression will be tested by Western blotting and by immunoprecipitation of ^{35}S-labelled proteins. Cells expressing tagged sting and piwi proteins will be transfected with dsRNA corresponding to cyclin E, and anti-T7 immunoprecipitates from these cells will be tested for the ability to degrade specifically cyclin E mRNA. We have extensive experience in the immunoprecipitation of enzymatically active complexes from cultured cells (see Brugarolas et al., 1995; Xiong et al., 1993) and anticipate no unusual difficulty with this approach. For example, we have already determined that active extracts can be prepared from S2 cells by lysis in non-ionic detergents and in buffers that we have used extensively in immunoprecipitation experiments (not shown).

The mut-7 gene is also a member of a multigene family, members of which have homology to both the Werner/reqQ family of helicases and to RNaseD, a bacterial RNA processing enzyme (Ketting et al., 1999). This has led to the proposal that mut-7 may provide catalytic activity to the (then hypothetical) sequence-specific nuclease complex. The Drosophila genomic sequence contains at least one potential homolog of mut-7/qde3. We are now in the process of obtaining a cDNA derived from this gene, and will test for association with the sequence-directed nuclease as described above.

Should either of these proteins prove to be a component of the enzyme, we will generate stable cell lines that express tagged derivatives. These will be used to prepare the nuclease for the biochemical experiments that are described below and for large-scale purification of the complex in Aim 3. Although the availability of an epitope-tagged enzyme will not completely eliminate the need for conventional purification procedures, it is likely to speed the process of isolating the enzyme. In addition, the availability of a tagged component of the complex will allow immunoprecipitation of i*n vivo* labeled nuclease complexes (both ^{35}S and ^{32}P) to identify potential protein and nucleic acid constituents of the enzyme.

Conventional purification

Work presented in **Preliminary Results** has suggested that the sequence-specific nuclease complex may be amenable to purification by conventional approaches. In addition to Q sepharose, we have tested a wide variety of resins for fractionation of the enzyme. To date, we find that substantial purification can be obtained by passage of crude extracts over Q sepharose, S sepharose, heparin agarose, gel filtration resins and hydroxylapetite. At present, we are beginning to link these columns in series to effect purification of the enzyme. In practice, each purification begins with transfection of 50-75 15cm tissue culture plates of S2 cells. These give rise to ~20-30 ml of extract. From this, a ribosomal pellet is prepared by high-speed centrifugation (see above). Activity is extracted in 300mM KOAc and ribosomal material is removed in a second spin, and the soluble nuclease is used for chromatography.

Thus far, we have been fortunate that the enzyme is quite stable in crude preparations. Lysates and partially purified nuclease can be kept on ice for days without measurable loss of activity. In addition, we have been quite successful in passing the enzyme over two sequential columns, however, as the purification proceeds beyond this point, we experience substantial loss of activity. We have attributed this to denaturation of the enzyme as the protein concentration of each fraction drops. We have attempted to combat this by inclusion of stabilizing agents, and recent data indicates that inclusion of octyl glucoside in all buffers can preserve activity. Based upon these data, we now feel ready to attempt purification of the enzyme on a large scale.

Biochemical analysis of the sequence-specific nuclease

Even without the scale-up that may be required for molecular cloning of the components of the complex, evidence suggests that sufficient material can be purified to permit biochemical characterization. The first series of questions will focus on the nature of the enzyme itself.

Because of the crude nature of the extract preparations, it has been impossible to determine as of yet whether digestion of the substrate occurs through an endonucleolytic mechanism, an exonucleolytic attack or both. This is particularly complicated since our current model suggests that the nuclease is not a single enzyme but a family of related enzymes incorporating distinct dsRNA-derived guide RNAs. We will use a number of modified substrates to discern whether endo- or exo-nucleolytic mechanisms account for mRNA destruction. If the RISC is solely an exonuclease, then substrate degradation must require a free end, and a circular RNA should be resistant. A circle can easily be prepared from a synthetic transcript containing 5' monophosphate and 3' OH ends. Circularization will occur inefficiently simply by treatment with RNA ligase. However, a more efficient ligation can be accomplished using DNA ligase by hybridization to a bridge oligonucleotide that will appose the two ends (Moore and Sharp, 1992). The circle can then be purified from unligated material based upon its gel mobility. If such circles are nuclease sensitive, then the complex must contain an endonuclease. The RISC may also contain an exonuclease activity. The simplest test of this possibility is to use substrates that extend beyond the region targeted by dsRNA. In crude

extracts, these appear to be degraded completely (see **Preliminary Results**); however, other nucleases are undoubtedly present. Thus, the sequence-specific enzyme may make an initial endonucleolytic cut and degradation may be completed by non-specific exonucleases. This should not be an issue with purified enzyme. Thus, if the RISC is solely an endonuclease, we should accumulate substrates that comprise approximately the portion of the synthetic transcript that was not represented in the transfected dsRNA. To eliminate the possibility that specificity may have migrated beyond the targeted region (for evidence counter to this see **Preliminary Results**), we will use a chimeric substrate in which 500 nt of cyclin E have been appended to sequences from lacZ.

A number of other issues will also be addressed with the purified enzyme. Although it is not possible, due to space limitation, to fully describe the experiments here, I will summarize a few key questions. Our preliminary results have shown that the RNAi nuclease prefers substrates of 200 or greater nt. in length. This is difficult to rationalize with the model that specificity is provided by a series of 25 nt. guide RNAs. It will therefore be critical to ask whether the requirement is for size alone or for the length of the homology (although see Fig 4). This can be addressed by creating a series of ~500 nucleotide chimeric RNAs in which combine portions derived from cyclin E and from lacZ. If only a small amount of cyclin E sequence is needed to trigger degradation of the chimera, we may assume that the requirement is for total length of the substrate molecule. If the length requirement is for homologous sequence, this will be much more puzzling and may indicate that the enzyme acts cooperatively, digesting the substrate only upon binding of multiple complexes. In addition, it will be of interest to determine whether the enzyme can digest structured RNAs, particularly since an RNA that is completely double-stranded is resistant. We will also determine whether ssDNA can be targeted and whether a response can be raised against cellular RNAs that are not mRNA – for example, rRNAs and snRNAs – both *in vivo* and *in vitro*. Finally, we may address the level of homology necessary for targeting by providing mutant versions of the substrate. Data from C. elegans indicate that genes that are homologous to those targeted by the dsRNA (~80% identity) can also be silenced by RNAi (reviewed in Sharp, 1999). However, it is unclear whether this reflects flexibility in the recognition process or arises from the fact that these genes have short regions of much higher identity. This question will be most easily addressed if the nuclease will attack a long substrate (~500 nt) that contains only a relatively short region of identity (<100 nt) since this would simplify chemical synthesis of the transcription template. However, if necessary, we can prepare longer synthetic transcription templates that are within the size range that is recognized by the enzyme.

The availability of a purified nuclease preparation will also allow some rudimentary kinetic analysis. Although we may not be able to precisely quantify the enzyme by determining the protein concentration of the purified fraction, we can achieve an estimate of enzyme concentration by quantifying the 25mer. This calculation requires two assumptions. The first is that we will have determined the molecular weight of the complex. This is not unreasonable considering the progress that we have already made toward purifying the activity. The second relates to the stoichiometry of the 25mer within the complex. The most reasonable estimates would be one or two molecules per nuclease, reflecting the presence of the 25mer in single stranded or double stranded form. Finally, we will not be able to determine precisely the fraction of total nuclease, as represented by the quantity of the 25 mer, that is catalytically active. Nevertheless, by looking at reaction kinetics, we may be able to discern a rough turnover number and gain insight into whether the enzyme acts cooperatively.

Summary

The experiments described in this section of the proposal are designed to provide information about the formation and properties of the dsRNA-induced, sequence-specific nuclease activity. We aim to determine whether the putative guide RNA is produced by processing of the dsRNA, by replication or by a combination of these. In addition, we will purify the nuclease complex both for the purpose of biochemical characterization and as a prelude to the molecular cloning of the components of the complex as described in the final section of this application.

Aim 3 Components of the RISC complex

In this section of the proposal, I outline our strategy for cloning the protein and RNA components of the complex. It should be noted, that the Principal Investigator has prior experience in purification and cloning of novel proteins (see for example, Xiong et al., 1993).

Source of material for large-scale purification

One of the major barriers to obtaining protein sequence from conventionally purified material is the ability to isolate sufficient mass for sequencing. This has changed somewhat with the introduction of tandem mass spectroscopy; however, in practice, 1 pmol of protein is required to maximize the probability of success. For purification trials, we have routinely prepared extracts from 50-75 plates of transfected cells. By estimating the amount of 25mer in fractionated enzyme from a single Q sepharose column, we feel that the material from this preparation would be sufficient, assuming no loss. Not only is this assumption unrealistic in general but we also have experienced losses as we purify through multiple chromatographic steps. Therefore, we estimate that approximately a 10 fold increase in starting material will be necessary to achieve yields sufficient for sequencing of purified nuclease subunits. This can be accomplished in two ways. First material can be stockpiled. The ongoing work that is related to this application is supported by a single technician who routinely transfects 120 plates of cells per week for extract preparation. Second, preparation of starting material would be aided greatly by the production of a stable cell line in which RNAi was provoked by constitutive dsRNA expression (see Aim 1).

Protein purification

Protein purification will follow the procedures established through work described in the prior **Specific Aim**. We recognize that an increase in starting material may necessitate some changes in the protocol, specifically the inclusion additional purification steps. However, we have identified a number of resins over which the nuclease can be effectively fractionated and feel confident that we will be able to accommodate problems that arise as we increase the scale of our protocols.

Identifying the protein components of the enzyme

Once the nuclease complex is purified in sufficient quantities, we will undertake a molecular cloning of the genes encoding the subunits of the enzyme. At CSHL, we have a well-established facility for protein sequencing that is headed by Ryuji Kobayashi, an Associate Professor with many years of experience in both protein sequencing and in the development of technology for protein analysis. Previously, I have collaborated with Dr. Kobayashi on the purification, sequencing and cloning of the CDK inhibitor, p21 (Xiong et al., 1993). Thus, I am experienced in both the principles and practice of the protein sequencing process. Since I last made use of the facility in 1993, capabilities have increased tremendously. The initial steps of the process remain the same. The activity is purified sufficiently so that candidate components can be identified on protein gels. Desired fractions are pooled, concentrated and electrophoresed in a single lane. The gel is then stained and delivered to the sequencing facility. Dr. Kobayashi excises the candidate bands and subjects them to

an in-gel digestion with agrobacter protease I, a protease that fragments the protein by cutting after lysine. The resulting peptides, separated by HPLC chromatography, can then be treated in one of two ways. The first is automated Edman degradation, the conventional sequencing process that I have used in the past. The second is analysis by LCQ deca electrospray mass spectroscopy. By optimizing the methodology, Dr. Kobayashi can routinely obtain protein sequence through standard methods starting with 2-3 pmol of protein. With mass spectroscopy less than 1 pmol is required, with recent data suggesting a sensitivity limit of ~100 fmol.

Once the sequence of peptide fragments is obtained, it is highly likely that we can make use of the Drosophila genome sequence to identify candidate proteins. At present, the majority of the genome is available, and release of the entire annotated sequence is anticipated within the next few months. Of course, an available EST would be most helpful. Otherwise, we must initially infer a coding sequence based upon predictions from the genome. Although predicting the protein sequence accurately may be problematic, we will enlist the aid of Dr. Michael Zhang, a faculty member in the CSHL bioinformatics group who specializes in predicting introns and transcription start sites in genomic sequence (Zhu and Zhang, 1999; Tabaska and Zhang, 1999; Zhang, 1997; Zhang and Marr, 1993, see attached letter). At a minimum, the genomic sequence should provide a start point for the cloning full-length cDNAs corresponding to each component of the nuclease complex. We have recently prepared a high quality cDNA library from S2 cells in the λ-ZapII vector. This will be used with a combination of PCR and conventional hybridization to isolate full-length cDNAs. It should be noted that the P.I. has extensive experience in library construction and screening.

The identification of the protein components of the complex will pave the way for numerous experiments. However, space limitations and a lack of precise information concerning the nature of these subunits preclude a complete description. Nevertheless, a brief outline can be given. In each case, we will prepare polyclonal antisera both against bacterially expressed proteins and against synthetic peptides. Again, the P.I. has extensive experience with these methodologies. Specific antisera will be used first to confirm association between each candidate and the nuclease activity. Co-immunoprecipitation experiments will provide useful information concerning the extent to which subunits are associated in naïve S2 cells that have not been exposed to dsRNA. If complexes do exist in non-transfected cells, this may indicate that RISC complexes are involved in the regulation of endogenous genes or transposons. The availability of an immunological reagent will allow isolation of these complexes, and the strategies outlined below can be used to identify guide RNAs that, in turn, identify the regulated genes. Antibodies can also be used for immunodepletion experiments to determine whether a given protein is essential for activity *in vitro*. Finally, the availability of molecular clones is essential for reconstitution experiments that will allow us to probe the role of each subunit within the complex.

The RNA components of the sequence-specific nuclease

Our results indicate that the sequence-specific nuclease contains at least one essential RNA component. The identification of the putative guide RNA provides one candidate; however, the enzyme may also contain additional RNA subunits. To investigate this possibility, we will assess the RNA composition of the purified enzyme fractions. T4 RNA ligase can transfer a radioactive label from pCp to RNAs that contain a 3' OH group. RNAs that contain 3' phosphate groups can be labeled by treating with phosphatase prior to pCp ligation. RNAs with unusual 3' ends can potentially be labeled at the 5' end by phosphatase treatment followed by transfer of phosphate from radioactively labeled ATP. In some cases, decapping may be required to allow 5' end labeling (Tharun and Parker, 1999; LaGrandeur and Parker, 1998). A combination of these procedures can be used to identify possible nucleic acid subunits of the enzyme.

We envision two possible outcomes. First, we may detect only the 25 nt. RNA in association with activity. Second, the enzyme may contain one or more additional RNA subunits. In either case, the labeling experiments described above will guide the design of cloning strategies. In general, potential RNA subunits will be gel isolated from purified fractions based upon the mobility of labeled RNAs electrophoresed in parallel. Unlabelled RNAs will be treated, if necessary, to expose a free 3' OH group. Using commercially available yeast polyA polymerase, a homopolymer tail will be added to each RNA species (Martin and Keller, 1998). This will serve as a primer for 5' RACE to yield a dsDNA that can be cloned and sequenced through conventional methods. So that the ends of the RNAs can be determined with less ambiguity, different homopolymers (at both the 5' and 3' ends) will be used for cloning in independent reactions.

With regard to the putative guide RNA, we anticipate gaining several pieces of information. First, we will learn its precise size and sequence composition. Our data indicate that the 25 mer that is observed on Northern blots represents a family of RNAs representing different parts of the input dsRNA. It will be of interest to determine whether the distribution is random or whether certain sections of the dsRNA are incorporated into the enzyme preferentially. This analysis will also reveal whether the putative guide RNA is perfectly homologous to the substrate or whether it contains either altered or additional sequence. For example, one way in which association of the guide RNA with the enzyme could be specified would be through addition of either a primary sequence tag or a specific modification. The former should emerge from sequence analysis while the latter may await purification of uniformly labeled guide RNAs from S2 cells (this would be greatly aided by antibody reagents that are specific to the complex – see previous section). Sequencing should also reveal immediately whether the dsRNA adenine deaminases have played a role in the generation of sequence specific nuclease. This would be indicated by the presence of characteristic mutations in the guide sequences (Maas et al., 1997; Bass, 1997; O'Connell, 1997). The information gained by sequence analysis may instruct either reconstitution experiments or attempts to reprogram the purified nuclease by providing exogenous guide RNAs i*n vitro*. Only in this way can we definitively establish that the substrate of the nuclease is determined by the sequence of these putative specificity subunits.

Any other discrete RNAs that are present in the nuclease preparation will be cloned via a similar approach. If another RNA (e.g. an scRNA) is detected, oligonucleotide-directed digestion with RNAse H may reveal the dependence of activity on this RNA. This possibility will be tested in purified fractions, in extracts of transfected cells and in naïve extracts.

Are nuclease subunits required for dsRNA-induced silencing in vivo?

One broad goal of our work is to understand not only the nature of the sequence specific nuclease but also the biological function of dsRNA-induced gene silencing. It will therefore be essential for each of the components of the sequence specific nuclease to probe the consequences of loss-of-function in intact animals. For this purpose, we have chosen two model systems, C elegans and Drosophila. Minimally, these studies will reveal whether any individual component is essential for dsRNA-induced gene silencing. Furthermore, loss-of-function mutations may begin to reveal biological functions of RNA interference and perhaps uncover additional modes by which dsRNA regulates gene expression. The latter might be indicated by a loss of nuclease activity without a complete loss of silencing. Such studies are clearly long-term, and both a lack of information regarding the nature of RISC subunits and space limitations preclude a complete description here. However, an overview of our plans may help to reveal the eventual direction of our studies.

Loss-of-function mutations in Drosophila

Methods for identifying loss-of-function mutations in Drosophila are well established. However, we have no experience with these approaches. For this reason, we have enlisted the cooperation of a colleague at CSHL, Tim Tully, who is an established Drosophila geneticist (see attached letter). His help will be essential not only for technical expertise but for providing access to the facilities and materials necessary for this aspect of the project.

The gene corresponding to each component of the nuclease will be mapped cytologically on the Drosophila genome. This can be done classically by *in situ* hybridization; however, by the time that this becomes relevant, the Drosophila genome sequence, complete with integrated cytology, is likely to be available. Based upon this information, we will search for known mutations, P-element insertions and/or chromosomal abnormalities that may affect the gene of interest. If previously generated mutations fail to map to the locus, our job becomes more difficult. In this case, a number of options are available. If, for example, a P-element exists nearby, we could attempt to generate a local hop into our gene of interest, using PCR to monitor the event.

Once mutations are identified, homozygous fly strains (provided that mutations are not lethal) will be prepared and tested for the ability to silence genes in response to dsRNA. These studies will be carried out by injection of the dsRNA into drosophila embryos (Kennerdell and Carthew, 1998). We will test for RNA interference using nautilus as the target, since the phenotype arising from silencing of this gene has been well established (Misquitta and Paterson, 1999) and using GFP since this provides a simple visual assay. We will also search for additional phenotypes arising from the disruption. For example, transposon mobilization has been observed in C. elegans upon disruption of genes that are required for silencing.

If the approaches described above fail, classical mutagenesis could be carried out. This could be based on any of a variety of readouts for loss of sensitivity to dsRNA-induced gene silencing in embryos. For example, using GFP-expressing embryos, we could screen for persistence of fluorescence following injection of GFP dsRNA. An aspect of this approach that is both an advantage and a disadvantage is that it is non-targeted. Thus, mutations may be selected not only in the particular gene of interest but also in other loci that are critical for RNAi.

We recognize that this could be a long and complex process, and in our inexperience, we will rely heavily on the goodwill of our collaborators throughout. However, we do feel that *in vivo* analysis of specific mutations in components of the nuclease is a necessary step in the long run toward understanding the biological relevance of dsRNA-induced gene silencing.

Loss-of-function mutations in C. elegans

Previous studies have demonstrated that RNA interference in C. elegans is accessible by genetic approaches (Tabara et al., 1999; Ketting et al., 1999). It is our presumption that the components of this process are likely to be conserved between worms and flies. Therefore, we will also take advantage of a genetic resource that has been created at CSHL that allows the rapid identification of worms carrying deletions within genes of interest.

Plasterk and colleagues have recently developed a strategy for rapidly identifying desired deletions within the C. elegans genome (Jansen et al., 1997). This relies on the use of chemical mutagens (EMS and TMP/UV) that are known to induce deletions. Mutagenized worms are stored in arrays of pools with corresponding frozen animals and DNA lysates for the identification of deletions. Mutations that include a given gene are identified by PCR. Primers are chosen such that amplification of the genome would give a fairly large fragment (> 3kB). Induced deletions within this region would produce a shorter PCR product that would indicate the presence of a desired mutation within a pool. Sensitivity is increased by choosing amplification conditions that favor short products.

Individual worms are chosen by continuous subdivision of positive pools and further rounds of PCR. In practice, all assays are done in duplicate to eliminate false positives. Michael Hengartner has used the Plasterk strategy to create a C. elegans deletion bank at CSHL. This resource contains approximately 400,000 mutant genomes in 20,000 pools.

Potential C. elegans homologs of nuclease components may already be represented by existing mutations – even in those that are expected to emerge continuously from genetic studies of RNA interference. However, if necessary, we will search the deletion bank for alterations that affect our target genes. Once identified, homozygous animals will be produced and, if viable, tested for the ability to carry out dsRNA-induced gene silencing. As an assay, we will target the unc-22 gene since interference with this locus produces an easily identifiable phenotype (Montgomery et al., 1998). Initially, silencing will be induced by feeding dsRNA-expressing E. coli, but all phenotypes will also be confirmed by dsRNA injection. In addition to effects on gene silencing, we will also assess effects on the regulation of endogenous transposons since genetic studies have linked the mutator phenotype to defects in dsRNA-induced gene silencing in a number of cases (Ketting et al., 1999).

Again, we are admittedly not experienced with the C. elegans system; however, Michael Hengartner has indicated a willingness to help with the studies proposed above (see attached letter). In fact, the C. elegans knockout facility is a component of the CSHL cancer center (of which I am a member) and a staff of two C. elegans geneticists is dedicated to aiding novices with the types of studies that are proposed herein.

Without any knowledge of what genes will be identified as components of the nuclease activity, it is difficult to anticipate the types of information that may be gained by loss-of-function studies in C. elegans. It is our hope that by combining biochemical, *in vitro* studies, with analysis of intact animals that we may contribute to an understanding of the biological role of RNA interference.

Summary

In this application, we propose a biochemical approach to deciphering the mechanisms that underlie dsRNA-induced gene silencing. RNA-interference allows an adaptive defense against both exogenous and endogenous dsRNAs, providing something akin to a dsRNA immune response. The primary goal of the work proposed in this application is to understand the mechanisms by which a cell can raise this response. We have presented evidence that RNA interference is accomplished, at least in part, through the action of a sequence-specific nuclease that is generated in response to dsRNA. Our data, and that of others (Hamilton and Baulcombe, 1999), is consistent with a model in which dsRNAs present in a cell are converted, in a manner analogous to antigen processing, into discrete, small RNAs that guide the nuclease in the choice of substrate. We propose to purify and characterize the nuclease and to clone the protein and RNA components of the enzyme. In addition, we propose to develop approaches that may allow the use of cultured Drosophila cells as a general tool for probing gene function. The combination of these studies may lead eventually to an ability to harness RNA interference as a genetic tool in other organisms, particularly mammals, in which analogous tools are presently lacking.

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EDUCATION

1976	Baccalaureate: Collège de Genève, Geneva, Switzerland
1980	Diploma in Biology: University of Geneva, Geneva, Switzerland
1983	Ph.D. in Molecular Biology: University of Heidelberg, Heidelberg, Germany
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PROFESSIONAL EXPERIENCE

Research

1980:	Diploma work with Dr. K. Illmensee, University of Geneva. "Caractérisation biochimique d'un tératome de la souris et comparaison avec plusieurs tissus normaux".
1980-1983	Ph.D. research in the laboratory of Dr. Walter Keller German Cancer Research Center and University of Heidelberg "mRNA splicing *in vitro* with HeLa cell extracts".
1983-1986	Post-doctoral research in the laboratory of Dr. Alan M. Weiner, Yale University, New Haven, Connecticut "Study of the 3' end formation of U1 RNA".
January 1987- June 1993	Senior Staff Investigator Cold Spring Harbor Laboratory Cold Spring Harbor, NY 11724
July 1993-August 2005	Professor Cold Spring Harbor Laboratory Cold Spring Harbor, NY 11724
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Teaching appointments

1988-2005	Member of the Genetics Program Stony Brook University, Stony Brook, NY 11794
August 1991-2005	Member of the Pharmacology Program Stony Brook University, Stony Brook, NY 11794
July 1993-2005	Member of the Molecular and Cellular Biology Program, Stony Brook University, Stony Brook, NY 11794
May 1994-April 95	Adjunct Assistant Professor, Dept. of Pharmacology, Stony Brook University, Stony Brook, NY 11794
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TEACHING EXPERIENCE

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Michael Sheldon	1993
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Tracy Kuhlman	1999
Setareh Sepehri	2000
Laura Schramm	2/2001
Ping Hu	1/2003
Farida Emra	8/2004
Ashish Saxena	2004
Chih-Chi Yuan	11/2006

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Susan M. Lobo-Ruppert	1987-1993	Asst. Prof., U. Alabama
Ratneswaran Ratnasabapathy	1989-1991	Asst. Prof., Long Island U.
Renu Mital	1992-1996	Post. Doc., Austria
Frank Pessler	1994-1996	M. D. Resident, New York Univ.
Mee-Wa Wong	1995-1997	Staff, Cellomics, Pittsburgh
William R. Henry	1992-1998	Asst. Prof., U. of Michigan.
Vivek Mittal	1994-1999	Asst. Prof., CSH Laboratory
P. Shannon Pendergrast	1992-2001	Staff Scientist, Archemix, Boston
Xinyang Zhao	1998-2002	Research Associate, Sloan Kettering Cancer Center
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MEETING ORGANIZATION AND SCIENTIFIC REVIEW

Meeting organization

Co-organizer:
1997: Cold Spring Harbor Conference on Mechanisms of Eucaryotic Transcription, CSH
1999: Cold Spring Harbor Conference on Mechanisms of Eucaryotic Transcription, CSH
2001: Cold Spring Harbor Conference on Mechanisms of Eucaryotic Transcription, CSH
2007: SKMB Gene Regulation Workshop, Lausanne
2008: SKMB Gene Regulation Workshop, Lausanne
2008: 2nd CIG Symposium: Metabolism and Cancer, Lausanne
2008: 8th EMBL Transcription Meeting, Heidelberg
2008: CIG Symposium: Metabolism and Cancer, Lausanne
2009: CIG Symposium: DNA repair and Human Health, Lausanne
2009: SKMB Gene Regulation Workshop, Lausanne
2010: CIG Symposium: Sensing the Environment, Lausanne

Editorial Board

01/1992- 10/1993	Editorial Board, Molecular and Cellular Biology
11/1993-06/1998	Editor, Molecular and Cellular Biology

Scientific review

February 1991	Ad Hoc reviewer for the Molecular Biology Study Section
June 1991	Ad Hoc reviewer for the Molecular Cytology Study Section
July 1997-July 2001	Member, Molecular Biology Study Section, NIH
May 2000	Panelist, Baltics, Central and Eastern Europe, and the Former Soviet Union Initiative of the International Research Scholar Program
September 2001-Aug. 2004	Board of Scientific Counselors for the Nat. Cancer Inst., NIH
April 2008	Chair, review of program "Systemic Analysis of Multifactorial Diseases", Helmholtz Association

FELLOWSHIPS, SCHOLARSHIPS, MEMBERSHIPS and HONORS

11/1983-11/1984	Swiss National Science Foundation post-doctoral fellowship
01/1985-12/1986	Damon Runyon-Walter Winchell Cancer Fund fellowship
1989-1994	Scholarship from the Rita Allen Foundation
2007	EMBO member
2007-present	Executive board, Swiss Academy of Sciences
2007	Prof. Dr. Max Cloëtta Foundation Prize

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2. Hernandez, N. and Keller, W. (1983). Splicing of in vitro synthesized messenger RNA precursors in HeLa cell extracts. Cell 35, 89-99.

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2. Hernandez, N. (1993). TBP, a universal eucaryotic transcription factor? Genes & Dev. 7, 1291-1308.

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4. Henry, R.W., Ford, E., Mital, R., Mittal, V., and Hernandez, N. (1998). Crossing the line between RNA polymerases: transcription of human snRNA genes by RNA polymerases II and III. In "Cold Spring Harbor Symposia on Quantitative Biology", Cold Spring Harbor Press, Volume LXIII, pp. 111-120.

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An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells

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In a diverse group of organisms that includes *Caenorhabditis elegans*, *Drosophila*, planaria, hydra, trypanosomes, fungi and plants, the introduction of double-stranded RNAs inhibits gene expression in a sequence-specific manner[1–7]. These responses, called RNA interference or post-transcriptional gene silencing, may provide anti-viral defence, modulate transposition or regulate gene expression[1,6,8–10]. We have taken a biochemical approach towards elucidating the mechanisms underlying this genetic phenomenon. Here we show that 'loss-of-function' phenotypes can be created in cultured *Drosophila* cells by transfection with specific double-stranded RNAs. This coincides with a marked reduction in the level of cognate cellular messenger RNAs. Extracts of transfected cells contain a nuclease activity that specifically degrades exogenous transcripts homologous to transfected double-stranded RNA. This enzyme contains an essential RNA component. After partial purification, the sequence-specific nuclease co-fractionates with a discrete, ~25-nucleotide RNA species which may confer specificity to the enzyme through homology to the substrate mRNAs.

Although double-stranded RNAs (dsRNAs) can provoke gene silencing in numerous biological contexts including *Drosophila*[11,12], the mechanisms underlying this phenomenon have remained mostly unknown. We therefore wanted to establish a biochemically tractable model in which such mechanisms could be investigated.

Transient transfection of cultured, *Drosophila* S2 cells with a *lacZ* expression vector resulted in β-galactosidase activity that was easily detectable by an *in situ* assay (Fig. 1a). This activity was greatly reduced by co-transfection with a dsRNA corresponding to the first 300 nucleotides of the *lacZ* sequence, whereas co-transfection with a control dsRNA (*CD8*) (Fig. 1a) or with single-stranded RNAs of either sense or antisense orientation (data not shown) had little or no effect. This indicated that dsRNAs could interfere, in a sequence-specific fashion, with gene expression in cultured cells.

To determine whether RNA interference (RNAi) could be used to target endogenous genes, we transfected S2 cells with a dsRNA corresponding to the first 540 nucleotides of *Drosophila cyclin E*, a gene that is essential for progression into S phase of the cell cycle. During log-phase growth, untreated S2 cells reside primarily in G2/M (Fig. 1b). Transfection with *lacZ* dsRNA had no effect on cell-cycle distribution, but transfection with the *cyclin E* dsRNA caused a G1-phase cell-cycle arrest (Fig. 1b). The ability of *cyclin E* dsRNA to provoke this response was length-dependent. Double-stranded RNAs of 540 and 400 nucleotides were quite effective, whereas

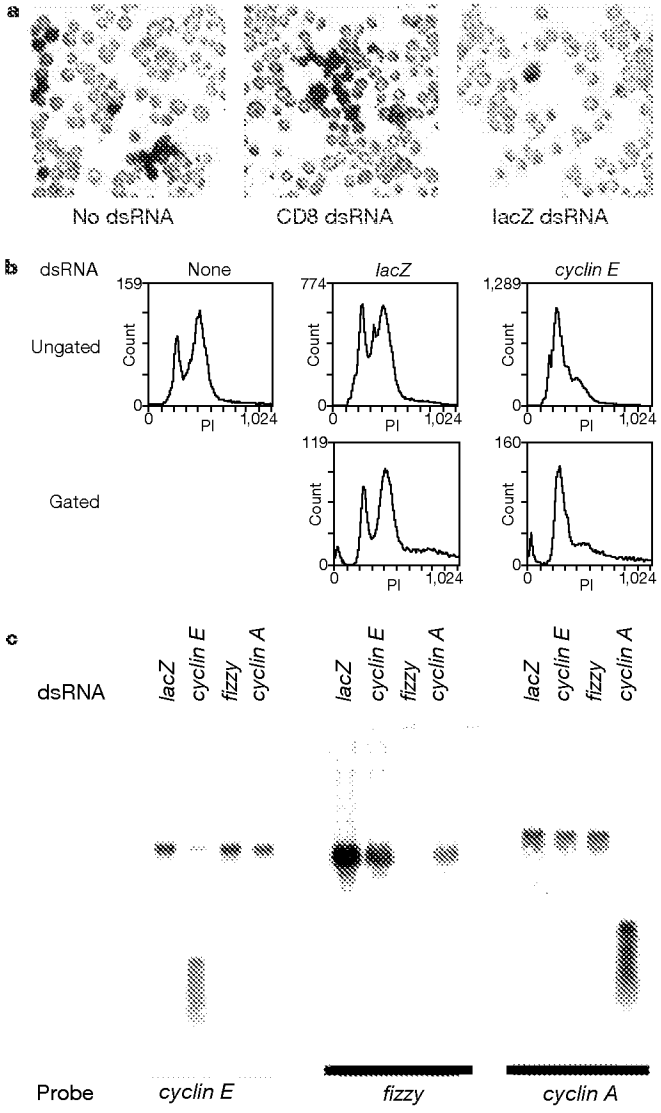

Figure 1 RNAi in S2 cells. **a**, *Drosophila* S2 cells were transfected with a plasmid that directs *lacZ* expression from the copia promoter in combination with dsRNAs corresponding to either human CD8 or lacZ, or with no dsRNA, as indicated. **b**, S2 cells were co-transfected with a plasmid that directs expression of a GFP–US9 fusion protein (12) and dsRNAs of either *lacZ* or *cyclin E*, as indicated. Upper panels show FACS profiles of the bulk population. Lower panels show FACS profiles from GFP-positive cells. **c**, Total RNA was extracted from cells transfected with *lacZ*, *cyclin E*, *fizzy* or *cyclin A* dsRNAs, as indicated. Northern blots were hybridized with sequences not present in the transfected dsRNAs.

dsRNAs of 200 and 300 nucleotides were less potent. Double-stranded *cyclin E* RNAs of 50 or 100 nucleotides were inert in our assay, and transfection with a single-stranded, antisense *cyclin E* RNA had virtually no effect (see Supplementary Information).

One hallmark of RNAi is a reduction in the level of mRNAs that are homologous to the dsRNA. Cells transfected with the *cyclin E* dsRNA (bulk population) showed diminished endogenous *cyclin E* mRNA as compared with control cells (Fig. 1c). Similarly, transfection of cells with dsRNAs homologous to *fizzy*, a component of the anaphase-promoting complex (APC) or *cyclin A*, a cyclin that acts in S, G2 and M, also caused reduction of their cognate mRNAs (Fig. 1c). The modest reduction in *fizzy* mRNA levels in cells transfected with *cyclin A* dsRNA probably resulted from arrest at a point in the division cycle at which *fizzy* transcription is low[14,15]. These results indicate that RNAi may be a generally applicable method for probing gene function in cultured *Drosophila* cells.

The decrease in mRNA levels observed upon transfection of specific dsRNAs into *Drosophila* cells could be explained by effects at transcriptional or post-transcriptional levels. Data from other systems have indicated that some elements of the dsRNA response may affect mRNA directly (reviewed in refs 1 and 6). We therefore sought to develop a cell-free assay that reflected, at least in part, RNAi.

S2 cells were transfected with dsRNAs corresponding to either *cyclin E* or *lacZ*. Cellular extracts were incubated with synthetic mRNAs of *lacZ* or *cyclin E*. Extracts prepared from cells transfected with the 540-nucleotide *cyclin E* dsRNA efficiently degraded the *cyclin E* transcript; however, the *lacZ* transcript was stable in these lysates (Fig. 2a). Conversely, lysates from cells transfected with the *lacZ* dsRNA degraded the *lacZ* transcript but left the *cyclin E* mRNA intact. These results indicate that RNAi ablates target mRNAs through the generation of a sequence-specific nuclease activity. We have termed this enzyme RISC (RNA-induced silencing complex).

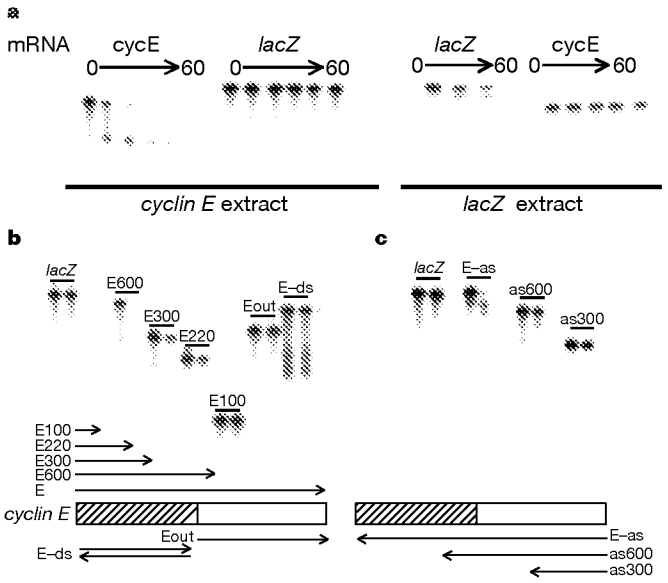

Figure 2 RNAi *in vitro*. **a**, Transcripts corresponding to either the first 600 nucleotides of *Drosophila cyclin E* (E600) or the first 800 nucleotides of *lacZ* (Z800) were incubated in lysates derived from cells that had been transfected with either *lacZ* or *cyclin E* (cycE) dsRNAs, as indicated. Time points were 0, 10, 20, 30, 40 and 60 min for *cyclin E* and 0, 10, 20, 30 and 60 min for *lacZ*. **b**, Transcripts were incubated in an extract of S2 cells that had been transfected with *cyclin E* dsRNA (cross-hatched box, below). Transcripts corresponded to the first 800 nucleotides of *lacZ* or the first 600, 300, 220 or 100 nucleotides of *cyclin E*, as indicated. Eout is a transcript derived from the portion of the *cyclin E* cDNA not contained within the transfected dsRNA. E-ds is identical to the dsRNA that had been transfected into S2 cells. Time points were 0 and 30 min. **c**, Synthetic transcripts complementary to the complete *cyclin E* cDNA (Eas) or the final 600 nucleotides (Eas600) or 300 nucleotides (Eas300) were incubated in extract for 0 or 30 min.

Although we occasionally observed possible intermediates in the degradation process (see Fig. 2), the absence of stable cleavage end-products indicates an exonuclease (perhaps coupled to an endonuclease). However, it is possible that the RNAi nuclease makes an initial endonucleolytic cut and that non-specific exonucleases in the extract complete the degradation process[16]. In addition, our ability to create an extract that targets *lacZ in vitro* indicates that the presence of an endogenous gene is not required for the RNAi response.

To examine the substrate requirements for the dsRNA-induced, sequence-specific nuclease activity, we incubated a variety of *cyclin-E*-derived transcripts with an extract derived from cells that had been transfected with the 540-nucleotide *cyclin E* dsRNA (Fig. 2b, c). Just as a length requirement was observed for the transfected dsRNA, the RNAi nuclease activity showed a dependence on the size of the RNA substrate. Both a 600-nucleotide transcript that extends slightly beyond the targeted region (Fig. 2b) and an ~1-kilobase (kb) transcript that contains the entire coding sequence (data not shown) were completely destroyed by the extract. Surprisingly, shorter substrates were not degraded as efficiently. Reduced activity was observed against either a 300- or a 220-nucleotide transcript, and a 100-nucleotide transcript was resistant to nuclease in our assay. This was not due solely to position effects because ~100-nucleotide transcripts derived from other portions of the transfected dsRNA behaved similarly (data not shown). As expected, the nuclease activity (or activities) present in the extract could also recognize the antisense strand of the *cyclin E* mRNA. Again, substrates that contained a substantial portion of the targeted region were degraded efficiently whereas those that contained a shorter stretch of homologous sequence (~130 nucleotides) were recognized inefficiently (Fig. 2c, as600). For both the sense and antisense strands, transcripts that had no homology with the transfected dsRNA (Fig. 2b, Eout; Fig. 2c, as300) were not degraded. Although we cannot exclude the possibility that nuclease specificity could have migrated beyond the targeted region, the resistance of transcripts that do not contain homology to the dsRNA is consistent with data from *C. elegans*. Double-stranded RNAs homologous to an upstream cistron have little or no effect on a linked downstream cistron, despite the fact that unprocessed, polycistronic mRNAs can be readily detected[17,18]. Furthermore, the nuclease was inactive against a dsRNA identical to that used to provoke the RNAi response *in vivo* (Fig. 2b). In the *in vitro* system, neither a 5′ cap nor a poly(A) tail was required, as such transcripts were degraded as efficiently as uncapped and non-polyadenylated RNAs.

Gene silencing provoked by dsRNA is sequence specific. A plausible mechanism for determining specificity would be incorporation of nucleic-acid guide sequences into the complexes that accomplish silencing[19]. In accord with this idea, pre-treatment of

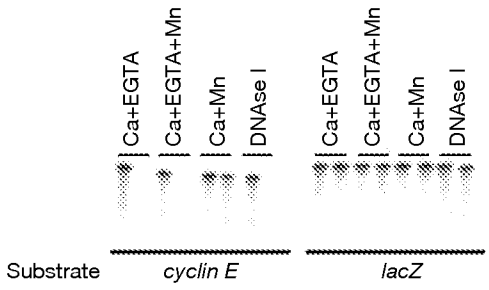

Figure 3 Substrate requirements of the RISC. Extracts were prepared from cells transfected with *cyclin E* dsRNA. Aliquots were incubated for 30 min at 30 °C before the addition of either the *cyclin E* (E600) or *lacZ* (Z800) substrate. Individual 20-μl aliquots, as indicated, were pre-incubated with 1 mM $CaCl_2$ and 5 mM EGTA, 1 mM $CaCl_2$, 5 mM EGTA and 60 U of micrococcal nuclease, 1 mM $CaCl_2$ and 60 U of micrococcal nuclease or 10 U of DNase I (Promega) and 5 mM EGTA. After the 30-min pre-incubation, EGTA was added to those samples that lacked it. Yeast tRNA (1 μg) was added to all samples. Time points were at 0 and 30 min.

extracts with a Ca^{2+}-dependent nuclease (micrococcal nuclease) abolished the ability of these extracts to degrade cognate mRNAs (Fig. 3). Activity could not be rescued by addition of non-specific RNAs such as yeast transfer RNA. Although micrococcal nuclease can degrade both DNA and RNA, treatment of the extract with DNAse I had no effect (Fig. 3). Sequence-specific nuclease activity, however, did require protein (data not shown). Together, our results support the possibility that the RNAi nuclease is a ribonucleoprotein, requiring both RNA and protein components. Biochemical fractionation (see below) is consistent with these components being associated in extract rather than being assembled on the target mRNA after its addition.

In plants, the phenomenon of co-suppression has been associated with the existence of small (~25-nucleotide) RNAs that correspond to the gene that is being silenced[19]. To address the possibility that a similar RNA might exist in *Drosophila* and guide the sequence-specific nuclease in the choice of substrate, we partially purified our activity through several fractionation steps. Crude extracts contained both sequence-specific nuclease activity and abundant, heterogeneous RNAs homologous to the transfected dsRNA (Figs 2 and 4a). The RNAi nuclease fractionated with ribosomes in a high-speed centrifugation step. Activity could be extracted by treatment with high salt, and ribosomes could be removed by an additional centrifugation step. Chromatography of soluble nuclease over an anion-exchange column resulted in a discrete peak of activity (Fig. 4b, *cyclin E*). This retained specificity as it was inactive against a heterologous mRNA (Fig. 4b, *lacZ*). Active fractions also contained an RNA species of 25 nucleotides that is homologous to the *cyclin E* target (Fig. 4b, northern). The band observed on northern blots may represent a family of discrete RNAs because it could be detected with probes specific for both the sense and antisense *cyclin E* sequences and with probes derived from distinct segments of the dsRNA (data not shown). At present, we cannot determine whether the 25-nucleotide RNA is present in the nuclease complex in a double-stranded or single-stranded form.

RNA interference allows an adaptive defence against both exogenous and endogenous dsRNAs, providing something akin to a dsRNA immune response. Our data, and that of others[19], is consistent with a model in which dsRNAs present in a cell are converted, either through processing or replication, into small specificity determinants of discrete size in a manner analogous to antigen processing. Our results suggest that the post-transcriptional component of dsRNA-dependent gene silencing is accomplished by a sequence-specific nuclease that incorporates these small RNAs as guides that target specific messages based upon sequence recognition. The identical size of putative specificity determinants in plants[19] and animals predicts a conservation of both the mechanisms and the components of dsRNA-induced, post-transcriptional gene silencing in diverse organisms. In plants, dsRNAs provoke not only post-transcriptional gene silencing but also chromatin remodelling and transcriptional repression[20,21]. It is now critical to determine whether conservation of gene-silencing mechanisms also exists at the transcriptional level and whether chromatin remodelling can be directed in a sequence-specific fashion by these same dsRNA-derived guide sequences. □

Note added in proof: Recently, Tuschl *et al.* have reported the development of cell-free extracts from *Drosophila* embryos that can carry out RNAi (T. Tuschl, P. D. Zamore, D. P. Bartel and P. A. Sharp, *Genes Dev.* **13,** 3191–3197; 1999). Their results also indicate that the RNAi is accomplished at least in part by nuclease degradation of targeted mRNAs.

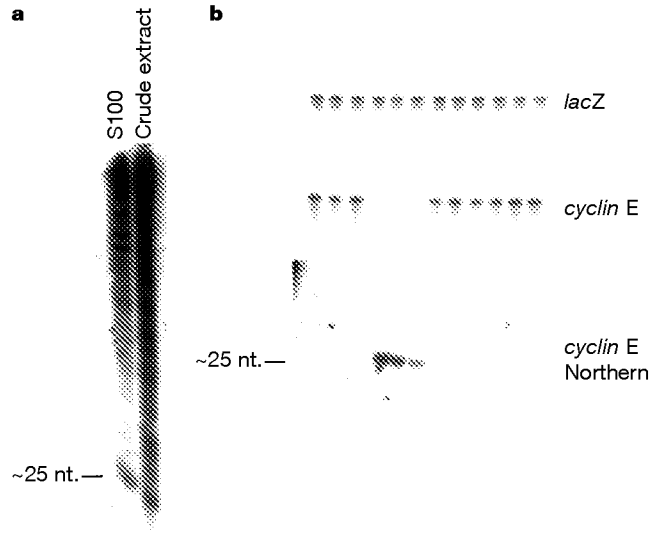

Figure 4 The RISC contains a potential guide RNA. **a**, Northern blots of RNA from either a crude lysate or the S100 fraction (containing the soluble nuclease activity, see Methods) were hybridized to a riboprobe derived from the sense strand of the *cyclin E* mRNA. **b**, Soluble *cyclin-E*-specific nuclease activity was fractionated as described in Methods. Fractions from the anion-exchange resin were incubated with the lacZ, control substrate (upper panel) or the cyclin E substrate (centre panel). Lower panel, RNA from each fraction was analysed by northern blotting with a uniformly labelled transcript derived from sense strand of the *cyclin E* cDNA. DNA oligonucleotides were used as size markers.

Methods

Cell culture and RNA methods

S2 (ref. 22) cells were cultured at 27 °C in 90% Schneider's insect media (Sigma), 10% heat inactivated fetal bovine serum (FBS). Cells were transfected with dsRNA and plasmid DNA by calcium phosphate co-precipitation[23]. Identical results were observed when cells were transfected using lipid reagents (for example, Superfect, Qiagen). For FACS analysis, cells were additionally transfected with a vector that directs expression of a green fluorescent protein (GFP)–US9 fusion protein[13]. These cells were fixed in 90% ice-cold ethanol and stained with propidium iodide at 25 μg ml^{-1}. FACS was performed on an Elite flow cytometer (Coulter). For northern blotting, equal loading was ensured by over-probing blots with a control complementary DNA (RP49). For the production of dsRNA, transcription templates were generated by polymerase chain reaction such that they contained T7 promoter sequences on each end of the template. RNA was prepared using the RiboMax kit (Promega). Confirmation that RNAs were double stranded came from their complete sensitivity to RNAse III (a gift from A. Nicholson). Target mRNA transcripts were synthesized using the Riboprobe kit (Promega) and were gel purified before use.

Extract preparation

Log-phase S2 cells were plated on 15-cm tissue culture dishes and transfected with 30 μg dsRNA and 30 μg carrier plasmid DNA. Seventy-two hours after transfection, cells were harvested in PBS containing 5 mM EGTA washed twice in PBS and once in hypotonic buffer (10 mM HEPES pH 7.3, 6 mM β-mercaptoethanol). Cells were suspended in 0.7 packed-cell volumes of hypotonic buffer containing *Complete* protease inhibitors (Boehringer) and 0.5 units ml^{-1} of RNasin (Promega). Cells were disrupted in a dounce homogenizer with a type B pestle, and lysates were centrifuged at 30,000*g* for 20 min. Supernatants were used in an *in vitro* assay containing 20 mM HEPES pH 7.3, 110 mM KOAc, 1 mM $Mg(OAc)_2$, 3 mM EGTA, 2 mM $CaCl_2$, 1 mM DTT. Typically, 5 μl extract was used in a 10 μl assay that contained also 10,000 c.p.m. synthetic mRNA substrate.

Extract fractionation

Extracts were centrifuged at 200,000*g* for 3 h and the resulting pellet (containing ribosomes) was extracted in hypotonic buffer containing also 1 mM $MgCl_2$ and 300 mM KOAc. The extracted material was spun at 100,000*g* for 1 h and the resulting supernatant was fractionated on Source 15Q column (Pharmacia) using a KCl gradient in buffer A (20 mM HEPES pH 7.0, 1 mM dithiothreitol, 1 mM $MgCl_2$). Fractions were assayed for nuclease activity as described above. For northern blotting, fractions were proteinase K/SDS treated, phenol extracted, and resolved on 15% acrylamide 8M urea gels. RNA was electroblotted onto Hybond N+ and probed with strand-specific riboprobes derived from cyclin E mRNA. Hybridization was carried out in 500 mM $NaPO_4$ pH 7.0, 15% formamide, 7% SDS, 1% BSA. Blots were washed in 1 × SSC at 37–45 °C.

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A genetic link between co-suppression and RNA interference in *C. elegans*

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Originally discovered in plants[1,2], the phenomenon of co-suppression by transgenic DNA has since been observed in many organisms from fungi[3] to animals[4–7]: introduction of transgenic copies of a gene results in reduced expression of the transgene as well as the endogenous gene. The effect depends on sequence identity between transgene and endogenous gene. Some cases of co-suppression resemble RNA interference (the experimental silencing of genes by the introduction of double-stranded RNA)[8], as RNA seems to be both an important initiator and a target in these processes[9–13]. Here we show that co-suppression in *Caenorhabditis elegans* is also probably mediated by RNA molecules. Both RNA interference[14,15] and co-suppression[16] have been implicated in the silencing of transposons. We now report that mutants of *C. elegans* that are defective in transposon silencing and RNA interference (*mut-2*, *mut-7*, *mut-8* and *mut-9*) are in addition resistant to co-suppression. This indicates that RNA interference and co-suppression in *C. elegans* may be mediated at least in part by the same molecular machinery, possibly through RNA-guided degradation of messenger RNA molecules.

We tested whether the MUT-7 protein, a putative 3′-5′ exoribonuclease required for transposon silencing and RNA interference (RNAi)[14], is also required for co-suppression in *C. elegans*. Co-suppression in *C. elegans* has been reported for a number of genes, including *fem-1*. As described previously[7], wild-type animals bearing a highly repetitive transgene containing multiple copies of the complete *fem-1* gene show a feminization of the germline, phenocopying loss-of-function mutations of the *fem-1* gene (Table 1). It has been shown that this effect depends on the presence of the *fem-1* promoter region[7]. When this region is not present, no feminization is observed, indicating that RNA is a mediator in co-suppression. We placed the same *fem-1* transgene in a *mut-7* mutant background and found that this feminization was no longer observed (Table 1). This result indicates that the RNA-mediated co-suppression effect of the *fem-1* transgene has a genetic basis and that it requires a protein (MUT-7) that is also involved in the processes of RNAi and transposon silencing. Thus, the aberrant RNA molecules that have been postulated in co-suppression[10,17] might be double-stranded RNA (dsRNA) molecules, also involved in RNAi[8].

To test whether the dependence of co-suppression on *mut-7* is general, we analysed two other genes for which co-suppression effects have been described: *gld-1* (ref. 6) and *mrt-2* (S. Ahmed and J. Hodgkin, personal communication). *Gld-1* co-suppression leads to an absence of oocytes and a tumorous germline, whereas *mrt-2* co-suppression results in hypersensitivity to ionizing radiation (which is consistent with the loss-of-function phenotypes of both genes[18,19]). Again, we find that the observed co-suppression effects

Table 1 Co-suppression of *fem-1*

Genotype	No. of animals with phenotype: Feminized	Wild type
Wild type; *pKEx1534*	28	2
mut-7(pk204); pkEx1534	0	27
rde-1(ne219); pkEx1539	30	1

Feminization of the germline by transgenes containing the *fem-1* gene. pkEx1534 was generated by injection of *fem-1* plasmid DNA into *mut7(pk204)* animals. This resulted in several non-co-suppressed transgenic lines (one containing the transgene pkEx1534). Restoration of *mut-7* gene function results in feminization of the germline. Injection of the same DNA into *rde-1(ne219)* animals results in lines displaying high levels of feminization.

Table 2 Co-suppression of *gld-1*

Genotype	No. of animals with phenotype: Tumorous germline	Wild type
mut-7(pk204)/+; pkEx1533	32	4
mut-7(pk204); pkEx1533	4	35
Complete promoter in wild type*	7	0
Complete promoter in *rde-1(ne219)**	4	0
Deleted promoter in wild type*	0	3†
Promoter only in wild type*	0	11

Induction of a 'tumorous germline' phenotype[18] by a *gld-1* multicopy transgene (pkEx1533), containing the complete *gld-1* promoter.
* The number of stable non-co-suppressed lines (designated wild type) or co-suppressed lines (tumorous germline) after injection is given; 31 (complete promoter in wild type), 22 (complete promoter in *rde-1(ne219)*), 32 (deleted promoter) and 107 (promoter only) F_1 transgenic animals were analysed.
† These three lines have no tumorous germline and produce oocytes. The strains produce some unfertilized eggs, indicative of a sperm defect, probably caused by a lower dosage of GLD-1 protein[18].

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Role for a bidentate ribonuclease in the initiation step of RNA interference

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RNA interference (RNAi) is the mechanism through which double-stranded RNAs silence cognate genes[1–5]. In plants, this can occur at both the transcriptional and the post-transcriptional levels[1,2,5]; however, in animals, only post-transcriptional RNAi has been reported to date. In both plants and animals, RNAi is characterized by the presence of RNAs of about 22 nucleotides in length that are homologous to the gene that is being suppressed[6–8]. These 22-nucleotide sequences serve as guide sequences that instruct a multicomponent nuclease, RISC, to destroy specific messenger RNAs[6]. Here we identify an enzyme, Dicer, which can produce putative guide RNAs. Dicer is a member of the RNase III family of nucleases that specifically cleave double-stranded RNAs, and is evolutionarily conserved in worms, flies, plants, fungi and mammals. The enzyme has a distinctive structure, which includes a helicase domain and dual RNase III motifs. Dicer also contains a region of homology to the RDE1/QDE2/ARGONAUTE family that has been genetically linked to RNAi[9,10].

Biochemical studies have suggested that post-transcriptional gene silencing (PTGS) is accomplished by a multicomponent nuclease that targets mRNAs for degradation[6,8,11]. The specificity of this complex may derive from the incorporation of a small guide sequence that is homologous to the mRNA substrate[6]. These ~22-nucleotide RNAs, originally identified in plants that were actively silencing transgenes[7], have been produced during RNAi *in vitro* using an extract prepared from *Drosophila* embryos[8]. Putative guide RNAs can also be produced in extracts from *Drosophila* S2 cells (Fig. 1a). To investigate the mechanism of PTGS, we have performed both biochemical fractionation and candidate gene approaches to identify the enzymes that execute each step of RNAi.

Our previous studies resulted in the partial purification of an enzyme complex, RISC, which is an effector nuclease for RNA interference[6]. This enzyme was isolated from *Drosophila* S2 cells in which RNAi had been initiated *in vivo* by transfection with double-stranded RNA (dsRNA). We first investigated whether the RISC enzyme, and the enzyme that initiates RNAi through processing of dsRNA into 22-nucleotide sequences, are distinct activities. RISC activity could be largely cleared from extracts by high-speed centrifugation (100,000*g* for 60 min), whereas the activity that produces 22-nucleotide sequences remained in the supernatant (Fig. 1b, c). This simple fractionation indicates that RISC and the 22-nucleotide sequence-generating activity may be separable. However, it seems probable that these enzymes interact at some point during the silencing process, and it remains possible that initiator and effector enzymes share common subunits.

RNase III family members are among the few nucleases that show specificity for dsRNA[12]. Analysis of the *Drosophila* and *Caenorhabditis elegans* genomes reveals several types of RNase III enzymes. First is the canonical RNase III, which contains a single RNase III signature motif and a dsRNA-binding domain (dsRBD; for example RNC_CAEEL). Second is a class represented by Drosha[13], a *Drosophila* enzyme that contains two RNase III motifs and a dsRBD (CeDrosha in *C. elegans*). A third class contains two RNase III signatures and an amino-terminal helicase domain (for example, *Drosophila* CG4792 and CG6493; *C. elegans* K12H4.8), which had been proposed as potential RNAi nucleases[14,20]. We tested representatives of all three classes for the ability to produce discrete RNAs of ~22 nucleotides from dsRNA substrates.

To test the dual RNase III enzymes, we prepared variants of Drosha and CG4792 tagged with the T7 epitope. These were expressed in transfected S2 cells and isolated by immunoprecipitation using antibody–agarose conjugates. Treatment of the dsRNA with the CG4792 immunoprecipitate yielded fragments of about 22 nucleotides, similar to those produced in either the S2 or embryo extracts (Fig. 2a). Neither the activity in extract nor that in immunoprecipitates depended on the sequence of the RNA substrate, as dsRNAs derived from several genes were processed equivalently (see Supplementary Information). Negative results

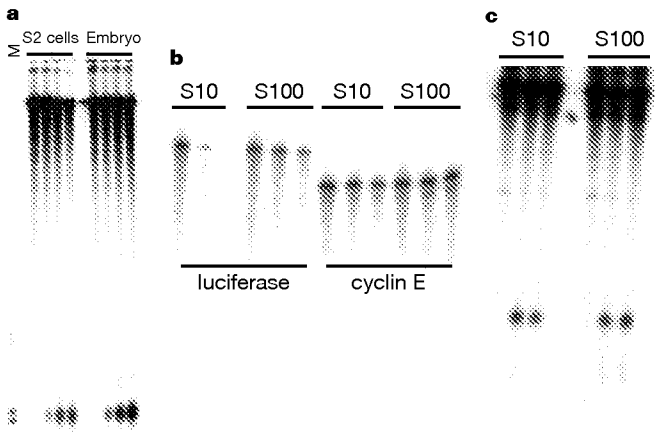

Figure 1 Generation of 22-nucleotide sequences and degradation of mRNA by distinct enzymatic complexes. **a**, Extracts prepared from for 0–12 h *Drosophila* embryos or *Drosophila* S2 cells. Extracts were incubated for 0, 15, 30 or 60 min (left to right) with a uniformly labelled dsRNA. A 22-nucleotide marker prepared by *in vitro* transcription of a synthetic template is indicated (M). **b**, Whole-cell extracts from S2 cells transfected with luciferase dsRNA. S10 represents our standard RISC extract[6]. S100 extracts were prepared by additional centrifugation of S10 extracts for 60 min at 100,000*g*. Assays for mRNA degradation[6] were performed for 0, 30 or 60 min (left to right in each set) with either a single-stranded luciferase mRNA or a single-stranded cyclin E mRNA, as indicated. **c**, S10 or S100 extracts incubated with cyclin E dsRNAs for 0, 60 or 120 min (left to right).

were obtained with Drosha and with immunoprecipitates of a DExH box helicase (Homeless[15]; see Fig. 2a and b). Western blotting confirmed that each of the tagged proteins was expressed and immunoprecipitated similarly (see Supplementary Information). Thus, we conclude that CG4792 may carry out the initiation step of RNAi by producing guide sequences of about 22 nucleotides from dsRNAs. Because of its ability to digest dsRNA into uniformly sized, small RNAs, we have named this enzyme Dicer (*Dcr*). *Dicer* mRNA is expressed in embryos, in S2 cells and in adult flies, which is consistent with the presence of functional RNAi machinery in all of these contexts (see Supplementary Information).

An antiserum directed against the carboxy terminus of the Dicer protein (Dicer-1, CG4792) could immunoprecipitate a nuclease activity from either the *Drosophila* embryo extracts or from S2 cell lysates that produced RNAs of about 22 nucleotides from dsRNA substrates (Fig. 2c). The putative guide RNAs that are produced by the Dicer-1 enzyme precisely co-migrate with 22-nucleotide sequences that are produced in extract, and with 22-nucleotide sequences that are associated with the RISC enzyme (Fig. 2d, f). The enzyme that produces guide RNAs in *Drosophila* embryo extracts is ATP dependent[8]. Depletion of this cofactor resulted in a roughly sixfold reduction of dsRNA cleavage rate and in the production of RNAs with a slightly lower mobility. Of note, both Dicer-1 immunoprecipitates and extracts from S2 cells require ATP for the production of ~22-nucleotide sequences (Fig. 2d). We did not observe the accumulation of lower-mobility products in these cases, although we did routinely observe these in ATP-depleted embryo extracts. The requirement of this nuclease for ATP is an unusual property, and may indicate that unwinding of guide RNAs by the helicase domain is required for the enzyme to act catalytically.

For efficient induction of RNAi in *C. elegans* and in *Drosophila*, the initiating RNA must be double-stranded and must also be several hundred nucleotides in length[4]. Similarly, Dicer was inactive against single-stranded RNAs regardless of length (see Supplementary Information). The enzyme could digest both 200- and 500-nucleotide dsRNAs, but was significantly less active with shorter substrates (see Supplementary Information). In contrast, *Escherichia coli* RNase III could digest to completion dsRNAs of 35 or 22 nucleotides (data not shown). This suggests that the substrate preferences of the Dicer enzyme may contribute to, but not wholly determine, the size dependence of RNAi.

To determine whether the Dicer enzyme is involved in RNAi *in vivo*, we depleted Dicer activity from S2 cells and tested the effect on dsRNA-induced gene silencing. Transfection of S2 cells with a

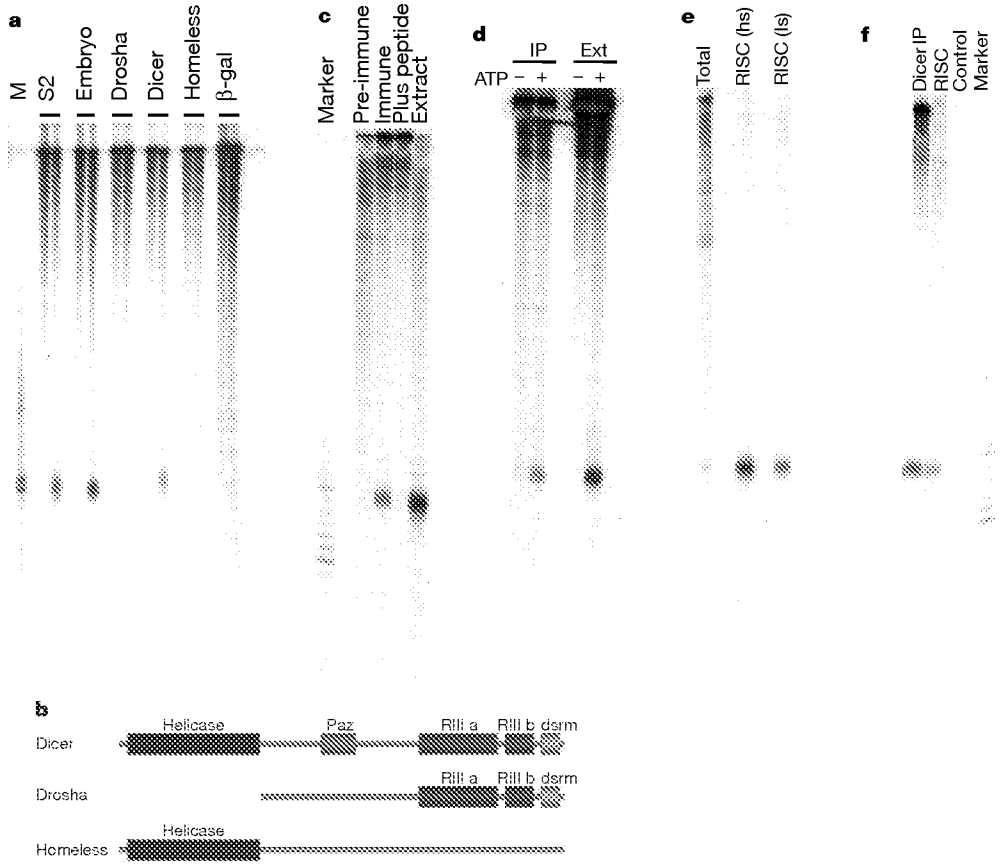

Figure 2 Production of 22-nucleotide sequences by CG4792/Dicer. **a**, *Drosophila* S2 cells transfected with plasmids that direct expression of T7-epitope-tagged versions of Drosha, CG4792/Dicer-1 and Homeless or untagged β-galactosidase. Proteins were immunoprecipitated and incubated with *cyclin E* dsRNA for 0 or 60 min. Reactions in *Drosophila* embryo and S2 cell extracts are shown. **b**, Domain structures of CG4792/Dicer-1, Drosha and Homeless. **c**, Immunoprecipitates prepared from detergent lysates of S2 cells using Dicer antiserum. As controls, similar preparations were made with a pre-immune serum and an immune serum that had been pre-incubated with an excess of antigenic peptide. Cleavage reactions in which each of these precipitates was incubated with a ~500 nucleotide fragment of *Drosophila* cyclin E are shown. An incubation of the substrate in *Drosophila* embryo extract is shown. **d**, Dicer immunoprecipitates incubated with dsRNA substrates in presence or absence of ATP. The same substrate was also incubated with ATP-added or ATP-depleted S2 extracts. **e**, *Drosophila* S2 cells transfected with uniformly, ^{32}P-labelled dsRNA corresponding to the first 500 nucleotides of GFP. RISC complex was affinity purified using a histidine-tagged version of *Drosophila* Ago-2, a component of the RISC complex (Hammond *et al.*, manuscript in preparation). RISC was isolated under ribosome-associated (ls, low salt) or soluble, ribosome-extracted (hs, high salt) conditions[6]. The spectrum of labelled RNAs in the total lysate is shown. **f**, Comparison of guide RNAs produced by incubation of dsRNA with a Dicer immunoprecipitate, with guide RNAs present in affinity-purified RISC complex. These co-migrate on a gel that has single-nucleotide resolution. The control lane shows an affinity selection for RISC from cells transfected with labelled dsRNA, but not with the epitope-tagged *Drosophila* Ago-2.

mixture of dsRNAs homologous to the two *Drosophila* Dicer genes (CG4792 and CG6493) resulted in a roughly 6–7-fold reduction of Dicer activity either in whole-cell lysates or in Dicer-1 immunoprecipitates (Fig. 3a and b). Transfection with a control dsRNA (murine caspase-9) had no effect. Qualitatively similar results were seen if Dicer mRNA was examined by northern blotting (data not shown). Depletion of Dicer substantially compromised the ability of cells to silence an exogenous, green fluorescent protein (GFP) transgene by RNAi (Fig. 3c). These results indicate that Dicer may be involved in RNAi *in vivo*. The lack of complete inhibition of silencing may result from an incomplete suppression of Dicer or may indicate that *in vivo* guide RNAs may be produced by more than one mechanism.

Our results indicate that the process of RNAi can be divided into at least two distinct steps. Initiation of PTGS would occur on processing of a dsRNA by Dicer into ~22-nucleotide guide sequences, although we cannot formally exclude the possibility that another Dicer-associated nuclease may participate in this process. These guide RNAs would be incorporated into a distinct nuclease complex (RISC) that targets single-stranded mRNAs for degradation. An implication of this model is that the guide sequences are themselves derived directly from the dsRNA that triggers the response. In accord with this model, we have shown that ^{32}P-labelled, exogenous dsRNAs that have been introduced into S2 cells by transfection are incorporated into the RISC enzyme as 22-nuclotide sequences (Fig. 2e).

A notable feature of the Dicer family is its evolutionary conservation. Homologues are found in *C. elegans* (K12H4.8), *Arabidopsis* (for example, CARPEL FACTORY[16], T25K16.4 and AC012328_1), mammals (Helicase-MOI[17]) and *Schizosaccharomyces pombe* (YC9A_SCHPO) (see Supplementary Information for comparisons). In fact, the human Dicer family member is capable of generating ~22-nucleotide RNAs from dsRNA substrates (see Supplementary Information), which indicates that these structurally similar proteins may all share similar biochemical functions. Exogenous dsRNAs can affect gene function in early mouse embryos[18], and our results suggest that this regulation may be accomplished by evolutionarily conserved RNAi machinery.

In addition to RNase III and helicase motifs, searches of the PFAM database indicate that each Dicer family member also contains a PAZ domain (see Supplementary Information)[19,20]. This sequence was defined on the basis of its conservation in the Zwille/ARGONAUTE/Piwi family that has been implicated in RNAi by mutations *in C. elegans* (Rde-1)[9] and *Neurospora* (Qde-2)[10]. Although the function of this domain is unknown, it is notable that this region of homology is restricted to two gene families that participate in dsRNA-dependent silencing. Both the ARGONAUTE and Dicer families have also been implicated in common biological processes, namely the determination of stem-cell fates. A hypomorphic allele of *carpel factory*, a member of the Dicer family in *Arabidopsis*, is characterized by increased proliferation in floral meristems[16]. This phenotype and a number of other characteristic features are also shared by *Arabidopsis ARGONAUTE* (*ago1-1*) mutants[21] (C. Kidner and R. Martiennsen, personal communication). These genetic analyses provide evidence that RNAi may be more than a defensive response to unusual RNAs, but may also have integral functions in the regulation of endogenous genes.

With the identification of Dicer as a potential catalyst of the initiation step of RNAi, we have begun to unravel the biochemical basis of this unusual mechanism of gene regulation. It is now important to determine whether the conserved family members from other organisms, particularly mammals, also have a function in dsRNA-mediated gene regulation.

Note added in proof: Yang *et al.*[22] have recently presented evidence that guide RNAs are derived directly from dsRNA in *Drosophila* embryos. Fagard *et al.*[23] have recently shown that *Arabidopsis* Ago1 is involved in PTGS. □

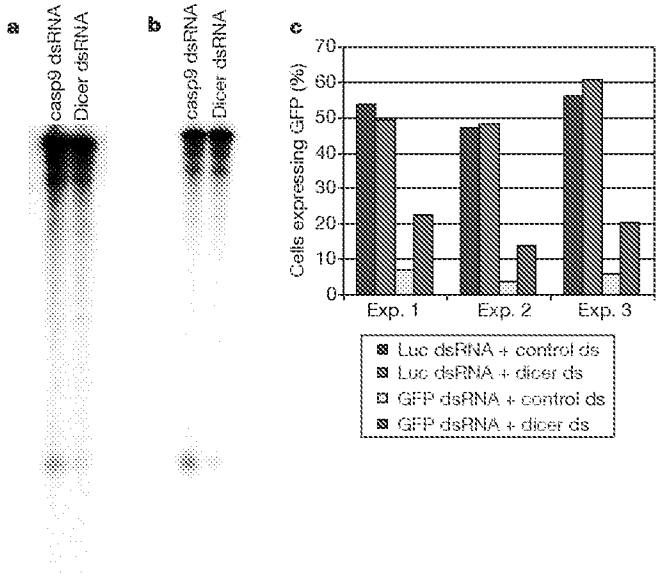

Figure 3 Dicer participates in RNAi. **a**, *Drosophila* S2 cells transfected with dsRNAs corresponding to the two *Drosophila* Dicers (CG4792 and CG6493) or control dsRNA corresponding to murine caspase-9 (casp9). Cytoplasmic extracts of these cells were tested for Dicer activity. Transfection with Dicer dsRNA reduces activity in lysates 7.4-fold. **b**, Dicer-1 antiserum (CG4792) used to prepare immunoprecipitates from S2 cells (treated as above). Dicer dsRNA reduces the activity of Dicer-1 6.2-fold. **c**, GFP expression of co-transfected cells. Three independent experiments were quantified by FACS. A comparison of the relative percentage of GFP-positive cells is shown for control (GFP plasmid plus luciferase dsRNA) or silenced (GFP plasmids plus GFP dsRNA) populations in cells that had previously been transfected with either control (caspase-9) or Dicer dsRNAs.

Methods

Plasmid constructs

A full-length complementary DNA encoding Drosha was obtained by polymerase chain raaction (PCR) from an expressed sequence tag sequenced by the Berkeley *Drosophila* genome project. The T7 epitope-tag was added to the N terminus of each cDNA by PCR, and the tagged cDNAs were cloned into pRIP—a retroviral vector designed specifically for expression in insect cells (E. B., unpublished observations). In this vector, expression is driven by the *Orgyia pseudotsugata* IE2 promoter (Invitrogen). As no cDNA was available for CG4792/Dicer, a genomic clone was amplified from a bac (bacterial artificial chromosome) (BACR23F10; obtained from the BACPAC Resource Center in the Deptartment of Human Genetics at the Roswell Park Cancer Institute). We added a T7 epitope tag at the N terminus of the coding sequence during amplification. We isolated the human *DICER* gene from a cDNA library prepared from HaCaT cells (G.J.H., unpublished observations). A T7-tagged version of the complete coding sequence was cloned into pCDNA3 (Invitrogen) for expression in human cells (LinX-A).

Cell culture and extract preparation

We cultured S2 cells at 27 °C in 5% CO_2 in Schneider's insect media supplemented with 10% heat-inactivated fetal bovine serum (Gemini) and 1% antibiotic–antimycotic solution (Gibco BRL). Cells were collected for extract preparation at 10^7 cells per ml. The cells were washed in PBS and resuspended in a hypotonic buffer (10 mM HEPES pH 7.0, 2 mM $MgCl_2$ and 6 mM β-mercaptoethanol) and lysed. We centrifuged cell lysates at 20,000*g* for 20 min. We stored extracts at −80 °C. We reared *Drosophila* embryos in fly cages by standard methodologies and collected them every 12 h. We dechorionated the embryos in 50% chlorox bleach and washed them thoroughly with distilled water. Lysis buffer (10 mM Hepes, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM EGTA, 10 mM β-glycerophosphate, 1 mM dithiothreitol (DTT) and 0.2 mM PMSF) was added to the embryos, and extracts were prepared by homogenization in a tissue grinder. Lysates were centrifuged for 2 h at 200,000*g*, and were frozen at −80 °C. LinX-A cells, a highly transfectable derivative of human 293 cells (L. Xie and G.J.H., unpublished observations) were maintained in DMEM/10% FCS.

Transfections and immunoprecipitations

We transfected S2 cells using a calcium phosphate procedure essentially as described[6]. Transfection rates were about 90%, as monitored in controls using an *in situ* β-galactosidase assay. We also transfected LinX-A cells by calcium phosphate co-precipitation. For immunoprecipitations, cells (~5×10^6 per immunoprecipitate) were

transfected with various clones, and lysed 3 d later in immunoprecipitate buffer (125 Mm KOAc, 1 mM MgOAc, 1 mM $CaCl_2$, 5 mM EGTA, 20 mM HEPES pH 7.0, 1 mM DTT and 1% Nonidet P40 plus complete protease inhibitors (Roche)). We centrifuged lysates for 10 min at 14,000*g*, and then added supernatants to T7 antibody-agarose beads (Novagen). We performed antibody binding for 4 h at 4 °C. Beads were centrifuged and washed three times in lysis buffer, and once in reaction buffer. The Dicer antiserum was raised in rabbits using a keyhole limpet haemocyanin-conjugated peptide corresponding to the C-terminal eight amino acids of *Drosophila* Dicer-1 (CG4792).

Cleavage reactions

Templates to be transcribed to dsRNA were generated by PCR with forward and reverse primers, each containing a T7 promoter sequence. RNAs were produced using Riboprobe kits (Promega) and were uniformly labelled during the transcription reaction with ^{32}P-labelled UTP. Single-stranded RNAs were purified from 1% agarose gels. For cleavage of dsRNA, 5 μl of embryo or S2 extracts were incubated for 1 h at 30 °C with dsRNA in a reaction containing 20 mM HEPES pH 7.0, 2 mM magnesium acetate, 2 mM DTT, 1 mM ATP and 5% Superasin (Ambion). Immunoprecipitates were treated similarly, except that a minimal volume of reaction buffer (including ATP and superasin) and dsRNA were added to beads that had been washed in reaction buffer. For ATP depletion, *Drosophila* embryo extracts were incubated for 20 min at 30 °C with 2 mM glucose and 0.375 U of hexokinase (Roche), before the addition of dsRNA.

Northern and western analysis

Total RNA was prepared from *Drosophila* embryos (0–12 h), from adult flies and from S2 cells using Trizol (Lifetech). We isolated mRNA by affinity selection using magnetic LIGO-dT beads (Dynal). RNAs were electrophoresed on denaturing formaldehyde/agarose gels, blotted and probed with randomly primed DNAs corresponding to Dicer. For western analysis, T7-tagged proteins were immunoprecipitated from whole-cell lysates in immunoprecipitate buffer using agarose-conjugated anti-T7 antibody. Proteins were released from the beads by boiling in Laemmli buffer, and were separated by 8% SDS–polyacrylamide gel electrophoresis. After transfer to nitrocellulose, proteins were visualized using an HRP-conjugated anti-T7 antibody (Novagen) and chemiluminescent detection (Supersignal, Pierce).

RNAi of Dicer

Drosophila S2 cells were transfected either with a dsRNA corresponding to mouse caspase-9 or with a mixture of two dsRNAs corresponding to *Drosophila* Dicer-1 and Dicer-2 (CG4792 and CG6493). Two days after the initial transfection, cells were again transfected with a mixture containing a GFP expression plasmid and either luciferase dsRNA or GFP dsRNA as described[6]. Cells were assayed for Dicer activity or fluorescence 3 d after the second transfection. Quantification of fluorescent cells was done on a Coulter EPICS cell sorter, after fixation. Control transfections indicated that Dicer activity was not affected by the introduction of caspase-9 dsRNA.

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Supplementary information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

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A model for SOS-lesion-targeted mutations in *Escherichia coli*

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The UmuD′$_2$C protein complex (*Escherichia coli* pol V)[1–3] is a low-fidelity DNA polymerase (pol) that copies damaged DNA in the presence of RecA, single-stranded-DNA binding protein (SSB) and the β,γ-processivity complex of *E. coli* pol III (ref. 4). Here we propose a model to explain SOS-lesion-targeted mutagenesis, assigning specific biochemical functions for each protein during translesion synthesis. (SOS lesion-targeted mutagenesis occurs when pol V is induced as part of the SOS response to DNA damage and incorrectly incorporates nucleotides opposite template lesions.) Pol V plus SSB catalyses RecA filament disassembly in the 3′ to 5′ direction on the template, ahead of the polymerase, in a reaction that does not involve ATP hydrolysis. Concurrent ATP-hydrolysis-driven filament disassembly in the 5′ to 3′ direction results in a bidirectional stripping of RecA from the template strand. The bidirectional collapse of the RecA filament restricts DNA synthesis by pol V to template sites that are proximal to the lesion, thereby minimizing the occurrence of untargeted mutations at undamaged template sites.

Lesions that block DNA replication persist in prokaryotic and eukaryotic cells despite the presence of base excision, nucleotide excision and postreplication repair[5]. A group of DNA polymerases have been discoverd (the UmuC/DinB/Rad30/Rev1 superfamily) whose function is to copy DNA template lesions[6]. The presence of *E. coli* pol V (UmuD′$_2$C) is essential for SOS-induced mutagenesis[5]. However, pol V cannot catalyse translesion synthesis (TLS) by itself; it requires the presence of RecA and single-stranded-DNA binding protein (SSB), and is stimulated by β-sliding clamp in a 'mutasomal' complex[7] (pol V Mut) to copy replication-blocking lesions[4].

Specific Aims

A central goal of the "war on cancer" is to gain a sufficiently detailed understanding of tumor cells to permit rational design of highly effective and specific anticancer therapies. During the past decades, our understanding of the molecular alterations that underlie the tumor development have proceeded at a rapid pace. Numerous oncogenes and tumor suppressor have been identified, and many of these have been fitted into molecular pathways that control cell growth and survival.

In a few cases such advances in our basic understanding of cancer cell biology has led to the development of novel therapies. Based upon these existing success stories (e.g. herceptin, gleevec), it is now clear that ongoing efforts to understand the biology underlying neoplastic transformation will lead to a pipeline of novel drug targets and ultimately to therapeutics. However, novel technologies will be essential if we are to accelerate the pace at which accumulating biological and genomic information moves from the discovery stage into the clinic.

For the past 5 years, Genetica and its collaborators have pursued the design and implementation of novel technologies for manipulating gene function in mammalian cells. It is our belief that the availability of facile genetic methods that can be applied in cell culture and ultimately in model organisms will help to relieve a critical bottleneck in the drug development process, namely the ability to validate potential targets by understanding the biological effects of creating loss-of-function mutations. Furthermore, we are committed to the use of forward genetic approaches to identify novel therapeutic targets that might not be obvious from existing data.

The central goal of this grant application is the design, validation and application of technologies that can be used to create stable loss-of-function mutations in mammalian cells and animals. Phase I of this project has seen substantial progress toward this goal. In Phase II, we will extend the foundation that we have built into a generally applicable system for probing gene function in human cancer cells. This will be accomplished through the following specific aims:

Aim 1. Creation of stable, loss-of-function mutations in embryonic cells using RNAi. We have demonstrated that RNA interference can be used to suppress gene function in embryonic cells. We propose to extend these studies by optimizing methods to permanently suppress gene expression in these cell types.

Aim 2. Creation of stable loss-of-function mutations in non-embyronic cell types. Long dsRNAs provoke a PKR response in differentiated cell types. We propose numerous strategies for bypassing this problem to create a loss-of-function technology that can be applied universally, not only in cell culture but ultimately also in model mammalian animals.

Aim 3. Execution of genetic screens using single cells. To date, our work, and that of others, requires that genetic elements that create phenotypes in mammalian cells be recovered from the pooled genomes of large numbers of cells. We propose methodologies that permit the recovery of exogenous genetic elements from single cells. This advance will greatly expand the range of phenotypic screens that can be performed using the loss-of-function technologies that will be developed in aims 1 and 2.

Exhibit D

It is not an overstatement to say that successful completion of these four specific aims would revolutionize the way in which we approach not only target validation for anti-cancer drugs but also more generally functional analysis in mammalian systems.

Background and Significance

The overall goal of this application is to develop novel methodologies to the creation of loss-of-function mutations in cultured mammalian cells and to apply these technologies to the identification of novel anti-cancer targets. Culture mammalian cells are the most commonly used model organism in both industrial and academic biology; however, the tools for manipulating this model have remained primitive as compared to genetic models such as yeast, flies and worms. This deficit is particularly acute with regard to the creation of loss-of-function mutations.

Over the past several years, we have devoted a great deal of effort to the use of antisense RNA as a regulator of gene function in mammalian cells (Carnero et al., 2000a) (Carnero et al., 2000b). Certainly, we have recorded numerous successes with this approach. However, antisense RNAs have not proven, either in our hands or in those of numerous other investigators, an effective and universal tool for creating loss-of-function phenotypes. This realization led to the decision several years ago to pursue alternative approaches.

RNA interference

In an evolutionarily diverse group of organisms ranging from plants to insects to nematodes and recently mammals, double-stranded RNA can act as a potent and specific inducer of gene silencing (reviewed in (Hammond et al., 2001)). The discovery of RNAi, per se, grew out of a desire to use antisense approaches to probe gene function in *C. elegans*. A desire to determine the function of the par-1 gene led Guo and Kemphues to inject antisense RNA into worms (Guo and Kemphues, 1995). This indeed created the expected phenotype, embryonic lethality. However, a serious paradox was raised by the observation that injection of the sense-orientation, control RNA created precisely the same phenotype. In hindsight, this was somewhat reminiscent of observations that had been made in plants in which increased gene dosage also caused loss of gene expression (see below). However, at the time, this result was interpreted as indicating an inhibition by saturation of factors needed for par-1 translation.

The key breakthrough came with the observations of Fire and Mello (Fire et al., 1998). Having also encountered a similar phenomenon in which either sense or antisense oriented RNAs inhibited gene function, these investigators asked whether co-injection of sense and antisense strands might give an additive, and thus more complete, effect. Shockingly, the mixture of sense and antisense strands silenced expression of a target gene roughly 10-fold more efficiently than either sense or antisense RNAs alone. Interpreting this dsRNA-induced effect as a novel phenomenon, they coined the term, RNA interference or RNAi, to describe the process. Since its discovery, RNAi has become an exceptionally powerful tool for manipulating gene expression, and consequently for analyzing gene function in a broad spectrum of model organisms (reviewed in (Hammond et al., 2001)).

In C. elegans, RNAi is the standard methodology for rapidly examining the consequences of loss-of-function mutations. The response can be provoked by injection of dsRNA into the worm gut, by soaking worms in dsRNA or by feeding worms bacteria that have been engineered

to express dsRNA (reviewed in (Hammond et al., 2001)). Having been provoked by any of these routes, dsRNA-induced silencing has a number of remarkable properties. First, it is systemic. Administration of dsRNA through the gut induces silencing throughout the recipient animal. Second, it is heritable. The penetrance of silencing is nearly complete in the F1 generation and wanes in following generations. Third, it is terrifically potent. Injection of animals with only a few molecules of dsRNA per cell causes complete silencing in both the parental worm and in its progeny.

These seminal observations have now yielded a tool that is being applied not only for the analysis of individual genes but for the analysis of entire genomes. Several independent groups have undertaken efforts to create RNAi-suppressed strains representing all 19,000 genes in the C. elegans genome (Fraser et al., 2000) (Maeda et al., 2001). A portion of these efforts have now been described in the literature, and based upon these preliminary studies (if you can call functional analysis of an entire chromosome preliminary), this approach will allow assignment of some function to approximately 14% of all worm genes. This is quite remarkable considering that so few potential phenotypic characters were examined in the work reported to date.

Double-stranded RNA-induced gene silencing was actually first discovered in plants as co-suppression (Jorgensen et al., 1996). In attempts to engineer more colorful petunias, Jorgensen and colleagues noted that not only did introduced transgenes fail to express but also induced silencing of homologous endogenous loci. As has become apparent from later genetic and biochemical studies, silencing was probably provoked when complex transgene arrays gave rise to inverted repeat transcripts which triggered an RNAi response. Silencing of endogenous genes in plants can also be provoked by infection with viruses that produce dsRNA as replication intermediates. This, so-called VIGS (virus-induced gene silencing) also proceeds via an RNAi mechanism. While RNAi has yet to become the standard tool for creating loss-of-function phenotypes in plants, large-scale screens are now underway which use VIGS to examine the consequences of silencing every gene in the Arabidopsis genome (Baulcombe, personal comunication).

RNAi has also proven to be an effective tool in Drosophila. This first became apparent with the observation that injection of dsRNA homologous to the Frizzled2 gene into embryos phenocopied the Frizzled2 null mutation (Kennerdell and Carthew, 1998). Subsequently, taking a cue from the fact that silencing could be provoked by stable transgenes in plants, the same investigators showed that heritable RNAi could be induced in Drosophila by ectopic expression of RNA hairpins (Kennerdell and Carthew, 2000). Similar stable suppression by RNAi has also been achieved using RNA hairpins in worms and plants (Smith et al., 2000).

Perhaps the greatest impact of RNAi has been made (and is yet to be made) in systems in which more conventional genetic approaches have been lacking. For example, those investigators who work in systems such as planaria, mosquitos and trypanosomes now have a facile tool with which to induced sequence-specific gene silencing (reviewed in (Hammond et al., 2001)). However, nowhere is the lack of good loss-of-function methodologies more acute felt than in mammalian cell culture systems and in mammalian animals.

Mammals have well-developed responses to dsRNA that have evolved to aid in antiviral defense (Williams, 1997). In most mammalian cells, an encounter with intracellular dsRNA activates a series of pathways, the best studied of which is PKR. This dsRNA-activated protein kinase phosphoylates eIF2α and consequently causes a non-specific shut-down of the

translational machinery. Also activated are NFkB pathways, which lead ultimately to cell-death and a nuclease system (2'-5'-oligoadenylate synthetase/RNAseL), which leads to degradation of viral and cellular RNAs. Thus, mammalian cells have been wired to respond to dsRNA by suicide, limiting viral spread by self-sacrifice.

The existence of such well-established pathways made it seem unlikely that seemingly alternative antiviral responses, such as RNAi, might exist in mammals and damped enthusiasm that RNAi might evolve into a tool for manipulating gene expression in mammals. However, this changed with the striking observation of Zernika-Goetz and colleages that injection of dsRNA into early-stage mouse embryos could provoke sequence-specific silencing (Wianny and Zernicka-Goetz, 2000) (Svoboda et al., 2000). Secondly, we found that many of the genes that we identified through our biochemical, mechanistic studies of RNAi were conserved in mammals (reviewed in (Hammond et al., 2001)). These observations led our group and several others to attempt to search for contexts and technologies that might allow RNAi to be used as a tool in mammalian systems.

As described in the Phase I Final Report, we have succeeded in this goal, using long dsRNAs to suppress gene expression in embryonic murine cells. Furthermore, Tuschl and colleagues have recently reported that RNAi could be provoked in a variety of human and mouse cell lines by introduction of the small RNAs (siRNAs, see below) that serve as specificity determinants for this process (Grishok et al., 2001) (Hutvagner et al., 2001);R. Ketting et al., submitted). However, even with these stunning accomplishments, the problem is not solved. Presently dsRNA responses in mammalian cell culture are quite transient, and no methodologies for provoking dsRNA-induced silencing have been described that have the potential to work in intact animals. Therefore it is the goal of this proposal to build upon the results of our group and the work of others to create a coherent system for probing gene function initially in human cancer cells, but also more broadly.

Phase I Final Report

SBIR phase I grant R43 CA83402-01

Beginning: 1-Aug-99
Ending : 31-Jul-01

Key Personnel involved in this project

Name	Title	Dates of Service	Hours
David Beach	P.I.	8/1/99 – 7/31/01	No pay
Lisa Molz	Sr. Staff Scientist	8/1/99 – 7/31/01	344
Scott Hammon	Staff Scientist	8/1/99 – 7/31/01	3771

Phase I specific aims

Aim 1. To construct retroviral vectors that will allow genetic selection of effective antisense RNAs. We will design retroviral vectors for antisense RNA expression. Since these will be based upon our MaRX system, they will be suitable for analysis of known genes or for use in genetic screens in mammalian cells.

Aim 2. To validate the use of antisense RNA as a genetic tool. Using a model system, p53-dependent growth arrest, we will select antisense fragments that inhibit p53 function.

Progress toward phase I specific aims

As will become apparent in the following sections, during the course of this Phase I application and subsequent no-cost extensions, we have dramatically shifted the focus of this project. However, before doing so, we did complete both of the original specific aims. Retroviral vectors were designed and optimized for antisense expression, and we validated the use of antisense RNA as a genetic tool. Vectors constructed at Genetica were provided to Amancio Carnero of the Institute for Child Heath in London, and his work, validating the utility of these vectors for genetic analysis has been reported in the literature. Because of the shift in the focus of this program, I will not describe these results in detail. Interested readers are referred to two manuscripts that have been published by Dr. Carnero and are appended to this application (Carnero et al., 2000a; Carnero et al., 2000b).

RNA interference in cultured Drosophila cells

As stated above, our work with antisense RNA has often been successful. However, it also became clear that the development of new alternatives for creating loss-of-function phenotypes in mammalian cells was essential. We therefore initiated a project designed to elucidate the mechanism of RNA interference and to contribute to the development of this phenomenon as a tool. All of the progress toward this goal has been accomplished as a very fruitful collaboration between Genetica and Dr. Gregory Hannon at Cold Spring Harbor Laboratory. It should be noted that this collaboration will continue and that Dr. Hannon will serve as a consultant for this proposal.

Despite the obvious importance of dsRNA-induced gene silencing, the mechanisms underlying this phenomenon were obscure. In a number of organisms, including C. elegans, evidence indicated that dsRNA provoked gene silencing at a post-transcriptional level (Montgomery and Fire, 1998). However, particularly in plants, there were also indications of a parallel, if not related, process that accomplishes gene silencing by modification of chromatin structure (see for example, Jones et al., 1998; Jones et al., 1999).

Recognizing the potential of cultured Drosophila cells for mechanistic studies of RNA interference and as a model for the use of RNAi in cell culture, we tested whether introduction of dsRNA into S2 or Kc cells affected gene expression in a sequence-specific fashion. We began by probing effects on an ectopically expressed gene. Transient transfection of cultured, *Drosophila* S2 cells with a vector that directs lacZ expression from the copia promter resulted in β-galactosidase activity that was easily detectable by an *in situ* assay (Fig. 1A).

Transfection of S2 cells with lacZ dsRNA almost completely suppressed β-galactosidase activity, whereas transfection with a control dsRNA (CD8) had no effect (Fig. 1A). This result was obtained irrespective of whether the plasmid DNA and the dsRNA were co-transfected or whether transfection with the dsRNA preceded introduction of the plasmid by 1-2 days (not shown).

To determine whether RNAi could also target endogenous genes, S2 cells were transfected with a dsRNA corresponding to the first 540 nucleotides of *Drosophila* cyclin E, a gene essential for progression into S phase (Knoblich et al., 1994; Richardson et al., 1995). During log-phase growth, untreated S2 cells reside primarily in G2/M (Fig. 1B). While transfection with lacZ dsRNA had no effect on the cell-cycle distribution, transfection with the cyclin E dsRNA caused a G1 phase cell-cycle arrest (Fig. 1B).

A. B. C.

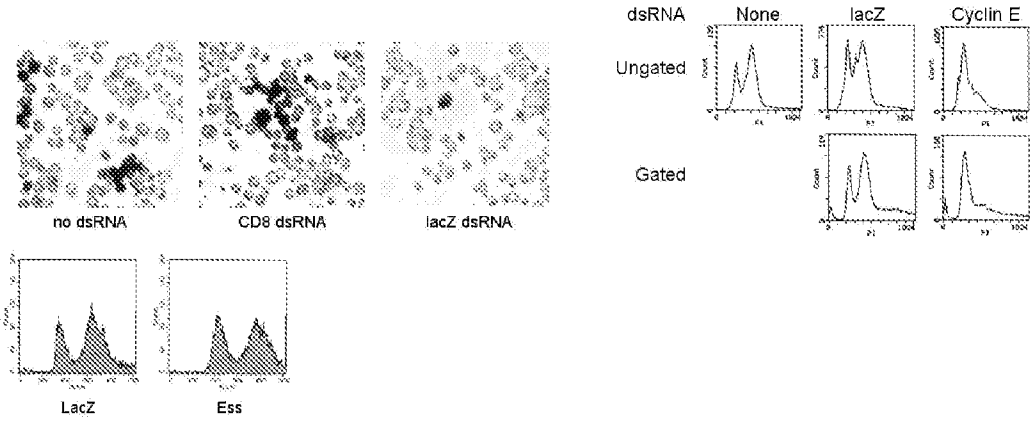

Figure 1. **A.** Drosophila S2 cells were transfected using a calcium phosphate protocol with a plasmid that directs lacZ expression from the copia promoter in combination with either no dsRNA or the indicated dsRNAs. Following optimization of transfection conditions, we achieve up to 50% (usually 20%) transfection rates using a phenotypic marker (e.g. LacZ or GFP expression) as a measure of efficiency. Identical results were obtained by transfection using lipid reagents (e.g. Superfect, Qiagen) and using Kc rather than S2 cells. **B**. Cells were transfected with dsRNAs corresponding to cyclin E or with a control dsRNA (lacZ), as indicated. Cells that successfully incorporated co-transfected DNA (lower panels, Gated) were marked using a plasmid that directs expression of a membrane linked GFP from the Drosophila actin promoter. This marker was chosen since the fluorescence of this fusion had been previously shown to survive fixation with ethanol (Kalejta et al., 1999). **C.** S2 cells were transfected either with a control dsRNA or with a single-stranded antisense RNA corresponding to the first 540 nucleotides of the cyclin E cDNA.

A simple model for the observed properties of RNA interference would be the induction by dsRNA of a nuclease activity that could specifically target cognate mRNAs. We therefore designed an assay to search for such an activity. S2 cells were transfected with dsRNAs corresponding to either cyclin E or lacZ, and whole-cell extracts were prepared by a simple, hypotonic lysis procedure. To test for the presence of nuclease activity, these extracts were incubated with ^{32}P-labelled, synthetic transcripts derived from either the cyclin E or the lacZ cDNAs.

Extracts prepared from cells transfected with cyclin E dsRNA efficiently degraded the cyclin E transcript; however, the lacZ transcript was stable in these lysates (Fig. 2). Conversely, lysates from cells transfected with the lacZ dsRNA degraded the lacZ transcript but left the cyclin E mRNA intact.

These results suggest that RNA interference reduces the level of target mRNAs, at least in part, through the generation of a sequence-specific nuclease activity. Although we occasionally observed possible intermediates in the degradation process (see Fig 2), the absence of stable cleavage end-products indicates an exonuclease (perhaps coupled to an endonuclease). However, it is possible that the RNAi nuclease makes an initial endonucleolytic cut and that non-specific exonucleases in the extract complete the degradation process. In

addition, our ability to create an extract that targets lacZ *in vitro* indicates that the presence of an endogenous gene is not required for the RNAi response.

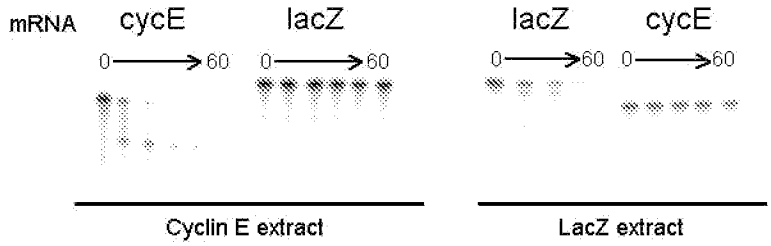

Figure 2. S2 cells were transfected with dsRNAs derived from either the cyclin E or lacZ cDNAs, as indicated. After 3 days, cells were tested for a successful dsRNA response by monitoring the cell cycle arrest induced by ablation of cyclin E. Extracts were prepared by havesting cells in 5mM EGTA/5mM EDTA. Cells were washed in PBS three times and in hypotonic lysis buffer (10 mM Hepes pH 7.3, 6 mM β-mercaptoethanol) once. Cells were disrupted by 20 strokes in a dounce homogenizer (type B pestle). The resulting lysates were centrifuged for 20 min. at 30,000xg, and supernatants were used in the degradation assay. Assays were carried out for the indicated times in a reaction buffer (20 mM hepes pH 7.3, 110 mM KOAc, 1 mM $Mg(OAc)_2$, 3 mM EGTA, 2 mM $CaCl_2$, 1 mM DTT). Samples were analyzed by electrophoresis on 8% denaturing polyacrylamide gels.

Gene silencing provoked by dsRNA is sequence-specific. A plausible mechanism for determining specificity would be incorporation of nucleic acid guide sequences into the complexes that accomplish silencing (Hamilton and Baulcombe, 1999) and reviewed in (Hammond et al., 2001; Sharp, 1999). A well-established method for testing the dependency of a process on a nucleic acid component is through use of a conditionally active nuclease (Krainer and Maniatis, 1985). The activity of micrococcal nuclease depends on Ca^{2+}. Thus, extracts can be treated with this nuclease in the presence of calcium to destroy endogenous RNA and DNA. The micrococcal nuclease can be inactivated by addition of EGTA, and the reaction in question can be carried out. Inhibition is interpreted as evidence for a nucleic acid requirement. Pre-treatment of S2 extracts with micrococcal nuclease abolished the ability of these extracts to degrade cognate mRNAs (Fig. 3A), indicating the requirement for a nucleic acid cofactor. This is likely to be an RNA since treatment of the extract with DNAse I had no effect (Fig 3A). Sequence-specific nuclease activity, however, did require protein (not shown).

One potential artifact in this type of experiment is a false-positive result that arises from the release of RNA binding proteins that could coat the substrate molecule and block access by the RNAi nuclease. In addition, micrococcal nuclease itself can bind RNA non-specifically in the absence of calcium. We therefore tested whether addition of non-specific competitor RNA following nuclease treatment could rescue activity. Neither yeast tRNA nor total S2 RNA had any effect. Considered together, our results support the possibility that the RNAi nuclease is an RNP, requiring both RNA and protein components. Biochemical fractionation (see below) is consistent with these components being associated in extract rather than being assembled on the target mRNA following its addition.

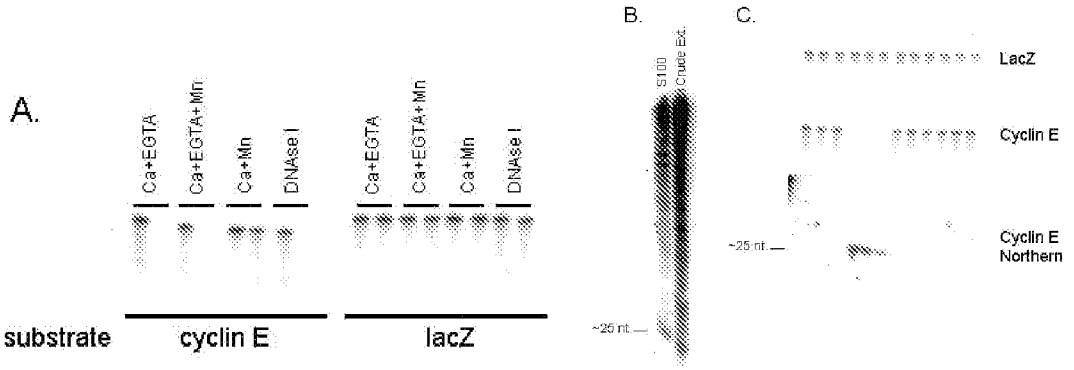

Figure 3. **A**. S2 cells were transfected with cyclin E dsRNA and extracts were prepared as described above. 30 μl aliquots of extract were treated with 60U of micrococcal nuclease (Mn, Worthington) for 30 min at 30°C in the presence of 1 mM Ca^{2+}. At the end of 30 minutes, EGTA was added to 5 mM. Controls included pretreatment with Ca^{2+} in the absence of nuclease (lanes Ca) and treatment with the nuclease in the absence of calcium (lanes Ca+EGTA+Mn). DnaseI (RQ1, Promega) was added to 2U per reaction. Following pretreatment, the aliquots of extract were incubated for 30 min with either the lacZ or cyclin E substrate as indicated. **B, C** S2 cells were transfected with cyclin E dsRNA and extracts were prepared as described above. **B**. RNA was prepared either from crude lysates or from S100 (ribosomal) pellets. This was electrophoresed on a 15% polyacrylamide/Urea gel and transferred to Hybond N+ by electroblotting. A probe was prepared by *in vitro* transcription and corresponded to the 540 nt cyclin E substrate (sense orientation). The blot was hybridized in a moderate stringency buffer (500 mM $NaPO_4$, pH 7.0, 15% Formamide, 7% SDS, 1% BSA) overnight at 45°C. Washing was at 37°C in 1X SSC. **C**. The activity was extracted from the S100 pellet with 300 mM KCl, diluted and chromatographed on a Q-sepharose column. Fractions surrounding the peak of activity are shown. The top panel demonstrates a lack of activity toward a control substrate, lacZ. The center panel shows activity toward the cognate substrate, cyclin E. The bottom panel shows a northern blot of RNAs contained in the fractions.

Data indicating the dependence of the RNAi nuclease on an essential RNA component prompted a search for the nature of the cofactor. In plants, the phenomenon of post-transcriptional gene silencing has been associated with the existence of small (~25nt) RNAs that correspond to the gene that is being silenced (Hamilton and Baulcombe, 1999). To address the possibility that a similar RNA might exist in *Drosophila* and guide the sequence-specific nuclease in the choice of substrate, we partially purified our activity and searched for co-fractionating RNAs that are homologous to the substrate.

We initially attempted to fractionate the activity by sedimentation through glycerol and sucrose density gradients. These indicated a very high molecular weight for the nuclease; however, examination of active fractions indicated that the nuclease was associated with ribosomes. This was of interest for several reasons, principle among which was the previously articulated notion that RNAi might work as a translational surveillance mechanism. However, we have not established definitively whether association with ribosomes is biologically relevant or whether our observations reflect an artifact of extract preparation.

In any case, it was necessary to dissociate the nuclease from ribosomes before any serious attempt at purification could be made. Numerous proteins that associate with ribosomes can be released by incubation at high salt concentrations (for example, reviewed in (Merrick, 1994)). Similarly, the RNAi nuclease can be quantitatively released from the ribosome fraction by incubation with 300 mM KCl. Gel filtration of the soluble nuclease indicates a size of between 200 and 500 kDa. (although this estimate is still quite crude). Chromatography of soluble nuclease over an anion exchange column (Q-sepharose) resulted in a discrete peak of activity that retained specificity since it was inactive against a heterologous mRNA.

Crude extracts contained both sequence-specific nuclease activity and abundant, heterogeneous RNAs homologous to the transfected dsRNA (Fig. 2,3). Active fractions contained a discrete RNA species of 25 nt that is homologous to the cyclin E target (Fig 3C, northern). This band is likely to represent a family of distinct RNA species since it could be detected with probes specific for both the sense and antisense cyclin E sequences and with probes derived from completely independent segments of the cyclin E dsRNA (not shown).

A manuscript describing these results was published in Nature in April, 2000 and is appended. Since the publication of this work, the use of RNAi in cultured Drosophila cells has evolved into a powerful tool that is being used broadly to investigate gene function.

Protein components of the interference process

Also with support from this grant, we have identified two of the proteins that execute RNAi in Drosophila cells. The first is a protein that catalyzes the first step, processing of long dsRNAs into 22 nt. guide RNAs. The second is a protein component of the RISC enzyme.

Dicer

Rnase III family members are the only known ribonucleases that are specifically active on dsRNAs. The genomes of worms, flies and plants contain several types of RNAseIII enzymes, including canonical RNAseIIIs and proteins that combine RNAseIII domains with other structural and functional units. We amplified the genes encoding representatives of each class of enzymes from the Drosophila genome, appended an epitope tag and expressed each protein in S2 cells. Immunoprecipitates of one family member, CG4792, could process long dsRNAs into 22mers in vitro. We raised polyclonal antisera against this protein and could immunoprecipitate activities from extracts that could also process dsRNAs into 22mers (Fig. 4A).

A. B.

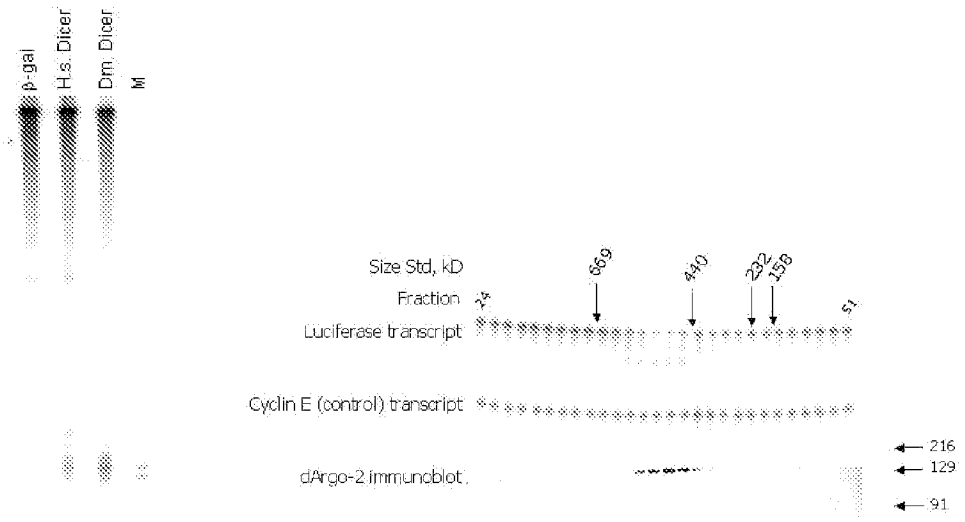

Figure 4. A. Double stranded RNA was treated in vitro with immunoaffinity purified Dicer from either human (H.s.) or Drosophila (D.M) or with a contro protein, b-galactosidase. Dicer proteins produce siRNAs of ~22nt. B. Fractionation of the RNAi effector complex, RISC, (as measured by its ability to degrade a cognate substrate, upper two panels) on a sizing matrix reveals a size

of ~500 KDa. Immunoblotting with the Argonaute2 antibody shows that this protein co-purifies with activity (lower panel).

CG4792 was renamed Dicer to reflect its ability to cleave dsRNA into small, uniform pieces. The Dicer gene encodes an enzyme of ~210 kDa, in agreement with the predicted size of this protein based upon column chromatography. The protein is conserved throughout evolution, with representatives in C. elegans (K12H4.8), Arabidopsis (CAF and others), Neurospora, S. pombe, and mammals. No Dicer protein can be identified in S. cerevisiae. We have demonstrated that the Dicer protein from human cells has biochemical activities analogous to those of Drosophila Dicer. All of these proteins share a characteristic arrangement of domains. All have an amino-terminal helicase domain, followed by a PAZ domain (see below). The catalytic portion resides in the c-terminus and comprises dual RNAseIII domains and one or more double-stranded RNA binding motifs. Of interest, the Dicer protein requires ATP for cleavage, both in extracts and in immunoaffinity purified material. This unusual requirement for a nuclease may reflect a mechanism of action that requires the helicase domain for translocation along its substrate and processive processing.

Reduction of Dicer activity by RNAi in S2 cells compromises the ability of these cells to silence genes in response to dsRNA. However, to demonstrate more definitively that Dicer plays a role in RNAi, we collaborated with Ronald Plasterk's laboratory in Holland and to identify a Dicer-mutant C. elegans. Indeed, this worm shows a defect in germline silencing of a GFP transgene in response to exogenous dsRNA. The worm also had a number of other interesting features that led to the discovery that RNAi regulates developmental timing.

A manuscript describing much of the above was published in Nature this January (2001) and is appended.

Argonaute

We have also pursued the protein machinery, which catalyzes the effector step of RNAi. Based upon a specific mRNA degradation assay, we purified the RISC complex to near homogeneity. Several proteins ranging in size from ~80 to ~200 kDa cofractionated with RISC. Protein microsequencing of two bands identified a Drosophila member of the Argonaute gene family as one of the co-fractionating bands. Of course, this was of immediate interest since members of the Argonaute family have been tied to RNAi through genetics in C. elegans (rde-1), Neurospora (qde-2) and Arabidopsis (Ago1) (reviewed in (Hammond et al., 2001)).

We raised a polyclonal anti-peptide antibody to the Drosophila Ago-2 protein and have demonstrated that this protein co-fractionates with RISC activity through 5 purification steps (Fig 4B). Furthermore, we have used a tagged version of the Argonaute protein to specifically recover the RISC complex, including 22 nt. siRNAs. Based upon this evidence, we feel quite confident that the Drosophila Argonaute 2 protein is a component of the effector nuclease.

Of interest, we have recently found that a portion of the Ago2 protein is associated with Dicer in cell extracts. We previously showed that Dicer and RISC are biochemically separable; however, we hypothesized that Dicer and RISC might associate at some point in the interference process. Although we have yet to definitively rule-out Dicer as a component of RISC, our working model is that transient association of Dicer and Argonaute proteins facilitates transfer of siRNAs into the RISC enzyme.

A manuscript describing this work (Hammond et al.) is in press at Science and is appended.

RNAi in mammals

Our experiments have suggested that the biochemical machinery of RNAi is conserved in mammals. Furthermore, several groups have reported specific interference by dsRNA in early mammalian embryos (Svoboda et al., 2000; Wianny and Zernicka-Goetz, 2000). We therefore tested whether embryonic, pluripotent murine cells might have an RNAi-like response.

We surveyed a number of cell lines of embryonic origin for the degree to which non-specific suppression of gene expression occurred upon introduction of dsRNA. As an assay, we tested the effects of non-specific dsRNA on the expression of GFP as measured *in situ* by counting fluorescent cells. As expected, in both human embryonic kidney cells (293) and mouse embryo fibroblasts GFP expression was virtually eliminated irrespective of the sequence of the co-transfected dsRNA (not shown). In some pluripotent teratocarcinoma and teratoma cell lines (e.g., N-Tera1, F9) the PKR response was attenuated but still evident (not shown); however, in striking contrast, transfection of non-specific dsRNAs had no effect on the expression of reporter genes either in mouse ES cells (not shown) or in p19 embryonal carcinoma cells, (Figure 5).

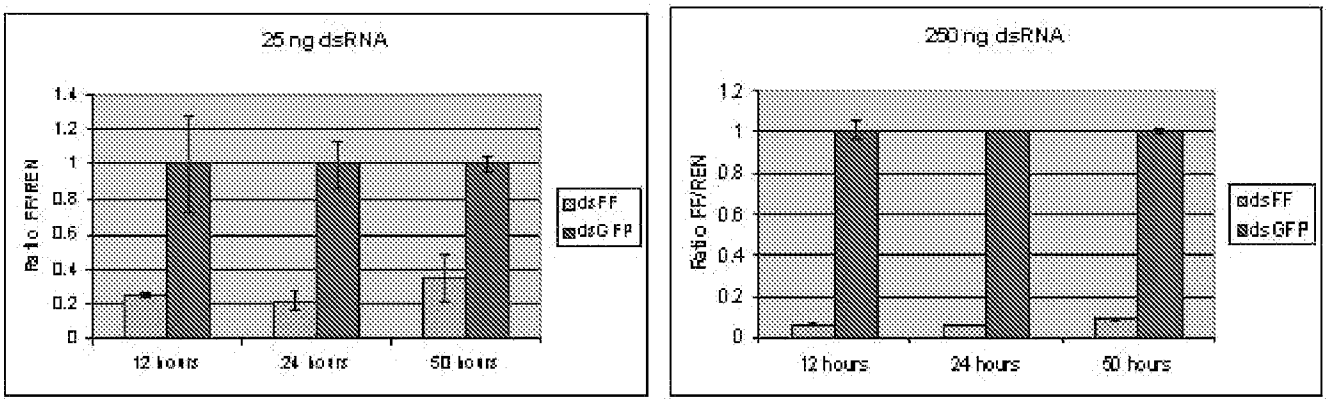

Figure 5. RNAi of firefly and *Renilla* luciferase in P19 cells. A. P19 cells transfected with plasmids that direct the expression of firefly and *Renilla* luciferases and dsRNAs 500mers (25 or 250ng, as indicated), that were homologous to either firefly luciferase mRNA (dsFF) or non-homologous (dsGFP). Luciferase activity were assayed at various times after transfection, as indicated. Ratios of firefly to renilla activity are normalized to dsGFP controls.

Transfection of P19 embryonal carcinoma cells with GFP in the present of cognate dsRNA corresponding to the first ~500 nucleotides of the GFP coding sequence had a strikingly different effect. GFP expression was eliminated in the vast majority of co-transfected cells, suggesting that these cultured murine cells might respond to dsRNA in a manner similar to that which we had previously demonstrated in cultured, Drosophila S2 cells (Hammond et al., 2000).

To quantify the extent to which dsRNA could induce sequence-specific gene silencing, we used a dual luciferase reporter assay similar to that which had first been used to demonstrate RNAi in Drosophila embryo extracts (Tuschl et al., 1999). P19 EC cells were transfected with a mixture of two plasmids that individually direct the expression of firefly luciferase and *Renilla* luciferase. These were co-transfected with no dsRNA, with dsRNA that

corresponds to the first ~500 nucleotides of the firefly luciferase or with dsRNA corresponding to the first ~500 nucleotides of GFP as a control. Co-transfection with GFP dsRNA gave luciferase activities that were similar to the no-dsRNA control, both in the firefly/renilla activity ratio and in the absolute values of both activities. In contrast, in cells that received the firefly luciferase dsRNA, the ratio of firefly to *Renilla* luciferase activity was reduced by up to 30-fold (250 ng, Figure 5). For comparison, we carried out an identical set of experiments in Drosophila S2 cells. Although qualitatively similar results were obtained, the silencing response was more potent. At equivalent levels of dsRNA, S2 cells suppressed firefly luciferase activity to virtually background levels (not shown).

The complementary experiment, in which dsRNA was homologous to *Renilla* luciferase, was also performed. Again, in this case, suppression of the expression of the *Renilla* enyzme was approximately 10-fold (not shown). Thus, the dsRNA response in P19 cells was flexible, and the silencing machinery was able to adapt to dsRNAs directed against any of the reporters that were tested.

We took two approaches to test whether this response was specific for dsRNA. Pre-treatment of the trigger with purified RNAse III, a dsRNA-specific ribonuclease, prior to transfection greatly reduced its ability to provoke silencing (not shown). Finally, transfection of cells with single-stranded antisense RNAs directed against either firefly or *Renilla* luciferase, had little or no effect on expression of the reporters. Considered together, these results provided a strong indication that double-stranded RNAs provoke a potent and specific silencing response in P19 embryonal carcinoma cells.

Efficient silencing could be provoked with relatively low concentrations of dsRNA (25 ng/ml of culture media; Figure 5). The response was concentration-dependent with maximal suppression of ~20-fold being achieved at a dose of 1.5 μg/ml of culture media (Figure 5)

Silencing was established rapidly and was evident by 12 hours post-transfection (the earliest time point examined). Furthermore, the response persisted without significant changes in the degree of suppression for up to 72 hours.

To assess whether the presence of a sequence-specific response to dsRNA was a peculiarity of P19 cells or whether it also extended to normal murine embryonic cells, we performed similar silencing assays in mouse embryonic stem cells. Co-transfection of ES cells with non-cognate dsRNAs (e.g. GFP), again, had no dramatic effect on either the absolute values or the ratios of *Renilla* and firefly luciferase activity (not shown). However, transfection with *Renilla* luciferase dsRNA dramatically suppressed *Renilla* luciferase expression.

A key feature of RNAi is that it exerts its effect at the post-transcriptional level by destruction of targeted mRNAs (reviewed in (Hammond et al., 2001)). To test whether dsRNAs induced silencing in mouse cells via post-transcriptional mechanisms, we used an assay identical to that, which was used initially to characterize RNAi responses in *Drosophila* embyo (Tuschl et al., 1999). We prepared lysates from P19 EC cells that were competent for *in vitro* translation of capped mRNAs corresponding to *Renilla* and firefly luciferase. Addition of non-specific dsRNAs to these extracts had no dramatic effect on either the absolute amount of luciferase expression or on the ratio of firefly to *Renilla* luciferase. In contrast, addition of dsRNA homologous to the firefly luciferase induced a dramatic and dose-dependent suppression of activity. Addition of RNA corresponding to only the antisense strand of the

dsRNA had no effect, comparable to a non-specific dsRNA control, and pre-treatment of the dsRNA silencing trigger with RNAse III greatly reduced its potential to induce silencing *in vitro*. Considered together, these results suggest that dsRNA can elicit a post-transcriptional gene silencing response in extract from mouse P19 cells.

Our results raise the possibility that, as in several model systems, RNAi might eventually be harnessed as a tool for probing gene function in mammalian cells. To date, suppression of gene expression is more complete in Drosophila cells than in mouse cells. Furthermore, it is as yet unclear whether RNAi in mammals will suffer from specificity problems similar to those that have been postulated for antisense RNAs. However, the finding that dsRNAs have sequence-specific silencing activity in pluripotent, embryonic murine cells could ultimately ignite a revolution in somatic cell genetics and in the methodologies used for engineering loss-of-function mutations in whole animals. It is this goal that the present proposal is designed to address.

Research Plan

Introduction

This submission is a revised application. The first phase II submission resulting from this Phase I grant was focused on the development of technologies for identifying genes necessary for the survival of human cancer cells. The reception for this proposal was lukewarm. Based upon the criticisms of the referees, we have completely changed both the focus and the substance of our Phase II submission.

The initial submission of this application followed very closely the receipt of funding for the Phase I proposal, and therefore reflected mainly progress that had been made during the review of the Phase I application and during the very early stages of the Phase I grant. During the course of pursuing the Phase I goals, and subsequent to the last submission, we have substantially changed the focus of our efforts. As detailed above, in the Phase I final report, Scott Hammond, then a postdoc and now a Staff Scientist at Genetica, joined the laboratory of Greg Hannon (one of the company's founders) as a Visiting Scientist. Through close collaborations between the Company and Dr. Hannon's group at CSHL, we have made substantial progress both toward understanding the mechanism of RNA interference and toward developing this biological phenomenon as a tool in several organisms. This research has now matured to a point at which a large-scale effort toward developing RNAi-based methodologies for stably manipulating gene expression in mammalian cells is warranted.

We now present a Phase II application that is completely altered from its last incarnation. It is, therefore, not relevant to respond to the criticisms of the prior referees, except to say that their lack of enthusiasm was taken to heart and that this revision follows the spirit of their recommendations. It is also worth noting that the goals of the original Phase I application – to devise methodologies for genetically manipulating tumor cell -- are much better reflected by the Specific Aims of the present Phase II proposal.

Experimental Procedures

<u>Aim 1. Creation of stable, loss-of-function mutations in embryonic cells using RNAi.</u>

As described above, RNAi can be induced in embryonic cells by transfection with dsRNAs of approximately 500 nt in length. In both embryonic and differentiated cell types, the 22 nt siRNAs/guide RNAs (the product of the first step of the RNAi pathway) can induce silencing (Elbashir et al., 2001). However, in both cases the effect is transient. Using long dsRNAs in P19 embryonal carcinoma cells or ES cells, we have achieved silencing that persists for approximately 3 days. In multiple cell systems, we have found that the duration of silencing with siRNAs is much shorter. Using synthetic 21mers, we find that suppression peaks at approximately 12 hours post-transfection and decays thereafter, reaching undetectable levels in most cell lines by 48 hours (our unpublished results).

This lack of a persistant effect reduces the utility of the presently available approach. Since RNAi operates on the mRNA, the development of a phenotype requires the natural decay of the encoded protein product. Thus, the given the limited time frame of the RNAi effect, this approach will be suitable only for the study of unstable proteins. Furthermore, many of the phenotypes, which are desirable to study, do not develop within a two-day time period. Thus, the potential limitations on the use of transient RNAi strongly argue for the development of strategies, which use dsRNA-induced silencing to create stable, loss-of-function in cultured cells and in animals. Given our documented successes in using RNAi in embryonic cell types, we will first focus on the creation of stable RNAi in these contexts. We have chosen to approach this goal by encoding dsRNA in the form of an inverted repeat or hairpin that can be expressed from a promoter of choice.

The strategy of inducing RNAi by hairpin expression has been successful in a number of systems including C. elegans, Drosophila and trypanosomes (reviewed in (Hammond et al., 2001)). However, in each case that has been reported in the literature, it is clear that one bottleneck is construction of the hairpin itself. In one case, it was reported that over 1000 bacterial colonies had to be screened in order to identify an un-rearranged clone. We have devised a methodology that simplifies hairpin cloning, and will use this approach throughout the experiments proposed in this application.

We have achieved the goal of simplified hairpin construction by dividing the process into two steps (Fig. 6). In the first step, we create a direct repeat by conventional cloning in a vector specifically designed for hairpin generation. The vector has been constructed such that the each of the repeats derived from a targeted gene flanks a selectable marker. This configuration promotes stability by selecting against recombination between the repeated sequences. Furthermore, the 3' repeat is flanked by loxP sites in a head-to-head configuration. Treatment with CRE recombinase inverts the sequences within the loxP sites to create a mixture of direct and inverted repeats. These are then distinguished by analysis of individual clones following transformation into E. coli.

A. B.

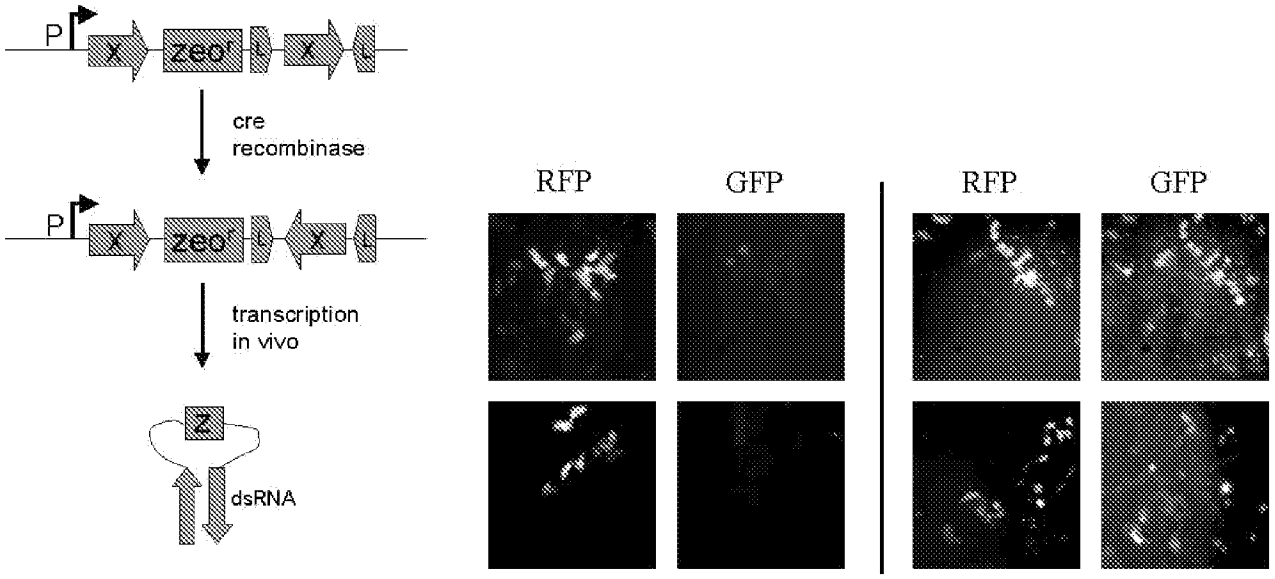

Figure 6. A. Strategy for the creation of hairpin RNAs for stable expression of dsRNA. B. P19 EC cells were stably transfected with a construct that directs expression of GFP dsRNA (left panels) or an unrelated control (right panels). Individual colonies expressing dsRNA were transfected with a combination of RFP (to identify transfected cells) and GFP (to monitor RNAi). In cells expressing GFP dsRNA, GFP expression was suppressed (left) but RFP expression was unaffected.

The instability of inverted repeats in E. coli derives from existence of a system, the SBC system, which recognizes and cleaves cruciform DNA (Connelly and Leach, 1996). We therefore took the additional step of performing all manipulations of inverted repeat constructs in sbcCD mutant cells (DL759). This not only simplifies recovery of products of the CRE reaction but also permits routine amplification of inverted repeat vectors without rearrangement.

As a test of whether inverted repeats could be used to create a stable RNAi phenotype in embryonic cells, we created a plasmid in which a ~500 nt inverted repeat was transcribed by the CMV promoter in the plasmid, pCDNA3. This was used to stably transfect p19EC cells, and 50 individual colonies were expanded following selection with G418. To test whether we had provoked stable silencing of GFP in any of these clones, they were transiently transfected with a mixture of plasmids encoding GFP and RFP, a red fluorescent protein that share no homology to GFP. In the case of stable silencing, the predicted result is that cells would be competent for RFP expression but would be unable to express GFP.

As shown in Figure 6, expression of a GFP hairpin RNA induced stable silencing of an exogenous GFP reporter in P19 cells. Approximately 50% of the clones, that were analyzed, showed highly effective silencing. The remainder appeared to have silenced to lesser degrees; however, these have not yet been investigated in detail.

Based upon such preliminary experiments, we feel that it is quite likely that we can achieve stable silencing using RNAi in embryonic cell types. However, two questions must be answered in the short term. First, we must determine if the mechanism of silencing is truly RNAi. Second, we must determine whether we can use stably expressed hairpins to silence endogenous genes.

RNAi and related processes have two key signatures. First, silencing is correlated with the production of small, ~22nt RNAs. Second, silencing occurs at the post-transcriptional level. This effector step occurs via the degradation of targeted mRNAs by a multicomponent nuclease, RISC, that incorporates the small RNAs as a guide to substrate selection. It should be noted, that we have contributed substantially to understanding the mechanism of RNAi (see Phase I final report and Appendicies). To determine the mechanism underlying dsRNA-induced silencing in mammalian cells, we will follow procedures quite similar to those that we have pioneered in Drosophila cells.

As stated above, the utility of RNAi in numerous model systems has come from the ability to stably silence endogenous genes. To extend our positive preliminary results with GFP to endogenous loci, we will prepare hairpin RNAs against two endogenous genes for which exists a positive selection for loss-of-function. One is HPRT, and the other is TK. Hypoxanthine-guanine Phosphoribosyl Transferase is a key enzyme in the production of thymidine via the salvage pathway. Treatment of HPRT+ cells with 6-thio-guanine causes incorporation of this toxic nucleoside into cellular DNA, and the resultant DNA damage induces cell death. Cells lacking HPRT activity are resistant to 6-TG (Wahl et al., 1975). Similarly, culture of cells that contain thymidine kinase (TK) activity in media containing any of a number of thymidine analogs (e.g. triflurothymidine) causes persistent DNA damage and cell death. It is, again, well documented that TK-/- cells are resistant to these compounds.

We will construct inverted repeat constructs comprising the first 500 nt of the HPRT and TK coding regions in pcDNA3, just as was done for GFP. These will be transfected into P19EC and ES cells, and G418 resistant colonies will be selected and expanded. For each, we will perform drug sensitivity curves, comparing to cells transfected with empty vector controls and to cells transfected with other hairpin constructs (e.g. GFP). Of course, we could test the efficacy of our approach also by assessing drug resistance in the primary transfected population or in pools of G418-resistant cells. However, we feel that more quantitative information will be gained from detailed studies of single cell clones.

In each case, we will correlate resistance to levels of expression from the hairpin constructs and to the abundance of ~22nt guide RNAs, if we find that hairpins are silencing through a conventional RNAi mechanism.

Should these experiments prove successful, we will test efficacy on a broader range of genes, including those involved in cancer development (e.g., p53, mdm2, p16, Rb etc). Studies in mouse embryos and in embryonic cell lines predict, for example, that loss of p53 will protect cells from the lethal effects of mdm2 disruption (Montes de Oca Luna et al., 1995). Recapitulating such a genetic epistasis will be an important step along the road to testing stable RNAi and a tool for investigating biological function.

Should these experiments succeed, it will be important to optimize the method of delivering the dsRNA. We envision a number of possibilities, including alternative delivery vehicles and expression from alternative promoters. It will also be critical to determine optimal parameters for suppression with respect to the configuration of the hairpin, including optimal length, placement within the mRNA sequence and degree of homology.

Genetica has made substantial efforts in the design and implementation of genetic systems based upon retroviral gene transfer. Therefore incorporation of RNAi triggers into our

existing retroviral platforms would be highly desirable. We will, of course, test the possibility that hairpin expression cassettes can be incorporated directly into retroviral vectors; however, we envision at least one potential problem. It is well established that extended secondary structures inhibit reverse transcription. Therefore, we feel that a 500 nt inverted repeat is likely to reduce retroviral replication and thus infection to almost undetectable levels. We will therefore test a modification of the strategy outlined above for the cloning of hairpin constructs for the expression of hairpins from retroviruses in vivo. Retrovirus vectors will be produced that carry direct repeats of the sequence to be suppressed. As described above, the 3' repeat will be flanked by loxP sites. Following integration into the genome, exposure to CRE recombinase will invert the 3' sequence leading to the production of a hairpin (at least in some percentage of cases, the maximal frequency being 50%). Cre can be delivered transiently either by transfection or through the use of a membrane permeable version of CRE (e.g. tat-CRE fusions).

We will begin by testing this strategy using positively selectable genes such as TK and HPRT; however, if we find this strategy to be workable, we will devise methodologies to positively select cells in which the 3' portion of the repeat has been inverted. Any number of modes, in which inversion activates a selectable marker (e.g. cell surface, fluorescent, or drug resistance) can be easily envisioned.

It will also be critical to determine whether transcription of the inverted repeat into a conventional mRNA transcript that is polyadenylated and capped is the most efficacious delivery method. It has previously been shown that transcription of antisense RNAs by either snRNA promoters (e.g., U1 or U6) or certain RNA polmyerase III promoters (e.g. VA1 RNA from adenovirus) produces greater effects than does transcription from mRNA promoters (reviewed in (Castanotto et al., 2000)). We have access to all necessary reagents to produce U1, U6 and VA-based expression vectors for delivery of hairpins, and we anticipate testing all of these strategies.

Of course, one can envision numerous combinations of promoter strategies and delivery methods, and these will be tested and created as the need arises.

Summary

The experiments proposed in this specific aim are designed to validate the use of RNAi to stably suppress gene expression in embryonic cells. The availability of such technology is not only a requisite precursor to the experiments contemplated in other cell types, below, but will open numerous doors to the determination of gene function through the study of loss-of-function phenotypes in cultured mammalian cells.

Aim 2. Creation of stable loss-of-function mutations in non-embyronic cell types.

Clearly, the development of strategies that permit the creation of stable loss-of-function mutations in embryonic cell types would permit a wide range of biological analyses that are presently difficult, if not impossible. However, our ultimate goal is to devise strategies that will permit the use of RNAi to suppress gene function in essentially any cell line, cell type, or cell of an intact organism.

Currently, the most significant barrier to achieving this goal is the PKR response. It is very likely the absence of such a response from embryonic cell types, such as P19 and ES cells, that has permitted the creation of stable loss-of-function phenotypes in the studies that we have executed thus far. Therefore, our goal is to devise strategies for presentation of the dsRNA trigger that allow it to elude PKR surveillance.

Expression Strategies

Triggering the PKR response depends upon three factors. First is the length of the dsRNA. PKR requires approximately 30 bp of contiguous double-stranded sequence to trigger dimerization and activation of the enzyme. Second is concentration. The PKR system evolved to respond to the high-level dsRNA production that might occur during viral infection. Third is subcellular localization. PKR is a cytoplasmic surveillance system, and dsRNA must appear in that compartment to trigger the response.

PKR is invariably triggered by transfection of cells with long dsRNAs (~500 bp). However, it is possible that presentation of long dsRNAs from endogenous promoters may evade the PKR response, either because such RNAs may be sequestered (e.g., in the nucleus) or because they may not reach sufficient cytoplasmic concentrations to trigger the response.

Building upon the work outlined in Aim 1, we can use the variety of expression vectors that will be constructed for optimization of RNAi in embryonic cell types to test whether these can induce RNAi without PKR in differentiated cell types. To begin, we will transfect NIH 3T3, 293, HeLa, U2OS, Rat 1 and C2C12 cells with expression vectors that direct expression of a 500 nt. hairpin corresponding to either the TK or HPRT genes, depending upon the outcome of experiments described above. Initially, transient transfections will be used to assess the ability of these constructs to trigger PKR, although we recognize that such studies may not reflect the responses that are triggered (or are not triggered) following stable integration into the genome. The pCDNA3 series of vectors will likely direct production of capped, polyadenylated RNA that is exported to the cytoplasm, unless the double-stranded nature of the transcript alters its localization. The series of plasmids that incorporate the U1 and U6 promoters will produce largely nuclear products. Although snRNAs are exported to the cytoplasm for assembly into snRNP particles, we will have removed the determinants of this active transport process from our expression vectors. Thus, a likely outcome of these studies is that CMV-driven hairpins may trigger PKR, should they achieve sufficient concentrations of dsRNA, and U1 and U6-driven constructs may fail to initiate a response, if transcripts are sufficiently confined to the nucleus.

It is not yet well established where in the cell the RNAi machinery resides. In plants, the response is at least partially cytoplasmic since PTGS suppresses RNA viruses that never enter the nuclear compartment. However, the initiator of the RNAi response, the Dicer enzyme contains a nuclear localization signal and appears to be at least partially nuclear by immunofluorescence (not shown). Therefore, it is likely that the nucleus will contain a sufficient complement of RNAi machinery to initiate the response, although this supposition has yet to be proved.

One potential caveat to the proposed experiments, is that in the process of selecting cell that express dsRNA hairpins, we may select a sub-population that tolerates hairpin expression for any of several reasons. For example, we could select cells that have secondary mutations. Alternatively, we could select cells, which have integrated expression constructs in such a way

that expression is restricted to low levels. In the former case, selection for such rare mutants could affect later phenotypic analysis. In the latter case, such a selection may benefit our studies or it may give a false-negative result, if the counter-selection against high expression levels reduces dsRNA to a point that is insufficient to trigger RNAi. Therefore, close attention will be paid to the rate at which stable transformants are isolated and to the expression levels of dsRNA that are achieved in these cells.

Should these studies succeed, the way forward is quite clear. We will exploit long dsRNAs as triggers of silencing for probing gene function in human cancer cells. One example of how this technology may be applied arose from the original submission of this Phase II application. We proposed the development of an improved secretion trap methodology for identifying secreted and cell surface proteins in tumor cells. This effort has moved forward to the point that Genetica now has a quite large database of trafficked proteins from breast cancer cells, and work with other tumors types is proceeding rapidly. In part, the goal is to develop these as potential diagnostics. However, we are also acutely interested in whether any of these proteins are specifically essential to the growth and survival of breast cancer cells since they may then be viable targets for therapeutic intervention. Stable RNAi strategies that are developed with the support of this application will be used to address this question both in cell culture and in xenograft models.

Short RNA hairpins

In most systems studied to date, including C. elegans, Drosophila, and plants, short RNAs are much less effective at triggering silencing than are long RNAs (reviewed in (Hammond et al., 2001)). For this reason, we have focused, so far, on the use of long dsRNAs as silencing triggers. However, it is clear that in both worms and plants, silencing can be triggered by dsRNAs as short as 28 nt (Parrish et al., 2000). Furthermore, Tuschl and colleagues have recently shown that short, synthetic RNAs that mimic our Dicer products can induce silencing upon transient delivery to numerous mammalian cell types (Elbashir et al., 2001). Of interest, all of these RNAs are below the cut-off for triggering PKR. We will therefore investigate whether the expression of short RNA hairpins can be used to induce efficient silencing.

These studies will proceed similarly to those described above. Short synthetic hairpins directed against GFP, TK and HPRT will be expressed from CMV, U1 and U6 promoter vectors in the cell types noted above. In preliminary studies, we will assess the ability of these RNAs delivered transiently (either as synthetic RNAs or via the expression vectors) to trigger the PKR response. Measurements will be made by assessing non-specific suppression of marker genes (e.g. luc) and by monitoring phosphorylation status of eIF2α.

One potential complication of such studies is that we have constructed hairpins, thus far, that contain the zeocin resistance gene as an ~350 bp loop. This has been neutral in the case of ~500 nt hairpins but is likely to be less so in the case of short hairpins. We must therefore modify our strategy to create hairpins with significantly shorter loops. There are numerous potential strategies to address this problem; however, none may involve removal of the loop by splicing, as is done in plant systems (Smith et al., 2000), since we plan on using non-mRNA promoters as potential expression vehicles. Instead, we intend to simply clone short hairpin sequences either as single, synthetic DNA fragments, or in two steps if hairpin formation in such synthetic oligonucleotides competes too vigorously with intermolecular hybridization to produce

clonable fragments. If we encounter problems with stability, we will disrupt DNA-DNA interactions without compromising RNA-RNA interactions by incorporation into the hairpins the ability to form G-U basepairs with target RNAs, which will, of course, not form in the DNA.

Retargeting a natural, endogenous trigger of RNAi

In plants, disruption of some genes that are involved in RNAi (e.g., argonaute, carpel factory) induces either embryo lethality or developmental abnormalities, depending upon the strength of the mutation (reviewed in (Hammond et al., 2001)). This was a strong suggestion that RNAi might regulate the expression of endogenous, protein coding genes. This hypothesis was recently confirmed by work from our group (specifically, our close collaborators at CSHL) and from two other laboratories (Grishok et al., 2001; Hutvagner et al., 2001).

Worms carrying mutations in the Dicer gene are indeed compromised for RNAi; however, they showed additional phenotypes that indicated a role for this enzyme in regulating developmental timing. Ultimately, the basis of this defect was identified as a lack of processing of small temoporal RNAs, such as let-7 and lin-4, which control the timing of developmental events in metazoans.

The let-7 RNA is a small, ~21 nt RNA that binds to and controls translation of several target genes. This mode of control creates coordinate regulation of numerous proteins that regulate developmental timing through the production of a single RNA molecule. The let-7 RNA is produced from a longer precursor of ~75 nt that forms a hairpin RNA. This hairpin is processed into mature let-7 RNA by Dicer (Grishok et al., 2001; Hutvagner et al., 2001), Ketting et al., submitted), and other elements of the RNAi machinery (e.g., argonaute) are required for let-7 to regulate its target genes (Grishok et al., 2001). This strongly suggests that the let-7 precursor is a natural, endogenous trigger of RNAi.

We propose to test the possibility that we may re-target let-7 at will by changing the composition of the let-7 hairpin. Comparative analysis of let-7 RNAs from worm, mammals and Drosophila show a conserved secondary structure that is an ~28 nt hairpin interrupted by a bulge. Indeed such helices are excellent substrates for the Dicer enzyme, being processed with disproportionate efficiency as compared to ~28 nt. perfect helices (our unpublished results).

We will begin by creating retargeted let-7 RNAs by in vitro transcription and testing the ability of these RNAs to suppress target genes by transient transfection into numerous cell types. Success in these experiments will prompt our proceeding to expression of let-7 RNAs for stable suppression.

Since the goal is to express the precise let-7 precursor, we cannot simply insert our retargeted let-7 genes into CMV vectors. Instead, we will take several independent strategies to synthesis of retargeted let-7 within a cell. The first will take advantage of the ability of T7 RNA polymerase to function in vivo in mammalian cells. Since this polymerase can be made to both initiate and terminate precisely, it may provide an excellent vehicle for let-7 production. As an alternative, we may use the U1 or U6 snRNA promoters, and use cis-linked hammerhead ribozymes to create the appropriate 3'end. Finally, we have begun to map the endogenous let-7 promoter. In both mouse and human genomes, let-7 genes are arranged in tandem, with ~200 nt. separating the expressed portions of the genes. This makes it extremely likely that the sequences required for let-7 expression lie within these boundaries. We propose to isolate and

alter a let-7 locus to retarget the let-7 product and use the endogenous promoter to present the regulatory RNA. Of course, this promoter is developmentally regulated, and this strategy may initially restrict utility. However, let-7 is expressed in most adult cell types, and any use of this promoter should allow broad, if not universal, application.

Summary

In this aim, we propose to extend the use of RNAi to create stable- loss-of-function mutations from embryonic to differentiated, adult cell types. Success in this aim would revolutionize the way in which we can approach investigation of biological function in mammalian cells, leading ultimately to efforts to assess the consequences of knocking-out every gene in the human and mouse genomes. Furthermore, the ability to establish RNAi without activation of the PKR response would permit the production of loss-of-function mutations in any model mammalian system in which transgenesis has been established.

Aim 3. Execution of genetic screens using single cells

As described above, the primary goal of Genetica is to develop technologies that permit the application of well-established genetic approaches to the discovery and validation of therapeutic targets in cultured mammalian cells. Our progress, so far, is embodied in the design, validation and implementation of the MaRX system. This is an optimized suite of retroviral vectors and packaging cell lines designed to enable the delivery of complex cDNA libraries to cultured cells, the selection of cells that display a particular phenotype, the recovery of the integrated virus and the re-infection of a second round of recipient cells for verification of phenotype, all without subcloning (see Figure 7). This system has been used in numerous genetic screens, however, two pieces of the puzzle have been missing. First is the ability to create effective loss-of-function phenotypes, and this problem is addressed by Aims 1 and 2. Second is the need to recover integrated viruses from relatively large cell numbers.

A. **B.**

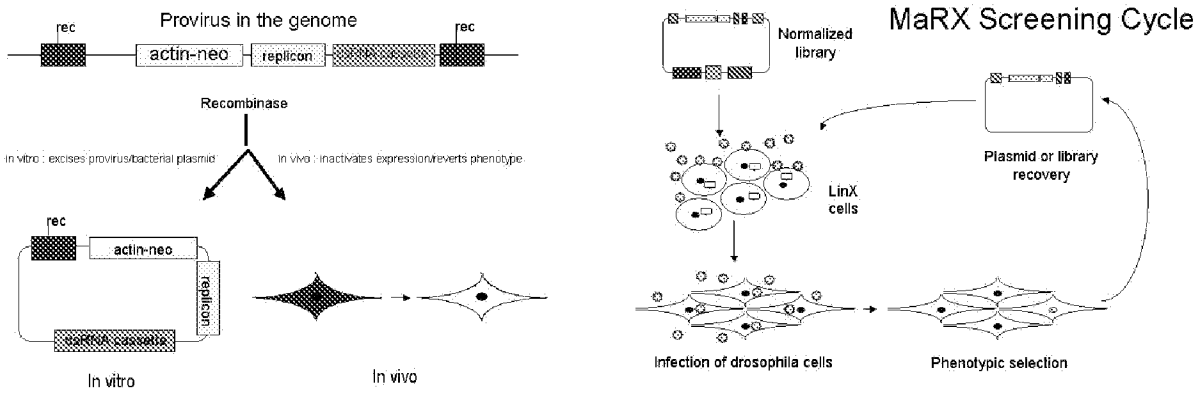

Figure 7. The MaRX cycle. We have created a retroviral gene transfer system that allows the isolation of genes from cDNA libraries based upon functional screens in cultured cells. A. Schematic diagram of a MaRX provirus. Inclusion of recombinase sites in the LTR allow excision and recovery of the provirus from the genome of infected cells. The excised plasmid can not only be propagated in bacteria but also be used to create infectious virus without intervening cloning steps. B. A schematic of the screening cycle. Plasmid libraries can be converted into high-titer retrovirus in LinX packaging cells. These are used to infect recipient cells. Infected cells are selected based upon the desired phenotype and the gene that presumably conferred the phenotype is rescued by in vitro excision. Recovered plasmids can be used in further rounds of screening or for confirmation of results.

To date, all of the genetic screens that we, and others, have performed in cultured mammalian cells have relied on the ability to recover relevant genetic elements from relatively large numbers of pooled genomes. Our goal is to devise methodologies that will permit recovery of a relevant genetic element from the genome of a single isolated cell. This will radically alter the types of loss-of-function screens that can be carried out using the aforementioned RNAi approaches. For example, we could perform screens aimed at inducing the differentiation or growth arrest of cancer cells, isolating cells with the appropriate phenotype using FACS (Genetica has dedicated significant effort to this type of selection using a Mo-Flo cytometer). The inducer of this phenotype could be recovered from each, individual cell using the procedures outlined below to identify potential therapeutic targets. Similar selections could be applied in synthetic lethality screens, in which the identification of apoptotic cells is the desired outcome. Combining single-cell genetics with RNAi-based loss-of-function approaches would represent a tremendous advance in our ability to harness the power of genetics for the discovery of potential therapeutic targets.

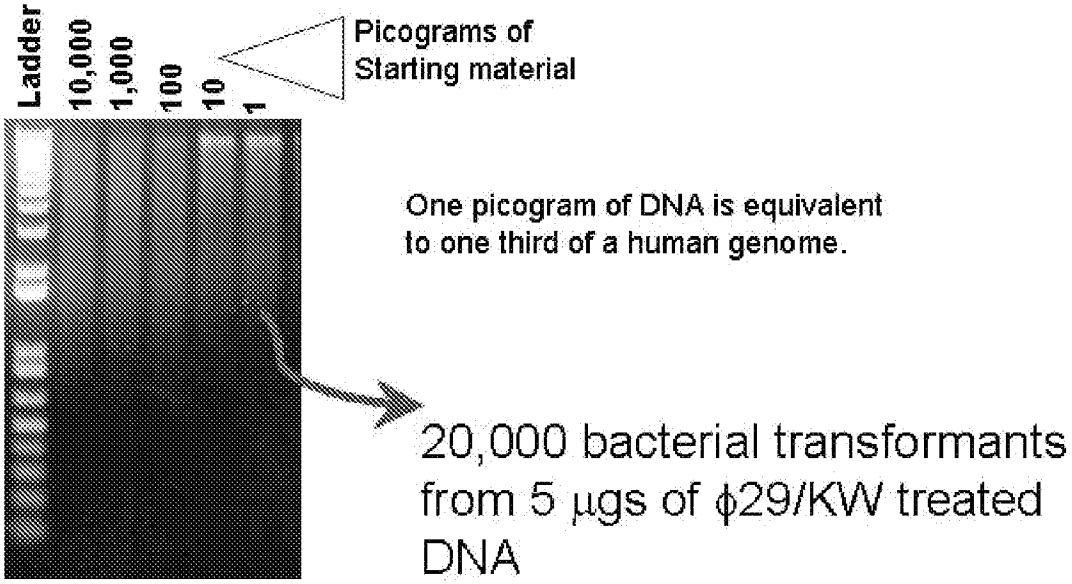

Figure 8. Recovery of MaRX from the equivalent of one mammalian genome.

Inducers of RNAi will be delivered to cells using the MaRX retroviral system. We have already used this system for induction of RNAi in Drosophila cells using a version of MaRX (pRIP) that is customized for insect cell expression. We are presently constructing the mammalian counterpart to this vector. Two approaches to the recovery of MaRX viruses from single cells can be envisioned. First is excision of the virus with CRE recombinase and amplification in vivo; however, we do not wish to limit the output of our screen to replication-competent, or for that matter, even viable cells. Second is to cause selective and accurate amplification of viral sequences in vitro. We have chosen the second approach.

Amplification in vitro could be carried out in any of several ways. Conventional PCR could be used to amplify relevant DNA fragments, but an integral aspect of the MaRX system is that all steps from the infection of the initial recipient cell to subsequent rounds of re-testing of

putative positive viruses is done without subcloning. A second possibility has emerged from recent work with rolling circle amplification.

Polymerases that replicate via a rolling circle mechanism offer the advantage of not requiring a thermal cycling step (Dean et al., 2001). In nature many circular DNA molecules such as plasmids or viruses replicate in this manner. In the laboratory the prolonged extension of an oligonucleotide primer annealed to a circular DNA template proceeds via linear rolling circle amplification (RCA) (Fire and Xu, 1995; Lizardi et al., 1998). The circle is replicated continuously as tandom copies are synthesized and displaced. Exponential amplification can be achieved by using a specific primer for each strand (Lizardi et al., 1998).

Recently, the ϕ29 DNA polymerase has been exploited to perform multiply-primed RCA (Dean et al., 2001). In this procedure random hexamers are used as primers on circular DNA templates which can then be amplified 10,000-fold in a few hours. This represents an improvement of 40 fold over linear RCA using two specific primers.

The ϕ29 DNA polymerase exhibits properties that make it an excellent candidate to perform multiply-primed RCA (Dean et al., 2001). It can perform strand displacement DNA synthesis for more than 70 kb without dissociating from the template. The enzyme is highly processive and can readily synthesize DNA strands of ~ 0.5 Mb in length. Also, it exhibits high fidelity with an error rate of 1 in 10^6-10^7, which is one to two logs better than Taq polymerase. Finally, the enzyme is very stable and demonstrates linear reaction kinetics at 30^0C for over 12 hours.

Our goal is to amplify the genome of a single cell integrated with MaRX DNA such that we can excise the vector, transform bacteria, and recover cDNA. We believe that we can achieve this using the ϕ29 DNA polymerase and random hexamer primers. We have demonstrated that it is possible to amplify 10 ngs of purified genomic DNA integrated with a MaRX-GFP vector approximately 350 fold (not shown approximately 3.5 ugs of total DNA quantitated for each reaction). Greater fold amplifications are seen with the addition of excess nucleotides and by increasing the reaction time. Reactions starting with 10 pgs of pUC DNA demonstrate amplifications of approximately 400,000 fold (approximately 4.0 ugs of total DNA quantitated for each reaction). Greater fold amplifications are always observed when comparing pUC DNA versus genomic DNA. This may be an indication that RCA is more efficienty performed from a circular template; however, it should be noted that excision of MaRX with CRE recombinase prior to amplification will produce a circular DNA which is predicted to be preferentially amplified as compared to the remainder of the genome.

When the amplified genomic MaRX DNA was excised with the KW recombinase, transformed and mini-preps an intact, excised MaRX virus was recovered. Importanly, these results demonstrate faithful synthesis of MaRX DNA during the ϕ29 DNA polymerase amplification process.

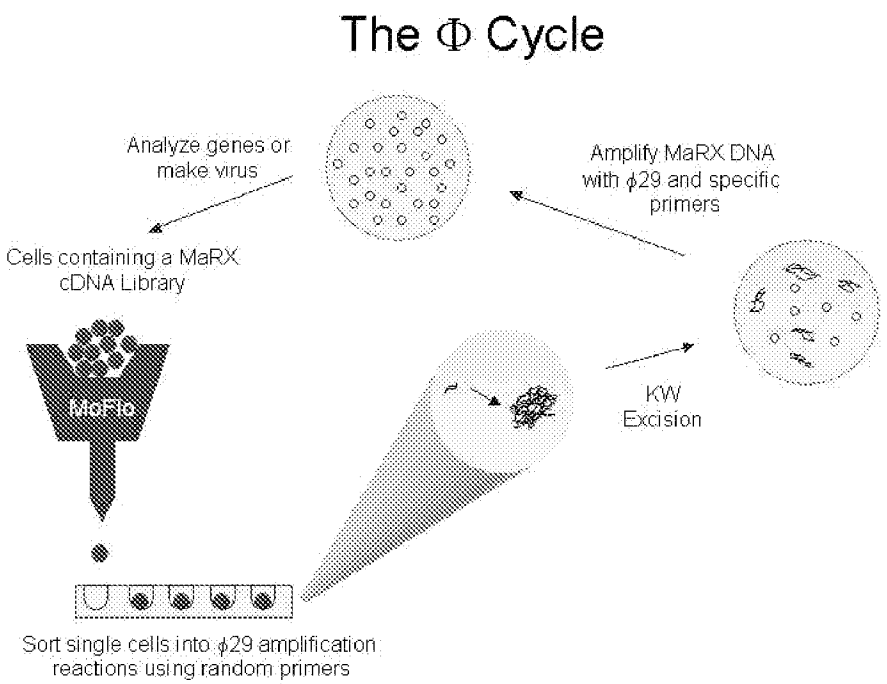

Figure 9. A diagrammatic representation of the MaRX screening cycle couple with phi-29 polymerase is shown.

Central to our screening scheme is the ability to recover an viable MaRX virus plasmid from a single isolated cell. The human genome contains 3.3 picograms of DNA. As a test of whether this goal is achievable, we teste amplification of MaRX-GFP DNA from 1 picogram of genomic DNA (figure 8). At the levels of DNA used for these experiments, the hexamers can actually self-prime and generate background amplification. However, this background does not interfere with the genomic amplification. After excision and transformation of the 1 picogram sample 20,000 bacterial transformants were obtained. These numbers are in the range of what we expect from a typical MaRX excision. These results provide strong evidence that we can amplify the amount of DNA present in a single cell to obtain quantities needed for excision and transformation.

The forgoing experiments set the groundwork for testing ϕ29 amplification in single cells. At our disposal is a flow cytometer, which we will use to sort cells for amplification reactions. There exists an established method to sort single cells for RT-PCR (Gaynor et al., 1996), which will provide us an effective starting point. Although our reactions will have different components and the temperature for polymerization will be isothermal we may be able to lyse cells under the same Nonidet P-40 (NP40) detergent lysis conditions established in previous studies. We will first test ϕ29 amplification of pUC DNA with increasing amounts of NP40 to determine if there is inhibition of the polymerase. Other detergents such as deoxycholate and triton-X100 will be tested as well. The concentration of magnesium and potassium ions will also have to be adjusted for optimal cell lysis and ϕ29 synthesis.

Our goal will be to establish a procedure in which minimal manipulation is involved. A single cell in a few nanoliters can be sorted directly into lysis conditions in one microliter. An appropriate volume of ϕ29 reaction buffer may be added such that any inhibitory action of the lysis buffer may be diluted away. The need for denaturation of the DNA can be tested before the addition of the ϕ29 reaction buffer. It is probable that a single cell in a microliter of water

may simply burst in the hypotonic environment such that there may be no need for lysis with detergents.

Successful amplification of genomic MaRX DNA from a single cell will be followed by KW excision and transformation. Because amplification of the pUC plasmid is very efficient we expect there will be little problem amplifying an excised circular MaRX vector. Amplification of excised vector may also eliminate the need for bacterial transformation because of the prolific nature of the ϕ29 DNA polymerase. Enough vector can be produced such that cDNA can be analyzed and/or it can be used directly to make virus.

With the success of genomic amplification from a single MaRX cell followed by KW excision we will have established a procedure that we call the ϕ Cycle (see figure 9). In essence, this procedure will further strengthen the effectiveness and efficiency of the MaRX cycle by uniting it with the extraordinary features of the ϕ29 DNA polymerase.

Summary

The goal of this proposal is to build generate novel approaches to the analysis of gene function in mammalian cells. We propose to couple the existing genetic approaches which we have devised over the last several years with effective loss-of-function phenotypes generate through RNAi and with the ability to operate genetic screens on single cells. Achieving this goal will greatly expand the range of problems that can be addressed using genetics in cultured mammalian cells. Furthermore, even the ability to create stable loss-of-function cell lines using RNAi will provide a powerful new tool in the effort to define and to validate potential therapeutic targets for a wide range of human diseases.

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HE:AB
N1:Cold Spring Harbor Lab
N2:1 Bungtown Road
N4:Cold Spring Harbor
N5:NY
N6:11724
HX:T
HZ:12

Exhibit E

*
D1:Greg Hannon
D2:HPRTHpaZeo1-5
D3:GCGTCGTGATTAGCGATGATGAACCAGGGTTAACCGGACCTGCAGCACGTGT
D4:50N
D7:DSL
*
D1:Greg Hannon
D2:HPRThpazeo1-3
D3:GCGTCGTGATTAGCGATGATGAACCAGGGTTAACTCAGTCCTGCTCCTCGGC
D4:50N
D7:DSL
*
D1:Greg Hannon
D2:HPRT2hpazeo2-5
D3:GGACCTCTCGAAGTGTTGGATACAGGCCGTTAACCGGACCTGCAGCACGTGT
D4:50N
D7:DSL
*
D1:Greg Hannon
D2:HPRT2hpazeo2-3
D3:GGACCTCTCGAAGTGTTGGATACAGGCCGTTAACTCAGTCCTGCTCCTCGGC
D4:50N
D7:DSL
*
D1:Greg Hannon
D2:luchpazeo5
D3:AACGGATTACCAGGGATTTCAGTCGATGGTTAACCGGACCTGCAGCACGTGT
D4:50N
D7:DSL
*
D1:Greg Hannon
D2:luchpazeo3
D3:AACGGATTACCAGGGATTTCAGTCGATGGTTAACTCAGTCCTGCTCCTCGGC
D4:50N
D7:DSL
*
D1:Greg Hannon
D2:gfphpazeo5
D3:CTCGCCGGACACGCTGAACTTGTGGCCGGTTAACCGGACCTGCAGCACGTGT
D4:50N
D7:DSL
*
D1:Greg Hannon
D2:gfphpazeo3
D3:CTCGCCGGACACGCTGAACTTGTGGCCGGTTAACTCAGTCCTGCTCCTCGGC
D4:50N
D7:DSL
*
D1:Greg Hannon
D2:tryo1hpazeo5
D3:TTCATAACATCCAAGGATCTGGGATATGGTTAACCGGACCTGCAGCACGTGT
D4:50N
D7:DSL
*
D1:Greg Hannon

D2:tyro1hpazeo3
D3:TTCATAACATCCAAGGATCTGGGATATGGTTAACTCAGTCCTGCTCCTCGGC
D4:50N
D7:DSL
*
D1:Greg Hannon
D2:tyro2hpazeo5
D3:GTGGATGACCGTGAGTCCTGGCCCTCTGGTTAACCGGACCTGCAGCACGTGT
D4:50N
D7:DSL
*
D1:Greg Hannon
D2:tyro2hpazeo3
D3:GTGGATGACCGTGAGTCCTGGCCCTCTGGTTAACTCAGTCCTGCTCCTCGGC
D4:50N
D7:DSL
+

Luciferase siRNA

Luciferase Let-7 like

Luciferase simple hairpin

Exhibit F

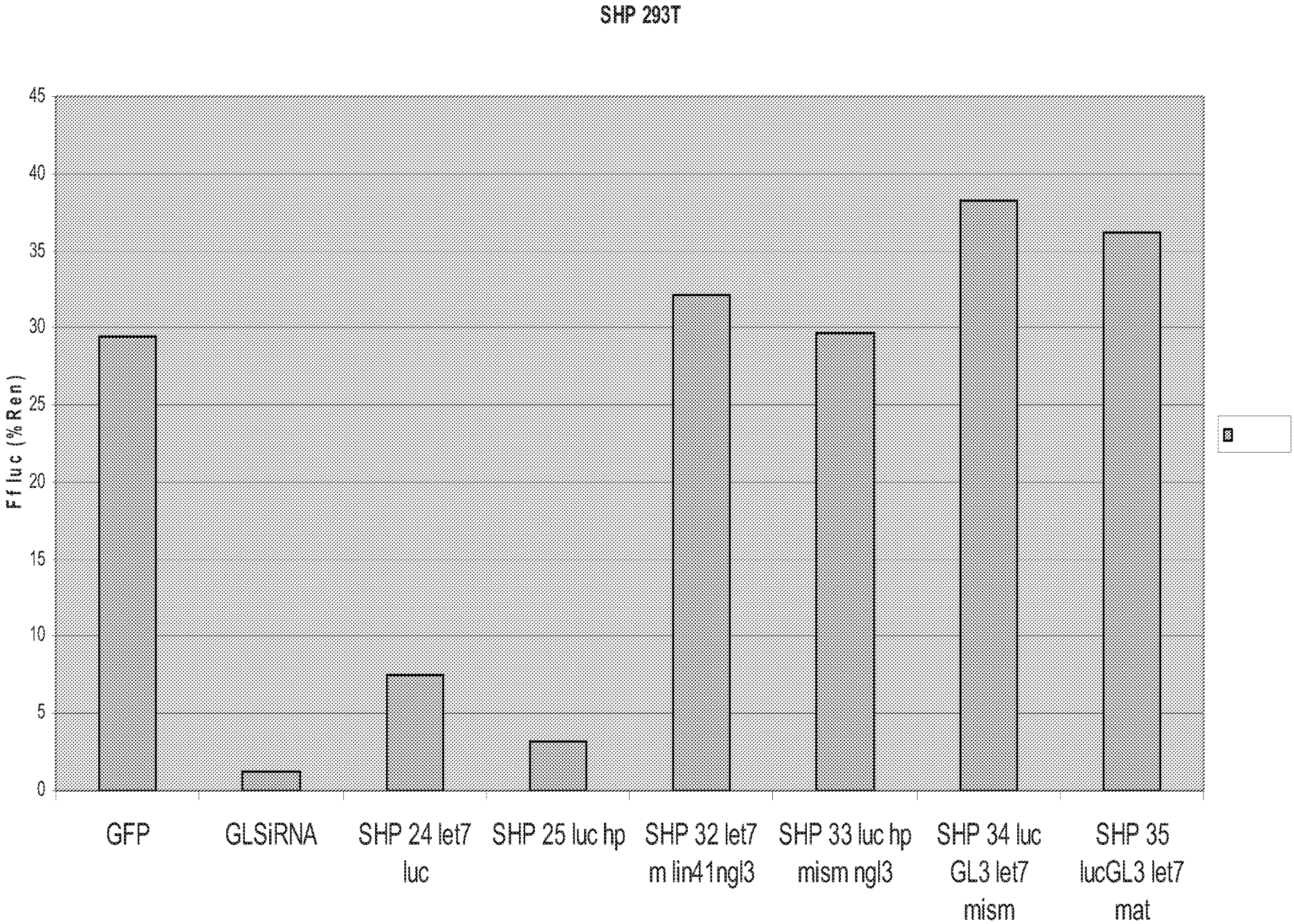

SHP 293T
Ff luc (% Ren)
45
40
35
30
25
20
15
10
5
0
GFP
GLSiRNA
SHP 24 let7 luc
SHP 25 luc hp
SHP 32 let7 m lin41ngl3
SHP 33 luc hp mism ngl3
SHP 34 luc GL3 let7 mism
SHP 35 lucGL3 let7 mat

Exhibit G

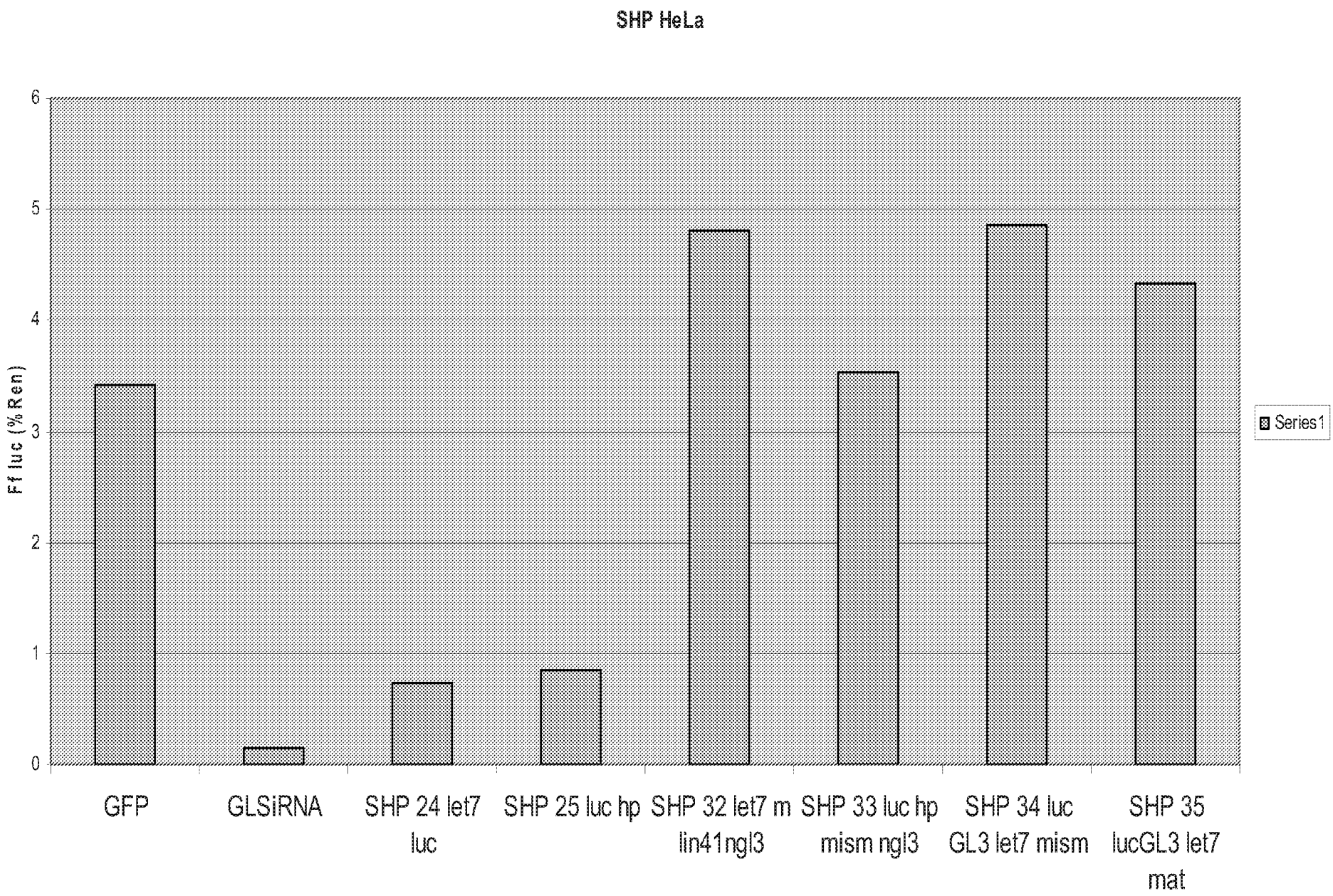

SHP HeLa
Ff luc (% Ren)
6
5
4
3
2
1
0
GFP
GLSiRNA
SHP 24 let7 luc
SHP 25 luc hp
SHP 32 let7 m lin41ngl3
SHP 33 luc hp mism ngl3
SHP 34 luc GL3 let7 mism
SHP 35 lucGL3 let7 mat
Series1

Exhibit H

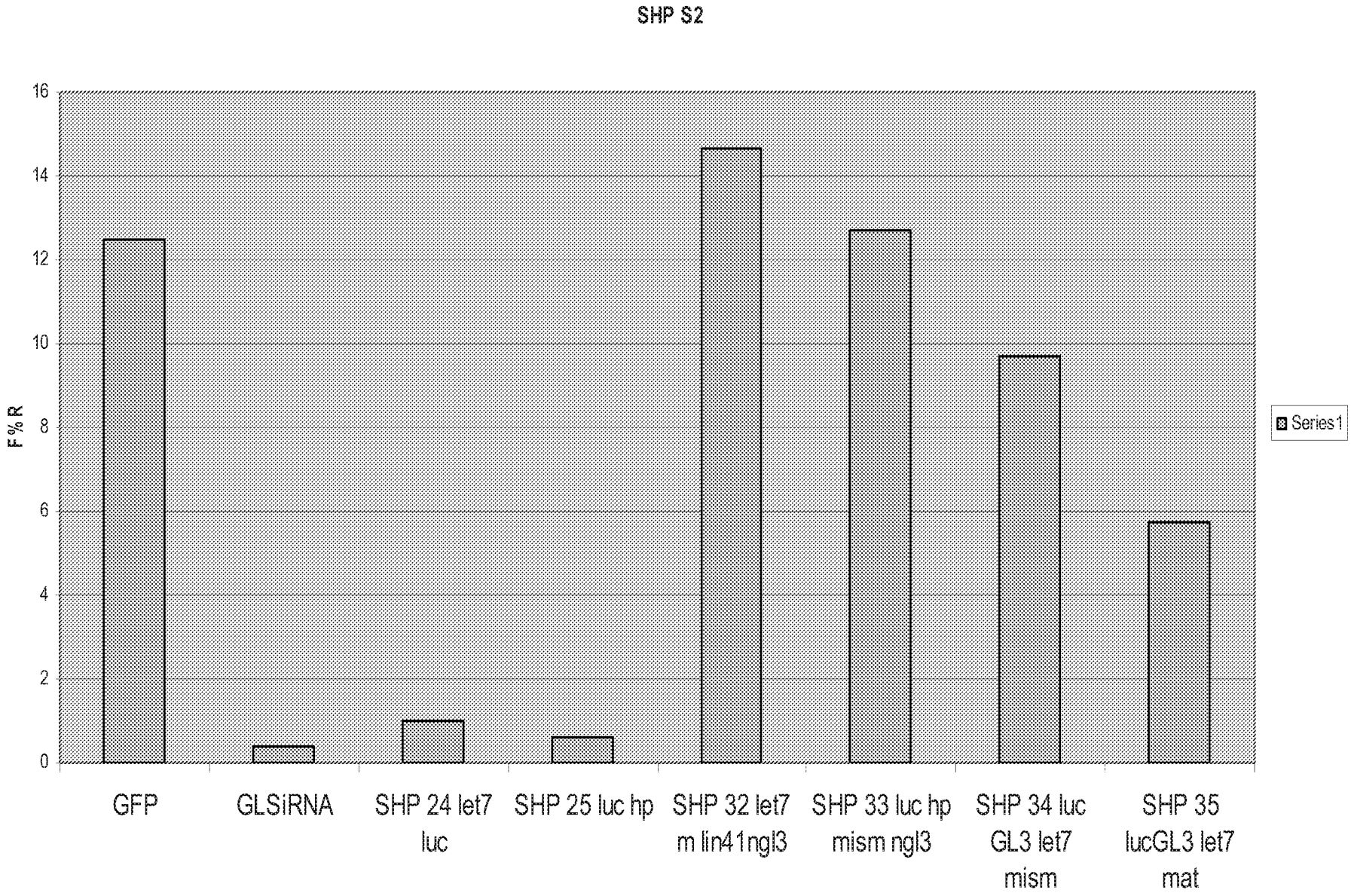
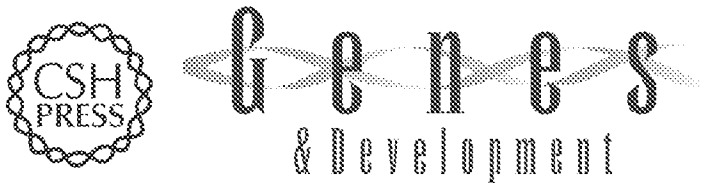

SHP S2
F%R
16
14
12
10
8
6
4
2
0
GFP
GLSiRNA
SHP 24 let7 luc
SHP 25 luc hp
SHP 32 let7 m lin41ngl3
SHP 33 luc hp mism ngl3
SHP 34 luc GL3 let7 mism
SHP 35 lucGL3 let7 mat
Series1

Exhibit I

Luciferase activity (% Renilla)
3
2.5
2
1.5
1
0.5
0
siRNA
Luc simple vector
U6 siOligo 1-2
siOligo 1-6
siOligo 1-10
siOligo 1-19
siOligo 2-23
siOligo 2-36

Exhibit J



Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells

Patrick J. Paddison, Amy A. Caudy, Emily Bernstein, et al.

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Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells

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RNA interference (RNAi) was first recognized in *Caenorhabditis elegans* as a biological response to exogenous double-stranded RNA (dsRNA), which induces sequence-specific gene silencing. RNAi represents a conserved regulatory motif, which is present in a wide range of eukaryotic organisms. Recently, we and others have shown that endogenously encoded triggers of gene silencing act through elements of the RNAi machinery to regulate the expression of protein-coding genes. These small temporal RNAs (stRNAs) are transcribed as short hairpin precursors (~70 nt), processed into active, 21-nt RNAs by Dicer, and recognize target mRNAs via base-pairing interactions. Here, we show that short hairpin RNAs (shRNAs) can be engineered to suppress the expression of desired genes in cultured *Drosophila* and mammalian cells. shRNAs can be synthesized exogenously or can be transcribed from RNA polymerase III promoters in vivo, thus permitting the construction of continuous cell lines or transgenic animals in which RNAi enforces stable and heritable gene silencing.

[*Key Words*: RNAi; gene silencing; miRNA; shRNA; siRNA]

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An understanding of the biological role of any gene comes only after observing the phenotypic consequences of altering the function of that gene in a living cell or organism. In many cases, those organisms for which convenient methodologies for genetic manipulation exist blaze the trail toward an understanding of similar genes in less tractable organisms, such as mammals. The advent of RNA interference (RNAi) as an investigational tool has shown the potential to democratize at least one aspect of genetic manipulation, the creation of hypomorphic alleles, in organisms ranging from unicellular parasites (e.g., Shi et al. 2000) to mammals (Svoboda et al. 2000; Wianny and Zernicka-Goetz 2000).

Although *Caenorhabditis elegans* has, for some time, been well developed as a forward genetic system, the lack of methodologies for gene replacement by homologous recombination presented a barrier to assessing rapidly the consequences of loss of function in known genes. In an effort to overcome this limitation, Mello and Fire (Fire et al. 1998), building on earlier studies (Guo and Kemphues 1995), probed the utility of antisense RNA as a method for suppressing gene expression in worms. Through these efforts, they found that double-stranded RNA (dsRNA) was much more effective than antisense RNA as an inducer of gene silencing. Subsequent studies have shown that RNAi is a conserved biological response that is present in many, if not most, eukaryotic organisms (for review, see Bernstein et al. 2001b; Hammond et al. 2001b).

As a result of biochemical and genetic approaches in several experimental systems, the mechanisms underlying RNAi have begun to unfold (for review, see Bernstein et al. 2001b; Hammond et al. 2001b). These suggest the existence of a conserved machinery for dsRNA-induced gene silencing, which proceeds via a two-step mechanism. In the first step, the dsRNA silencing trigger is recognized by an RNase III family nuclease called Dicer, which cleaves the dsRNA into ~21–23-nt siRNAs (small interfering RNAs). These siRNAs are incorporated into a multicomponent nuclease complex, RISC, which identifies substrates through their homology to siRNAs and targets these cognate mRNAs for destruction.

Although it was clear from the outset that RNAi would prove a powerful tool for manipulating gene expression in invertebrates, there were several potential impediments to the use of this approach in mammalian cells. Most mammalian cells harbor a potent antiviral response that is triggered by the presence of dsRNA viral

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replication intermediates. A key component of this response is a dsRNA-activated protein kinase, PKR, which phosphorylates EIF-2α, inducing, in turn, a generalized inhibition of translation (for review, see Williams 1997; Gil and Esteban 2000). In addition, dsRNA activates the 2′5′ oligoadenylate polymerase/RNase L system and represses IκB. The ultimate outcome of this set of responses is cell death via apoptosis.

Therefore, it came as a welcome surprise that dsRNA could induce sequence-specific silencing in mammalian embryos, which apparently lack generalized responses to dsRNA (Svoboda et al. 2000; Wianny and Zernicka-Goetz 2000). Indeed, microinjection of dsRNA into mouse zygotes could specifically silence both exogenous reporters and endogenous genes to create anticipated phenotypes. Subsequently, these observations were extended to embryonic cell lines, such as embryonic stem cells and embryonal carcinoma cells, which do not show generic translational repression in response to dsRNA (Billy et al. 2001; Yang et al. 2001; Paddison et al. 2002). However, restriction of conventional RNAi to these few embryonic and cell culture systems would place a significant limitation on the utility of this approach in mammals.

Tuschl and colleagues first showed that short RNA duplexes, designed to mimic the products of the Dicer enzyme, could trigger RNA interference in vitro in *Drosophila* embryo extracts (Tuschl et al. 1999; Elbashir et al. 2001b,c). This observation was extended to mammalian somatic cells by Tuschl and coworkers (Elbashir et al. 2001a) and by Fire and colleagues (Caplen et al. 2001), who showed that chemically synthesized siRNAs could induce gene silencing in a wide range of human and mouse cell lines. The use of synthetic siRNAs to transiently suppress the expression of target genes is quickly becoming a method of choice for probing gene function in mammalian cells.

Dicer, the enzyme that normally produces siRNAs in vivo, has been linked to RNA interference both through biochemistry and through genetics (Bernstein et al. 2001a; Grishok et al. 2001; Ketting et al. 2001; Knight and Bass 2001). Indeed, *C. elegans* animals that lack Dicer are RNAi-deficient, at least in some tissues. However, these animals also have additional phenotypic abnormalities. Specifically, they are sterile and show a number of developmental abnormalities that typify alterations in developmental timing. Indeed, the phenotypes of the *Dicer* mutant animals were similar to those previously observed for animals carrying mutations in the *let-7* gene (Reinhart et al. 2000).

The *let-7* gene encodes a small, highly conserved RNA species that regulates the expression of endogenous protein-coding genes during worm development. The active RNA species is transcribed initially as an ~70-nt precursor, which is posttranscriptionally processed into a mature ~21-nt form (Reinhart et al. 2000). Both in vitro and in vivo data from *C. elegans* (Grishok et al. 2001; Ketting et al. 2001; Knight and Bass 2001) and human cells (Hutvagner et al. 2001) have pointed to Dicer as the enzyme responsible for *let-7* maturation and for the maturation of a similar small RNA, *lin-4* (Grishok et al. 2001). Thus, at least some components of the RNAi machinery respond to endogenously encoded triggers to regulate the expression of target genes.

Recent studies have placed *let-7* and *lin-4* as the founding members of a potentially very large group of small RNAs known generically as micro-RNAs (miRNAs). Nearly 100 potential miRNAs have now been identified in *Drosophila*, *C. elegans*, and mammals (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001). Although the functions of these diverse RNAs remain mysterious, it seems likely that they, like *let-7* and *lin-4*, are transcribed as hairpin RNA precursors, which are processed to their mature forms by Dicer (Lee and Ambros 2001; E. Bernstein, unpubl.).

Since the realization that small, endogenously encoded hairpin RNAs could regulate gene expression via elements of the RNAi machinery, we have sought to exploit this biological mechanism for the regulation of desired target genes. Here we show that short hairpin RNAs (shRNAs) can induce sequence-specific gene silencing in mammalian cells. As is normally done with siRNAs, silencing can be provoked by transfecting exogenously synthesized hairpins into cells. However, silencing can also be triggered by endogenous expression of shRNAs. This observation opens the door to the production of continuous cells lines in which RNAi is used to stably suppress gene expression in mammalian cells. Furthermore, similar approaches should prove efficacious in the creation of transgenic animals and potentially in therapeutic strategies in which long-term suppression of gene function is essential to produce a desired effect.

Results

Short hairpin RNAs trigger gene silencing in Drosophila *cells*

Several groups (Grishok et al. 2001; Hutvagner et al. 2001; Ketting et al. 2001; Knight and Bass 2001) have shown that endogenous triggers of gene silencing, specifically small temporal RNAs (stRNAs) *let-7* and *lin-4*, function at least in part through RNAi pathways. Specifically, these small RNAs are encoded by hairpin precursors that are processed by Dicer into mature, ~21-nt forms. Moreover, genetic studies in *C. elegans* have shown a requirement for Argonaute-family proteins in stRNA function. Specifically, alg-1 and alg-2, members of the EIF2c subfamily, are implicated both in stRNA processing and in their downstream effector functions (Grishok et al. 2001). We have recently shown that a component of RISC, the effector nuclease of RNAi, is a member of the Argonaute family, prompting a model in which stRNAs may function through RISC-like complexes, which regulate mRNA translation rather than mRNA stability (Hammond et al. 2001a).

We wished to test the possibility that we might retarget these small, endogenously encoded hairpin RNAs to regulate genes of choice with the ultimate goal of sub-

verting this regulatory system for manipulating gene expression stably in mammalian cell lines and in transgenic animals. Whether triggered by long dsRNAs or by siRNAs, RNAi is generally more potent in the suppression of gene expression in *Drosophila* S2 cells than in mammalian cells. We therefore chose this model system in which to test the efficacy of short hairpin RNAs (shRNAs) as inducers of gene silencing.

Neither stRNAs nor the broader group of miRNAs that has recently been discovered form perfect hairpin structures. Indeed, each of these RNAs is predicted to contain several bulged nucleotides within their rather short (~30-nt) stem structures. Because the position and character of these bulged nucleotides have been conserved throughout evolution and among at least a subset of miRNAs, we sought to design retargeted miRNA mimics to conserve these predicted structural features. Only the *let-7* and *lin-4* miRNAs have known mRNA targets (Wightman et al. 1993; Slack et al. 2000). In both cases, pairing to binding sites within the regulated transcripts is imperfect, and in the case of *lin-4*, the presence of a bulged nucleotide is critical to suppression (Ha et al. 1996). We therefore also designed shRNAs that paired imperfectly with their target substrates. A subset of these shRNAs is depicted in Figure 1A.

To permit rapid testing of large numbers of shRNA variants and quantitative comparison of the efficacy of suppression, we chose to use a dual-luciferase reporter system, as previously described for assays of RNAi in both *Drosophila* extracts (Tuschl et al. 1999) and mammalian cells (Caplen et al. 2001; Elbashir et al. 2001a). Cotransfection of firefly and *Renilla* luciferase reporter plasmids with either long dsRNAs or with siRNAs homologous to the firefly luciferase gene yielded an ~95% suppression of firefly luciferase without effect on *Renilla* luciferase (Fig. 1B; data not shown). Firefly luciferase could also be specifically silenced by cotransfection with homologous shRNAs. Surprisingly, those shRNAs modeled most closely on the *let-7* paradigm were the least effective inducers of silencing (data not shown). The inclusion of bulged nucleotides within the shRNA stem caused only a modest reduction in potency; however, the presence of mismatches with respect to the target mRNA essentially abolished silencing potential. The most potent inhibitors were those composed of simple hairpin structures with complete homology to the sub-

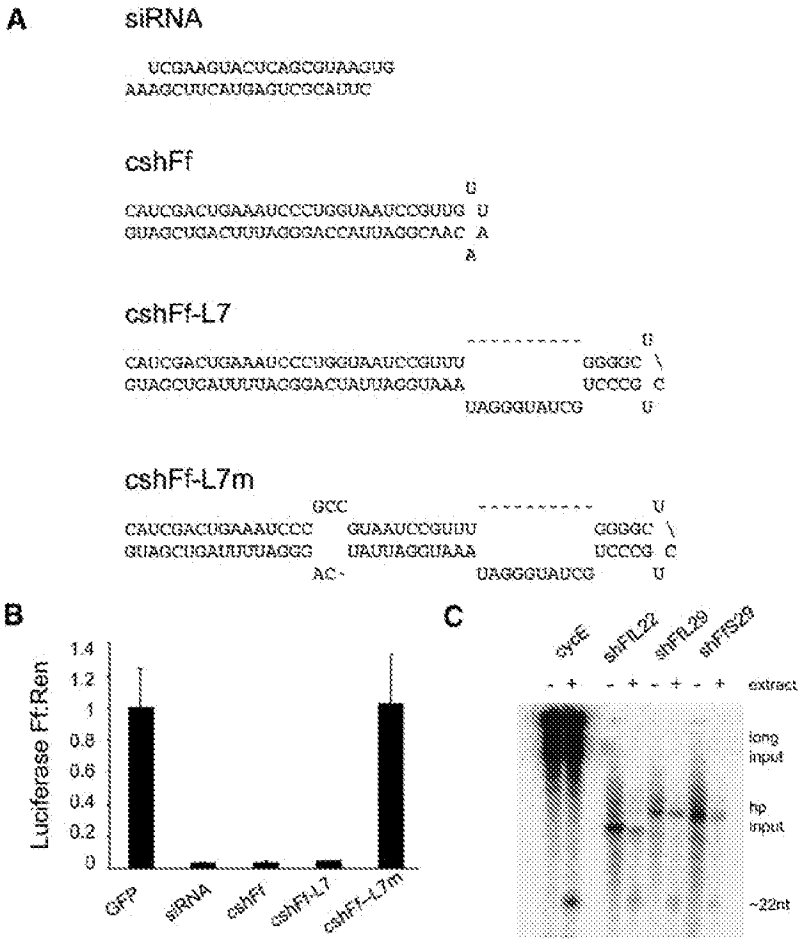

Figure 1. Short hairpins suppress gene expression in *Drosophila* S2 cells. (*A*) Sequences and predicted secondary structure of representative chemically synthesized RNAs. Sequences correspond to positions 112–134 (siRNA) and 463–491 (shRNAs) of Firefly luciferase carried on pGL3-Control. An siRNA targeted to position 463–485 of the luciferase sequence was virtually identical to the 112–134 siRNA in suppressing expression, but is not shown. (*B*) Exogenously supplied short hairpins suppress expression of the targeted Firefly luciferase gene in vivo. Six-well plates of S2 cells were transfected with 250 ng/well of plasmids that direct the expression of firefly and *Renilla* luciferase and 500 ng/well of the indicated RNA. Luciferase activities were assayed 48 h after transfection. Ratios of firefly to *Renilla* luciferase activity were normalized to a control transfected with an siRNA directed at the green fluorescent protein (GFP). The average of three independent experiments is shown; error bars indicate standard deviation. (*C*) Short hairpins are processed by the *Drosophila* Dicer enzyme. T7 transcribed hairpins shFfL22, shFfL29, and shFfS29 were incubated with (+) and without (–) 0–2-h *Drosophila* embryo extracts. Those incubated with extract produced ~22-nt siRNAs, consistent with the ability of these hairpins to induce RNA interference. A long dsRNA input (cyclin E 500-mer) was used as a control. Cleavage reactions were performed as described in Bernstein et al. (2001a).

strate. Introduction of G-U basepairs either within the stem or within the substrate recognition sequence had little or no effect (Fig. 1A,B; data not shown). Similarly, varying either the loop size from ~4 to 23 bases or the loop sequence (e.g., to mimic *let-7*) also proved neutral (data not shown).

These results show that short hairpin RNAs can induce gene silencing in *Drosophila* S2 cells with potency similar to that of siRNAs (Fig. 1B). However, in our initial observation of RNA interference in *Drosophila* S2 cells, we noted a profound dependence of the efficiency of silencing on the length of the dsRNA trigger (Hammond et al. 2000). Indeed, dsRNAs of fewer than ~200 nt triggered silencing very inefficiently. Silencing is initiated by an RNase III family nuclease, Dicer, that processes long dsRNAs into ~22-nt siRNAs. In accord with their varying potency as initiators of silencing, long dsRNAs are processed much more readily than short RNAs by the Dicer enzyme (Bernstein et al. 2001a). We therefore tested whether shRNAs were substrates for the Dicer enzyme.

We had noted previously that *let-7* (Ketting et al. 2001) and other miRNAs (E. Bernstein, unpubl.) are processed by Dicer with an unexpectedly high efficiency as compared with short, nonhairpin dsRNAs. Similarly, Dicer efficiently processed shRNAs that targeted firefly luciferase, irrespective of whether they were designed to mimic a natural Dicer substrate (*let-7*) or whether they were simple hairpin structures (Fig. 1C). These data suggest that recombinant shRNAs can be processed by Dicer into siRNAs and are consistent with the idea that these short hairpins trigger gene silencing via an RNAi pathway.

Short hairpin activated gene silencing in mammalian cells

RNAi is developing into an increasingly powerful methodology for manipulating gene expression in diverse experimental systems. However, mammalian cells contain several endogenous systems that were predicted to hamper the application of RNAi. Chief among these is a dsRNA-activated protein kinase, PKR, which effects a general suppression of translation via phosphorylation of EIF-2α (Williams 1997; Gil and Esteban 2000). Activation of these, and other dsRNA-responsive pathways, generally requires duplexes exceeding 30 bp in length, possibly to permit dimerization of the enzyme on its allosteric activator (e.g., Clarke and Mathews 1995).

Small RNAs that mimic Dicer products, siRNAs, presumably escape this limit and trigger specific silencing, in part because of their size. However, short duplex RNAs that lack signature features of siRNAs can efficiently induce silencing in *Drosophila* S2 cells but not in mammalian cells (A.A. Caudy, unpubl.). Endogenously encoded miRNAs may also escape PKR surveillance because of their size but perhaps also because of the discontinuity of their duplex structure. Given that shRNAs of <30 bp were effective inducers of RNAi in *Drosophila* S2 cells, we tested whether these RNAs could also induce sequence-specific silencing in mammalian cells.

Human embryonic kidney (HEK293T) cells were cotransfected with chemically synthesized shRNAs and with a mixture of firefly and *Renilla* luciferase reporter plasmids. As had been observed in S2 cells, shRNAs were effective inducers of gene silencing. Once again, hairpins designed to mimic *let-7* were consistently less effective than were simple hairpin RNAs, and the introduction of mismatches between the antisense strand of the shRNA and the mRNA target abolished silencing (Fig. 2A; data not shown). Overall, shRNAs were somewhat less potent silencing triggers than were siRNAs. Whereas siRNAs homologous to firefly luciferase routinely yielded ~90%–95% suppression of gene expression, suppression levels achieved with shRNAs ranged from 80%–90% on average. As we also observe with siRNAs, the most important determinant of the potency of the silencing trigger is its sequence. We find that roughly 50% of both siRNAs and shRNAs are competent for suppressing gene expression. However, neither analysis of the predicted structures of the target mRNA nor analysis of alternative structures in siRNA duplexes or shRNA hairpins has proved of predictive value for choosing effective inhibitors of gene expression.

We have adopted as a standard, shRNA duplexes containing 29 bp. However, the size of the helix can be reduced to ~25 nt without significant loss of potency. Duplexes as short as 22 bp can still provoke detectable silencing, but do so less efficiently than do longer duplexes. In no case do we observe a reduction in the internal control reporter (*Renilla* luciferase) that would be consistent with an induction of nonspecific dsRNA responses.

The ability of shRNAs to induce gene silencing was not confined to 293T cells. Similar results were also obtained in a variety of other mammalian cell lines, including human cancer cells (HeLa), transformed monkey epithelial cells (COS-1), murine fibroblasts (NIH 3T3), and diploid human fibroblasts (IMR90; Fig. 2; data not shown).

Synthesis of effective inhibitors of gene expression using T7 RNA polymerase

The use of siRNAs to provoke gene silencing is developing into a standard methodology for investigating gene function in mammalian cells. To date, siRNAs have been produced exclusively by chemical synthesis (e.g., Caplen et al. 2001; Elbashir et al. 2001a). However, the costs associated with this approach are significant, limiting its potential utility as a tool for investigating in parallel the functions of large numbers of genes. Short hairpin RNAs are presumably processed into active siRNAs in vivo by Dicer (see Fig. 1C). Thus, these may be more tolerant of terminal structures, both with respect to nucleotide overhangs and with respect to phosphate termini. We therefore tested whether shRNAs could be prepared by in vitro transcription with T7 RNA polymerase.

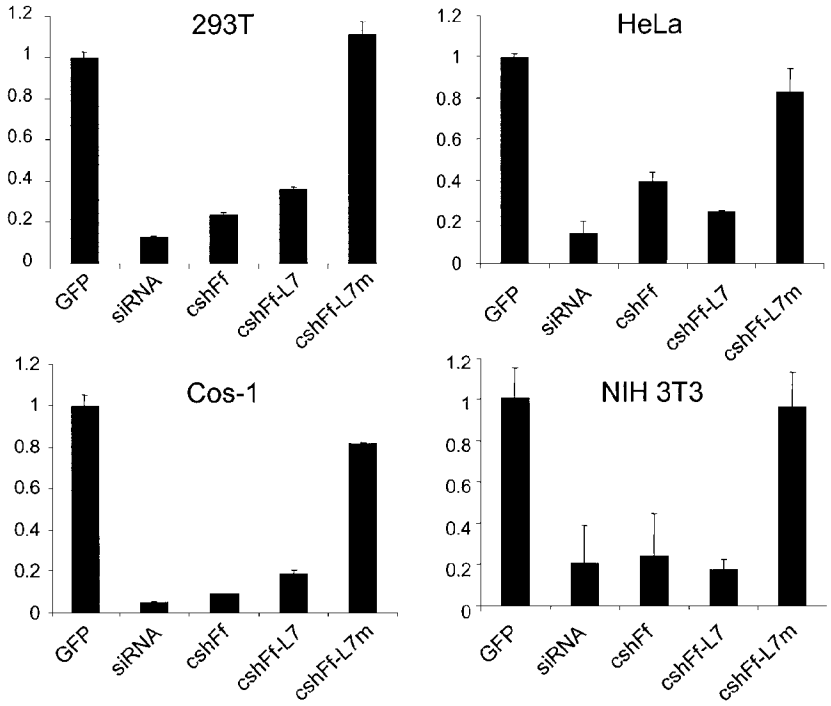

Figure 2. Short hairpins function in mammalian cells. HEK 293T, HeLa, COS-1, and NIH 3T3 cells were transfected with plasmids and RNAs as in Figure 1 and subjected to dual luciferase assays 48 h posttransfection. The ratios of firefly to *Renilla* luciferase activity are normalized to a control transfected with an siRNA directed at the green fluorescent protein (GFP). The average of three independent experiments is shown; error bars indicate standard deviation.

Transcription templates that were predicted to generate siRNAs and shRNAs similar to those prepared by chemical RNA synthesis were prepared by DNA synthesis (Fig. 3A,C). These were tested for efficacy both in S2 cells (data not shown) and in human 293 cells (Fig. 3B,D). Overall, the performance of the T7-synthesized hairpin or siRNAs closely matched the performance of either produced by chemical synthesis, both with respect to the magnitude of inhibition and with respect to the relative efficiency of differing sequences. Because T7 polymerase prefers to initiate at twin guanosine residues, however, it was critical to consider initiation context when designing in vitro transcribed siRNAs (Fig. 3B). In contrast, shRNAs, which are processed by Dicer (see Fig. 1C), tolerate the addition of these bases at the 5′ end of the transcript.

Studies in *Drosophila* embryo extracts indicate that siRNAs possess 5′ phosphorylated termini, consistent with their production by an RNase III family nuclease (Bernstein et al. 2001a; Elbashir et al. 2001b). In vitro, this terminus is critical to the induction of RNAi by synthetic RNA oligonucleotides (Elbashir et al. 2001c; Nykanen et al. 2001). Chemically synthesized siRNAs are nonphosphorylated, and enzymatic addition of a 5′ phosphate group in vitro prior to transfection does not increase the potency of the silencing effect (A.A. Caudy, unpubl.). This suggests either that the requirement for phosphorylated termini is less stringent in mammalian cells or that a kinase efficiently phosphorylates siRNAs in vivo. RNAs synthesized with T7 RNA polymerase, however, possess 5′ triphosphate termini. We therefore explored the possibility of synthesizing siRNAs with T7 polymerase followed by treatment in vitro with pyrophosphatase to modify the termini to resemble those of siRNAs. Surprisingly, monophosphorylated siRNAs (data not shown) were as potent in inducing gene silencing as transcription products bearing triphosphate termini (Fig. 3B). This may suggest either that the requirement for monophosphorylated termini is less stringent in mammalian cells or that siRNAs are modified in vivo to achieve an appropriate terminal structure.

Considered together, our data suggest that both shRNAs and siRNA duplexes can be prepared by synthesis with T7 RNA polymerase in vitro. This significantly reduces the cost of RNAi in mammalian cells and paves the way for application of RNAi on a whole-genome scale.

Transcription of shRNAs in vivo by RNA polymerase III

Although siRNAs are an undeniably effective tool for probing gene function in mammalian cells, their suppressive effects are by definition of limited duration. Delivery of siRNAs can be accomplished by any of a num-

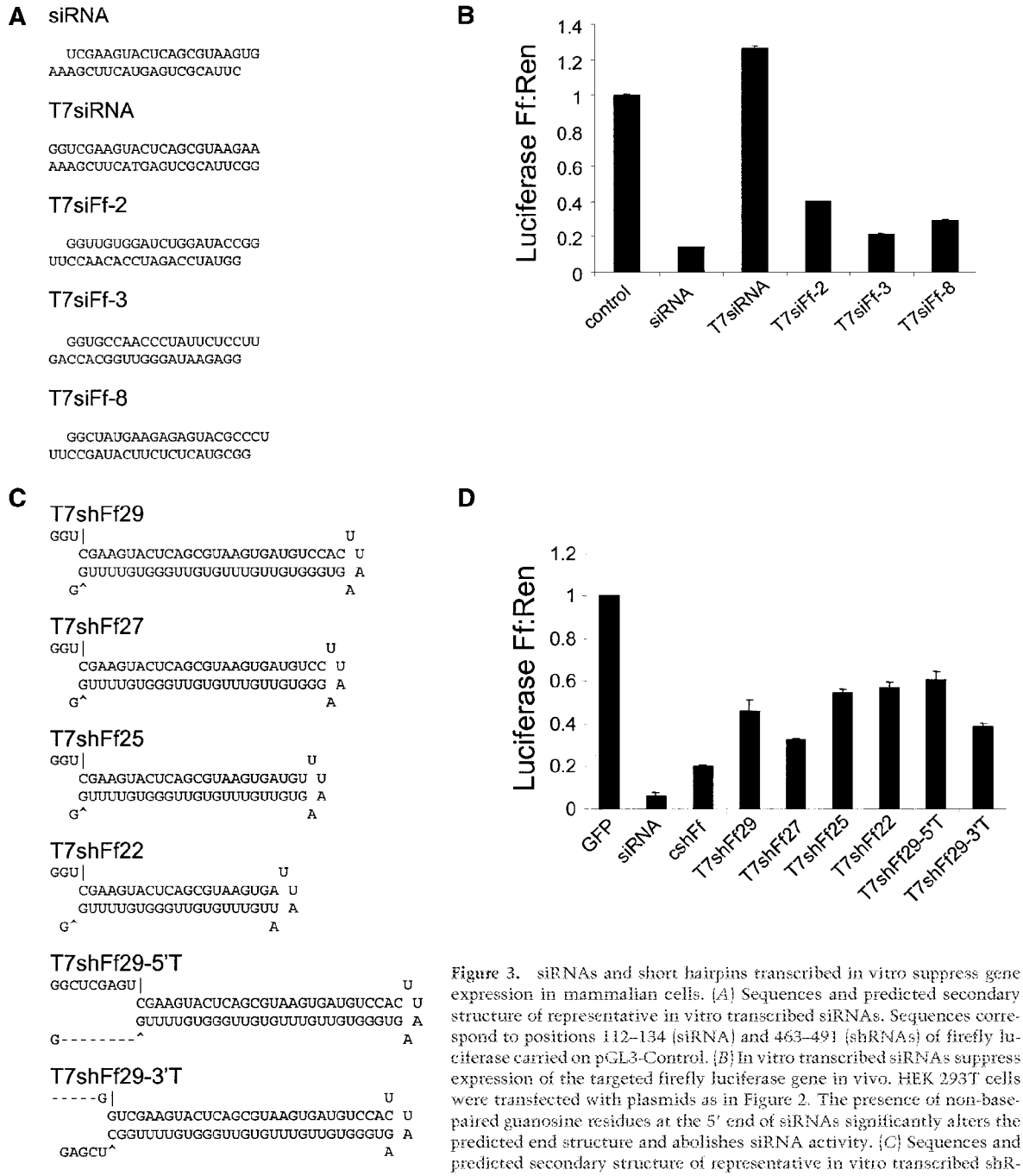

Figure 3. siRNAs and short hairpins transcribed in vitro suppress gene expression in mammalian cells. (*A*) Sequences and predicted secondary structure of representative in vitro transcribed siRNAs. Sequences correspond to positions 112–134 (siRNA) and 463–491 (shRNAs) of firefly luciferase carried on pGL3-Control. (*B*) In vitro transcribed siRNAs suppress expression of the targeted firefly luciferase gene in vivo. HEK 293T cells were transfected with plasmids as in Figure 2. The presence of non-base-paired guanosine residues at the 5′ end of siRNAs significantly alters the predicted end structure and abolishes siRNA activity. (*C*) Sequences and predicted secondary structure of representative in vitro transcribed shRNAs. Sequences correspond to positions 112–141 of firefly luciferase carried on pGL3-Control. (*D*) Short hairpins transcribed in vitro suppress expression of the targeted firefly luciferase gene in vivo. HEK 293T cells were transfected with plasmids as in Figure 2.

ber of transient transfection methodologies, and both the timing of peak suppression and the recovery of protein levels as silencing decays can vary with both the cell type and the target gene (Y. Seger and E. Bernstein, unpubl.). Therefore, one limitation on siRNAs is the development of continuous cell lines in which the expression of a desired target is stably silenced.

Hairpin RNAs, consisting of long duplex structures, have been proved as effective triggers of stable gene silencing in plants, in *C. elegans*, and in *Drosophila* (Ken-

nerdell and Carthew 2000; Smith et al. 2000; Tavernarakis et al. 2000). We have recently shown stable suppression of gene expression in cultured mammalian cells by continuous expression of a long hairpin RNA (Paddison et al. 2002). However, the scope of this approach was limited by the necessity of expressing such hairpins only in cells that lack a detectable PKR response. In principle, shRNAs could bypass such limitations and provide a tool for evoking stable suppression by RNA in mammalian somatic cells.

To test this possibility, we initially cloned sequences encoding a firefly luciferase shRNA into a CMV-based expression plasmid. This was predicted to generate a capped, polyadenylated RNA polymerase II transcript in which the hairpin was extended on both the 5′ and 3′ ends by vector sequences and poly(A). This construct was completely inert in silencing assays in 293T cells (data not shown).

During our studies on chemically and T7-synthesized shRNAs, we noted that the presence of significant single-stranded extensions (either 5′ or 3′ of the duplex) reduced the efficacy of shRNAs (data not shown). We therefore explored the use of alternative promoter strategies in an effort to produce more defined hairpin RNAs. In particular, RNA polymerase III promoters have well-defined initiation and termination sites and naturally produce a variety of small, stable RNA species. Although many Pol III promoters contain essential elements within the transcribed region, limiting their utility for our purposes, class III promoters use exclusively non-transcribed promoter sequences. Of these, the U6 snRNA promoter and the H1 RNA promoter have been well studied (Lobo et al. 1990; Hannon et al. 1991; Chong et al. 2001).

By placing a convenient cloning site immediately behind the U6 snRNA promoter, we have constructed pShh-1, an expression vector in which short hairpins are harnessed for gene silencing. Into this vector either of two shRNA sequences derived from firefly luciferase were cloned from synthetic oligonucleotides. These were cotransfected with firefly and *Renilla* luciferase expression plasmids into 293T cells. One of the two encoded shRNAs provoked effective silencing of firefly luciferase without altering the expression of the internal control (Fig. 4C). The second encoded shRNA also produced detectable, albeit weak, repression. In both cases, silencing was dependent on insertion of the shRNA in the correct orientation with respect to the promoter (Fig. 4C; data not shown). Although the shRNA itself is bilaterally symmetric, insertion in the incorrect orientation would affect Pol III termination and is predicted to produce a hairpin with both 5′ and 3′ single-stranded extensions. Similar results were also obtained in a number of other mammalian cell lines including HeLa, COS-1, NIH 3T3, and IMR90 (Fig. 4; data not shown). pShh1-Ff1 was, however, incapable of effecting suppression of the luciferase reporter in *Drosophila* cells, in which the human U6 promoter is inactive (data not shown).

As a definitive test of whether the plasmid-encoded shRNAs brought about gene silencing via the mammalian RNAi pathway, we assessed the dependence of suppression on an essential component of the RNAi pathway. We transfected pShh1-Ff1 along with an siRNA homologous to human *Dicer*. Figure 5 shows that treatment of cells with *Dicer* siRNAs is able to completely depress the silencing induced by pShh1-Ff1. Addition of an unrelated siRNA had no effect on the magnitude of suppression by pShh1-Ff1 (data not shown). Importantly, *Dicer* siRNAs had no effect on siRNA-induced silencing of firefly luciferase (data not shown). These results are consistent with shRNAs operating via an RNAi pathway similar to those provoked by stRNAs and long dsRNAs. Furthermore, it suggests that siRNA-mediated silencing is less sensitive to depletion of the Dicer enzyme.

The ultimate utility of encoded short hairpins will be in the creation of stable mutants that permit the study of the resulting phenotypes. We therefore tested whether we could create a cellular phenotype through stable suppression. Expression of activated alleles of the *ras* oncogene in primary mouse embryo fibroblasts (MEFs) induces a stable growth arrest that resembles, as a terminal phenotype, replicative senescence (Serrano et al. 1997). Cells cease dividing and assume a typical large, flattened morphology. Senescence can be countered by mutations that inactivate the p53 tumor suppressor pathway (Serrano et al. 1997). As a test of the ability of vector-encoded shRNAs to stably suppress an endogenous cellular gene, we generated a hairpin that was targeted to the mouse *p53* gene. As shown in Figure 6, MEFs transfected with pBabe-RasV12 fail to proliferate and show a senescent morphology when cotransfected with an empty control vector. As noted previously (Serrano et al. 1997), the terminally arrested state is achieved in 100% of drug-selected cells in culture by 8 d posttransfection. However, upon cotransfection of an activated *ras* expression construct with the pShh-p53, cells emerged from drug selection that not only fail to adopt a senescent morphology but also maintain the ability to proliferate for a minimum of several weeks in culture (Fig. 6). These data strongly suggest that shRNA expression constructs can be used for the creation of continuous mammalian cell lines in which selected target genes are stably suppressed.

Discussion

The demonstration that short dsRNA duplexes can induce sequence-specific silencing in mammalian cells has begun to foment a revolution in the manner in which gene function is examined in cultured mammalian cells. These siRNAs (Elbashir et al. 2001a) mimic the products generated by Dicer (Bernstein et al. 2001a) in the initiation step of RNAi and presumably enter the silencing pathway without triggering nonspecific translational suppression via PKR. siRNAs can be used to examine the consequences of reducing the function of virtually any protein-coding gene and have proved effective in provoking relevant phenotypes in numerous somatic cell types from both humans and mice. However, a significant dis-

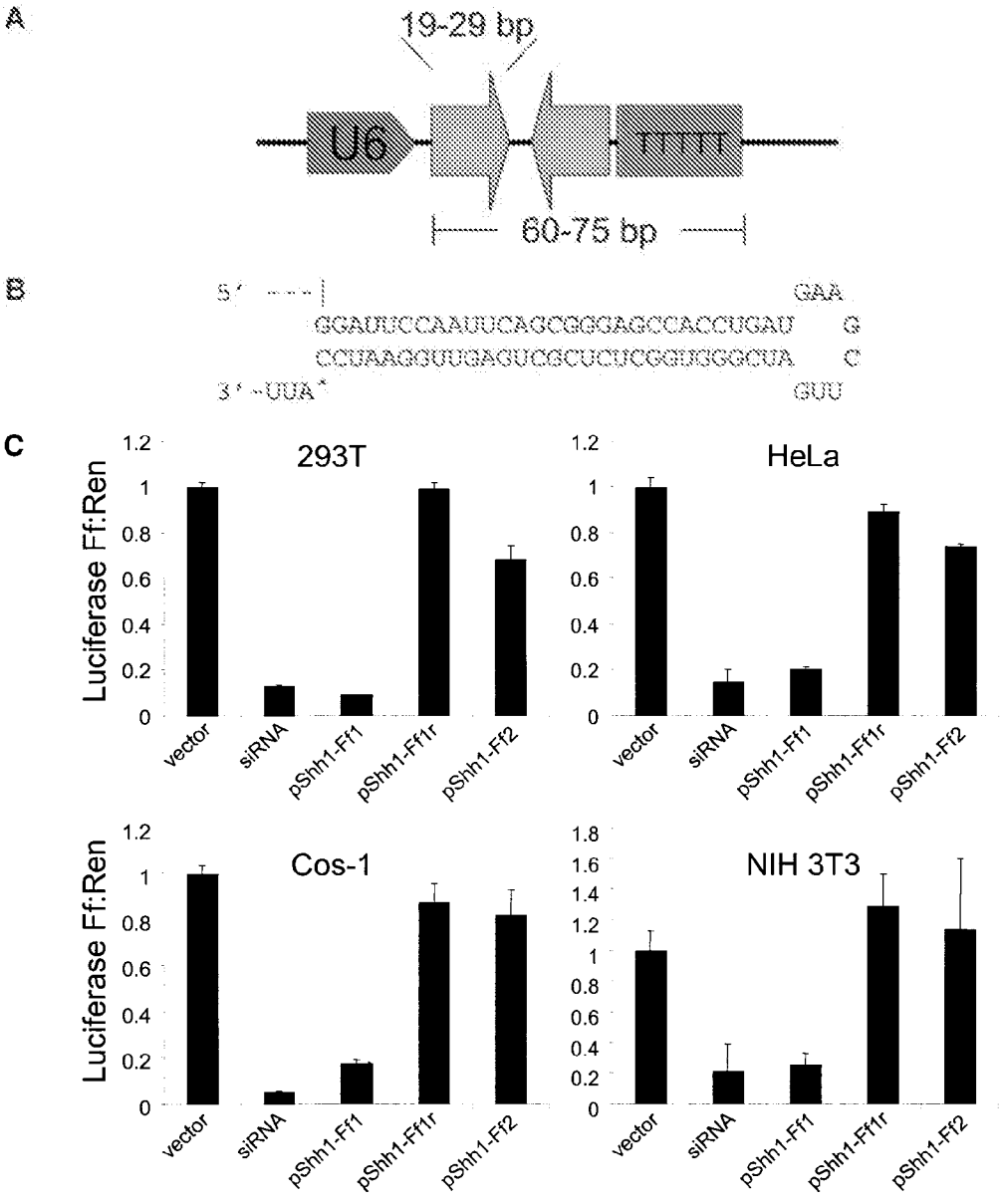

Figure 4. Transcription of functional shRNAs in vivo. (*A*) Schematic of the pShh1 vector. Sequences encoding shRNAs with between 19 and 29 bases of homology to the targeted gene are synthesized as 60–75-bp double-stranded DNA oligonucleotides and ligated into an *Eco*RV site immediately downstream of the U6 promoter. (*B*) Sequence and predicted secondary structure of the Ff1 hairpin. (*C*) An shRNA expressed from the pShh1 vector suppresses luciferase expression in mammalian cells. HEK 293T, HeLa, COS-1, and NIH 3T3 cells were transfected with reporter plasmids as in Figure 1, and pShh1 vector, firefly siRNA, or pShh1 firefly shRNA constructs as indicated. The ratios of firefly to *Renilla* luciferase activity were determined 48 h after transfection and represent the average of three independent experiments; error bars indicate standard deviation.

advantage of siRNAs is that their effects are transient, with phenotypes generated by transfection with such RNAs persisting for ~1 wk. In *C. elegans*, RNAi has proved to be such a powerful tool, in part, because silencing is both systemic and heritable, permitting the consequences of altering gene expression to be examined throughout the development and life of an animal. We have therefore sought to expand the utility of RNAi in mammalian systems by devising methods to induce stable and heritable gene silencing. Previously, we have shown that expression of long (~500-nt) dsRNAs could produce stable silencing in embryonic mammalian cells

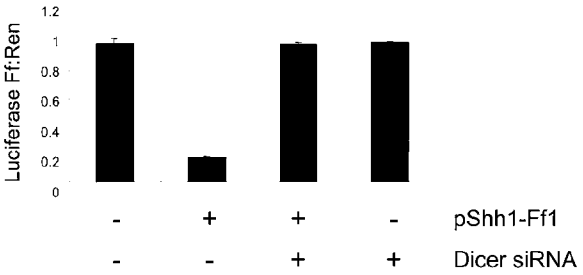

Figure 5. Dicer is required for shRNA-mediated gene silencing. HEK 293T cells were transfected with luciferase reporter plasmids as well as pShh1-Ff1 and an siRNA targeting human Dicer either alone or in combination, as indicated. The Dicer siRNA sequence (TCA ACC AGC CAC TGC TGG A) corresponds to coordinates 3137–3155 of the human *Dicer* sequence. The ratios of firefly to *Renilla* luciferase activity were determined 26 h after transfection and represent the average of three independent experiments; error bars indicate standard deviation.

(Paddison et al. 2002); however, the utility of this approach was limited by its restriction to cells that lack endogenous, nonspecific responses to dsRNA, such as PKR.

Recently, a number of laboratories (Grishok et al. 2001; Hutvagner et al. 2001; Ketting et al. 2001; Knight and Bass 2001) have shown that there exist endogenously encoded triggers of RNAi-related pathways, which are transcribed as short hairpin RNAs (stRNAs, or generically miRNAs). Here, we have shown that short hairpin RNAs, modeled conceptually on miRNAs, are potent experimental tools for inducing gene silencing in mammalian somatic cells. These shRNAs can be provided exogenously or can be synthesized in vivo from RNA polymerase III promoters. Not only does this enable the creation of continuous cell lines in which suppression of a target gene is stably maintained by RNAi, but similar strategies may also be useful for the construction of transgenic animals. Thus, short-hairpin-activated gene silencing (SHAGging) provides a complement to the use of siRNAs in the study of gene function in mammalian cells. Finally, the ability to encode a constitutive silencing signal may permit the marriage of shRNA-induced silencing with in vivo and ex vivo gene delivery methods for therapeutic approaches based on stable RNAi in humans.

Materials and methods

Cell culture

HEK 293T, HeLa, COS-1, MEF, and IMR90 cells were cultured in DMEM (GIBCO BRL) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution (GIBCO BRL). NIH 3T3 cells were cultured in DMEM supplemented with 10% heat-inactivated calf serum and 1% antibiotic/antimycotic solution.

RNA preparation

Both shRNAs and siRNAs were produced in vitro using chemically synthesized DNA oligonucleotide templates (Sigma) and the T7 Megashortscript kit (Ambion). Transcription templates

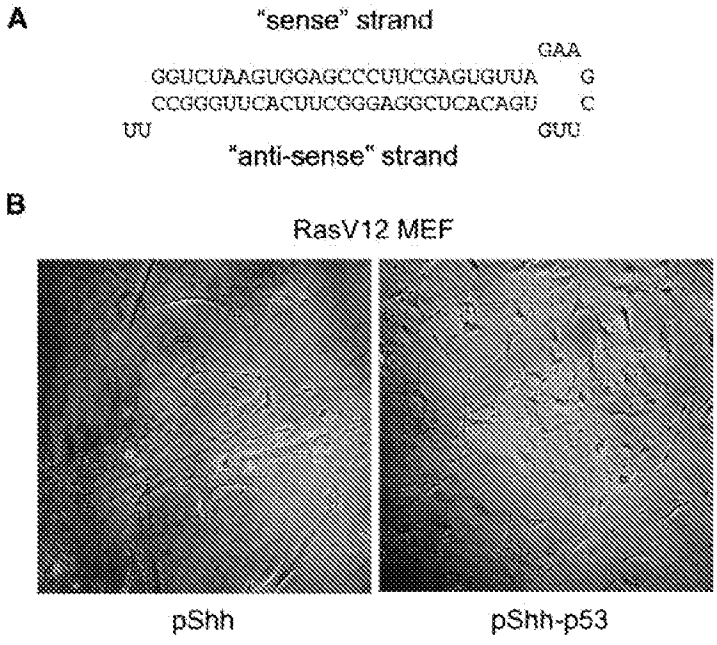

Figure 6. Stable shRNA-mediated gene silencing of an endogenous gene. (*A*) Sequence and predicted secondary structure of the *p53* hairpin. The 5′ shRNA stem contains a 27-nt sequence derived from mouse *p53* (nucleotides 166–192), whereas the 3′ stem harbors the complimentary antisense sequence. (*B*) Senescence bypass in primary mouse embryo fibroblasts (MEFs) expressing an shRNA targeted at *p53*. Wild-type MEFs, passage 5, were transfected with pBabe-RasV12 with control plasmid or with p53hp (5 μg each with FuGENE; Roche). Two days after transfection, cells were trypsinized, counted, and plated at a density of 1×10^5/10-cm plate in media containing 2.0 μg/mL of puromycin. Control cells cease proliferation and show a senescent morphology (*left* panel). Cells expressing the *p53* hairpin continue to grow (*right* panel). Photos were taken 14 d posttransfection.

were designed such that they contained *T7* promoter sequences at the 5′ end. shRNA transcripts subjected to in vitro Dicer processing were synthesized using a Riboprobe kit (Promega). Chemically synthesized RNAs were obtained from Dharmacon, Inc.

Transfection and gene silencing assays

Cells were transfected with indicated amounts of siRNA, shRNA, and plasmid DNA using standard calcium phosphate procedures at 50%–70% confluence in 6-well plates. Dual luciferase assays (Promega) were carried out by cotransfecting cells with plasmids containing firefly luciferase under the control of the SV40 promoter (pGL3-Control, Promega) and *Renilla* luciferase under the control of the SV40 early enhancer/promoter region (pSV40, Promega). Plasmids were cotransfected using a 1:1 ratio of pGL3-Control (250 ng/well) to pRL-SV40. RNAi in S2 cells was performed as previously described (Hammond et al. 2000). For stable silencing, primary MEFs (a gift from S. Lowe, Cold Spring Harbor Laboratory, NY) were cotransfected using Fugene 6 with pBabe-Ha-rasV12 and pShh-p53 (no resistance marker), according to the manufacturer's recommendations. Selection was for the presence of the activated *HarasV12* plasmid, which carries a puromycin-resistance marker. The pShh-p53 plasmid was present in excess, as is standard in a cotransfection experiment. We have now generated a version of the U6 promoter vector (pSHAG-1) that is compatible with the GATEWAY system (Invitrogen), and this can be used to transport the shRNA expression cassette into a variety of recipient vectors that carry *cis*-linked selectable markers. Furthermore, we have validated delivery of shRNAs using retroviral vectors. Updated plasmid information can be obtained at http://www.cshl.org/public/science/hannon.html.

Plasmids expressing hairpin RNAs

The U6 promoter region from −265 to +1 was amplified by PCR, adding 5′ *Kpn*I and 3′ *Eco*RV sites for cloning into pBSSK+. A linker/terminator oligonucleotide set bearing the U6 terminator sequence and linker ends of 5′ *Eco*RV and 3′ *Not*I was cloned into the promoter construct, resulting in a U6 cassette with an *Eco*RV site for insertion of new sequences. This vector has been named pShb1. Blunt-ended, double-stranded DNA oligonucleotides encoding shRNAs with between 19 and 29 bases of homology to the targeted gene were ligated into the *Eco*RV site to produce expression constructs. The oligonucleotide sequence used to construct Ff1 was: TCCAATTCAGCGGGAGCCACC TGATGAAGCTTGATCGGGTGGCTCTCGCTGAGTTGGA ATCCATTTTTTTT. This sequence is preceded by the sequence GGAT, which is supplied by the vector, and contains a tract of more than five Ts as a Pol III terminator.

In vitro Dicer assays

In vitro assays for Dicer activity were performed as described (Bernstein et al. 2001a).

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Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells

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Abstract

RNA interference was first recognized in *C. elegans* as a biological response to exogenous double-stranded RNA (dsRNA), which induces sequence-specific gene silencing (Fire et al. 1998). RNAi represents a conserved regulatory motif, which is present in a wide range of eukaryotic organisms (reviewed in Bernstein et al. 2001b; Hammond et al. 2001b). Recently, we (Ketting et al. 2001) and others (Grishok et al. 2001; Hutvagner et al. 2001; Knight and Bass 2001) have demonstrated that endogenously encoded triggers of gene silencing act through elements of the RNAi machery to regulate the expression of protein coding genes. These stRNAs are transcribed as small hairpin precursors (~70 nt) (Reinhart et al. 2000), processed into active, 21nt RNAs by Dicer (Grishok et al. 2001; Hutvagner et al. 2001; Ketting et al. 2001; Knight and Bass 2001) and recognize target mRNAs via base-pairing interactions (Wightman et al. 1993; Ha et al. 1996; Slack et al. 2000). Here, we show that small hairpin RNAs (shRNAs) can be engineered to suppress the expression of desired genes in cultured *Drosophila* and mammalian cells. shRNAs can be synthesized exogenously or can be transcribed from RNA polymerase III promoters *in vivo*, thus permitting the construction of continuous cell lines or transgenic animals in which RNAi enforces stable and heritable gene silencing.

Introduction

An understanding of the biological role of any gene comes only after observing the phenotypic consequences of altering the function of that gene in a living cell or organism. In many cases, those organisms for which convenient methodologies for genetic manipulation exist blaze the trail toward an understanding of similar genes in less tractable organisms, such as mammals. The advent of RNA interference (RNAi) as an investigational tool has demonstrated the potential to democratize at least one aspect of genetic manipulation, the creation of hypomorphic alleles, in organisms ranging from unicellular parasites (e.g., Shi et al. 2000) to mammals (Svoboda et al. 2000; Wianny and Zernicka-Goetz 2000).

While C. elegans has, for some time, been well developed as a forward genetic system, the lack of methodologies for gene replacement by homologous recombination presented a barrier to assessing rapidly the consequences of loss-of-function in known genes. In an effort to overcome this limitation, Mello and Fire (Fire et al. 1998), building on earlier studies (Guo and Kemphues 1995), probed the utility of antisense RNA as a method for suppressing gene expression in worms. Through these efforts, they found that dsRNA was much more effective than antisense RNA as an inducer of gene silencing. Subsequent studies have demonstrated that RNAi is a conserved biological response that is present in many, if not most, eukaryotic organisms (reviewed in Bernstein et al. 2001b; Hammond et al. 2001b).

As a result of biochemical and genetic approaches in several experimental systems, the mechanisms underlying RNAi have begun to unfold (reviewed in Bernstein et al. 2001b; Hammond et al. 2001b). These suggest the existence of a conserved machinery for dsRNA-induced gene silencing, which proceeds via a two-step mechanism. In the first step, the dsRNA silencing trigger is recognized by an RNAseIII-family nuclease, Dicer, which cleaves the dsRNA into ~21-23 nt. siRNAs (small interfering RNAs). These siRNAs are incorporated into a multi-component nuclease complex, RISC, which identifies substrates through their homology to siRNAs and targets these cognate mRNAs for destruction.

Although it was clear from the outset that RNAi would prove a powerful tool for manipulating gene expression in invertebrates, there were several potential impediments to the use of this approach in mammalian cells. Most mammalian cells harbor a potent antiviral response that is triggered by the presence of dsRNA viral replication intermediates. A key component of this response is a dsRNA-activated protein kinase, PKR, which phosphorylates EIF-2α, inducing, in turn, a generalized inhibition of translation (reviewed in Williams 1997; Gil and Esteban 2000). In addition, dsRNA activates the 2'5' oligoadenylate polymerase/RNAseL system and represses IκB. The ultimate outcome of this set of responses is cell death via apoptosis.

Thus, it came as a welcome surprise that dsRNA could induce sequence-specific silencing in mammalian embryos, which apparently lack generalized responses to dsRNA (Svoboda et al. 2000; Wianny and Zernicka-Goetz 2000). Indeed, microinjection of dsRNA into mouse zygotes could specifically silence both exogenous reporters and endogenous genes to create anticipated phenotypes. Subsequently, these observations were extended to embryonic cell lines, such as embryonic stem cells and embryonal carcinoma cells, which do not exhibit generic translational repression in response to dsRNA (Billy et al. 2001; Yang et al. 2001; Paddison et al. 2002). However, restriction of conventional RNAi to these few embryonic and cell culture systems would place a significant limitation on the utility of this approach in mammals.

Tuschl and colleagues first demonstrated that short RNA duplexes, designed to mimic the products of the Dicer enzyme, could trigger RNA interference *in vitro* in Drosophila embryo extracts (Tuschl et al. 1999; Elbashir et al. 2001b; Elbashir et al. 2001c). This observation was extended to mammalian somatic cells by Tuschl and co-workers (Elbashir et al. 2001a) and by Fire and colleagues (Caplen et al. 2001), who demonstrated that chemically synthesized siRNAs could induce gene silencing in a wide range of human and mouse cell lines. The use of synthetic siRNAs to transiently suppress the expression of target genes is quickly becoming a method-of-choice for probing gene function in mammalian cells.

Dicer, the enzyme that normally produces siRNAs *in vivo*, has been linked to RNA interference both through biochemistry and through genetics (Bernstein et al. 2001a; Grishok et al. 2001; Ketting et al. 2001; Knight and Bass 2001). Indeed, C. elegans that lack Dicer are RNAi-deficient, at least in some tissues. However, these animals also have additional phenotypic abnormalities. Specifically, they are sterile and display a number of developmental abnormalities that typify alterations in developmental timing . Indeed, the phenotypes of the Dicer mutant animals were similar to those previously observed for animals carrying mutations in the let-7 gene (Reinhart et al. 2000).

Let-7 encodes a small, highly conserved RNA species, which regulates the expression of endogenous protein coding genes during worm development. The active RNA species is transcribed initially as an ~70 nt. precursor, which is post-transcriptionally processed into a mature ~21 nt. form (Reinhart et al. 2000). Both *in vitro* and *in vivo* data from C. elegans (Grishok et al. 2001; Ketting et al. 2001; Knight and Bass 2001) and human cells (Hutvagner et al. 2001) have pointed to Dicer as the enzyme responsible for let-7 maturation and for the maturation of a similar small RNA, lin-4 (Grishok et al. 2001). Thus, at least some components of the RNAi machinery respond to endogenously encoded triggers to regulate the expression of target genes.

Recent studies have placed let-7 and lin-4 as the founding members of a potentially very large group of small RNAs known generically as miRNAs (micro-

RNAs). Nearly 100 potential miRNAs have been now been identified in Drosophila, C. elegans and mammals (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001). While the functions of these diverse RNAs remain mysterious, it seems likely that they, like let-7 and lin-4, are transcribed as hairpin RNA precursors, which are processed to their mature forms by Dicer (Lee and Ambros 2001), and our unpublished data).

Since the realization by our laboratory and by others that small, endogenously encoded hairpin RNAs could regulate gene expression via elements of the RNAi machinery, we have sought to exploit this biological mechanism for the regulation of desired target genes. Here we show that small hairpin RNAs (shRNAs) can induce sequence-specific gene silencing in mammalian cells. As is normally done with siRNAs, silencing can be provoked by transfecting exogenously synthesized hairpins into cells. However, silencing can also be triggered by endogenous expression of shRNAs. This observation opens the door to the production of continuous cells lines in which RNAi is used to suppress stably gene expression in mammalian cells. Furthermore, similar approaches should prove efficacious in the creation of transgenic animals and potentially in therapeutic strategies in which long-term suppression of gene function is essential to produce a desired effect.

Results and Discussion

Short Hairpin RNAs trigger gene silencing in Drosophila cells

Several groups (Grishok et al. 2001; Hutvagner et al. 2001; Ketting et al. 2001; Knight and Bass 2001) have previously shown that endogenous triggers of gene silencing, specifically that stRNAs, let-7 and lin-4 RNAs, function, at least in part through RNAi pathways. Specifically, these small RNAs are encoded by hairpin precursors that are processed by Dicer into mature, ~21 nt. forms. Moreover, genetic studies in C. elegans have demonstrated a requirement for Argonaute-family proteins in stRNA function. Specifically, alg-1 and alg-2, members of the EIF2c subfamily are implicated both in stRNA processing and in their downstream effector functions (Grishok et al. 2001). We have recently shown that a component of RISC, the effector nuclease of RNAi, is a member of the Argonaute family, prompting a model in which stRNAs may function through RISC-like complexes, which regulate mRNA translation rather than mRNA stability (Hammond et al. 2001a).

We wished to test the possibility that we might re-target these small, endogenously encoded hairpin RNAs to regulate genes of choice with the ultimate goal of subverting this regulatory system for manipulating gene expression stably in mammalian cell lines and in transgenic animals. Whether triggered by long dsRNAs or by siRNAs, RNAi is generally more potent in the suppression of gene expression in Drosophila S2 cell than in mammalian cells.

We therefore chose this model system in which to test the efficacy of small hairpin RNAs (shRNAs) as inducers of gene silencing.

Neither stRNAs nor the broader group of miRNAs that has recently been discovered form perfect hairpin structures. Indeed, each of these RNAs is predicted to contain several bulged nucleotides within their rather short (~30 nt) stem structures. Since the position and character of these bulged nucleotides has been conserved throughout evolution and amongst at least a subset of miRNAs, we sought to design re-targeted miRNA mimics to conserve these predicted structural features. Only the let-7 and lin-4 miRNAs have known mRNA targets (Wightman et al. 1993; Slack et al. 2000). In both cases, pairing to binding sites within the regulated transcripts is imperfect, and in the case of lin-4, the presence of a bulged nucleotide is critical to suppression (Ha et al. 1996). We therefore also designed shRNAs, which paired imperfectly with their target substrates. A subset of these shRNAs is depicted in Figure 1A.

To permit rapid testing of large numbers of shRNA variants and quantitative comparison of the efficacy of suppression, we chose to use a dual-luciferase reporter system, as previously described for assays of RNAi in both Drosophila extracts (Tuschl et al. 1999) and mammalian cells (Caplen et al. 2001; Elbashir et al. 2001a). Co-transfection of firefly and Renilla luciferase reporter plasmids with either long dsRNAs or with siRNAs homologous to the firefly luciferase gene yielded an approximately 95 percent suppression of firefly

luciferase without effect on Renilla luciferase (Figure 1B and not shown). Firefly luciferase could also be specifically silenced by co-transfection with homologous shRNAs. Surprisingly, those shRNAs modeled most closely on the let-7 paradigm were the least effective inducers of silencing (data not shown). The inclusion of bulged nucleotides within the shRNA stem caused only a modest reduction in potency; however, the presence of mismatches with respect to the target mRNA essentially abolished silencing potential. The most potent inhibitors were those composed of simple hairpin structures with complete homology to the substrate. Introduction of G-U basepairs either within the stem or within the substrate recognition sequence had little or no effect (Figure 1A,B and not shown). Similarly, varying either the loop size from approximately 4 to 23 bases or the loop sequence (e.g. to mimic let-7) also proved neutral (not shown).

These results demonstrate that short hairpin RNAs can induce gene silencing in Drosophila S2 cells with potency similar to that of siRNAs (Figure 1B). However, in our initial observation of RNA interference in Drosophila S2 cells, we noted a profound dependence of the efficiency on silencing on the length of the dsRNA trigger (Hammond et al. 2000). Indeed dsRNAs of fewer than ~200 nt. triggered silencing very inefficiently. Silencing is initiated by and RNAse III family nuclease, Dicer, that processes long dsRNAs into ~22 nt. siRNAs. In accord with their varying potency as initiators of silencing, long dsRNAs are processed much more readily than short RNAs by the Dicer enzyme

(Bernstein et al. 2001a). We therefore tested whether shRNAs were substrates for the Dicer enzyme.

We had previously noted that let-7 (Ketting et al. 2001), and other miRNAs (E.B., unpublished), are processed by Dicer with an unexpectedly high efficiency as compared to short, non-hairpin dsRNAs. Similarly, Dicer efficiently processed shRNAs that targeted firefly luciferase, irrespective of whether they were designed to mimic a natural Dicer substrate (let-7) or whether they were simple hairpin structures (Figure 1C). This data suggests that recombinant shRNAs can be processed by Dicer into siRNAs and is consistent with the notion that these small hairpins trigger gene silencing via an RNAi pathway.

Short Hairpin Activated Gene Silencing in Mammalian Cells

RNAi is developing into an increasingly powerful methodology for manipulating gene expression in diverse experimental systems. However, mammalian cells contain several endogenous systems that were predicted to hamper the application of RNAi. Chief among these is a dsRNA-activated protein kinase, PKR, which effects a general suppression of translation via phosphorylation of EIF-2α (Williams 1997; Gil and Esteban 2000). Activation of these, and other dsRNA-responsive pathways, generally requires duplexes exceeding 30 bp in length, possibly to permit dimerization of the enzyme on its allosteric activator (e.g. (Clarke and Mathews 1995).

Small RNAs that mimic Dicer products, siRNAs, presumably escape this limit and trigger specific silencing, in part because of their size. However, short duplex RNAs that lack signature features of siRNAs can efficiently induce silencing in Drosophila S2 cells but not in mammalian cells (A.A.C., unpublished). Endogenously encoded miRNAs may also escape PKR surveillance because of their size but perhaps also because of the discontinuity of their duplex structure. Given that shRNAs of fewer than 30 bp were effective inducers of RNAi in Drosophila S2 cells, we tested whether these RNAs could also induce sequence-specific silencing in mammalian cells.

Human embryonic kidney (HEK293T) cells were co-transfected with chemically synthesized shRNAs and with a mixture of firefly and Renilla luciferase reporter plasmids. As had been observed in S2 cells, shRNAs were effective inducers of gene silencing. Once again, hairpins designed to mimic let-7 were consistently less effective than were simple hairpin RNAs, and the introduction of mismatches between the antisense strand of the shRNA and the mRNA target abolished silencing (Fig 2A and not shown). Overall, shRNAs were somewhat less potent silencing triggers than were siRNAs. While siRNAs homologous to firefly luciferase routinely yielded ~90-95% suppression of gene expression, suppression levels achieved with shRNAs ranged from 80-90%, on average. As we also observe with siRNAs, the most important determinant of the potency of the silencing trigger is its sequence. We find that roughly 50% of both

siRNAs and shRNAs are competent for suppressing gene expression. However, neither analysis of the predicted structures of the target mRNA nor analysis of alternative structures in siRNA duplexes or shRNA hairpins has proven of predictive value for choosing effective inhibitors of gene expression.

We have adopted as a standard, shRNA duplexes containing 29 base pairs. However, the size of the helix can be reduced to approximately 25 nucleotides without significant loss of potency. Duplexes as short as 22 base pairs can still provoke detectable silencing but do so less efficiently than do longer duplexes. In no case do we observe a reduction in the internal control reporter (Renilla luciferase) that would be consistent with an induction of non-specific dsRNA responses.

The ability of shRNAs to induce gene silencing was not confined to 293T cells. Similar results were also obtained in a variety of other mammalian cell lines, including human cancer cells (HeLa), transformed monkey epithelial cells (COS-1), murine fibroblasts (NIH 3T3), and diploid human fibroblasts (IMR90) (Figure 2 and not shown).

Synthesis of effective inhibitors of gene expression using T7 RNA polymerase

The use of siRNAs to provoke gene silencing is developing into a standard methodology for investigating gene function in mammalian cells. To

date, siRNAs have been produced exclusively by chemical synthesis (e.g., (Caplen et al. 2001; Elbashir et al. 2001a). However, the costs associated with this approach are significant, limiting its potential utility as a tool for investigating in parallel the function of large numbers of genes. Short hairpin RNAs are presumably processed into active siRNAs *in vivo* by Dicer (see Figure 1C). Thus, these may be more tolerant of terminal structures, both with respect to nucleotide overhangs and with respect to phosphate termini. We therefore tested whether shRNAs could be prepared by *in vitro* transcription with T7 RNA polymerase.

Transcription templates that were predicted to generate siRNAs and shRNAs similar to those prepared by chemical RNA synthesis were prepared by DNA synthesis (Figure 3A, 3C). These were tested for efficacy both in S2 cells (not shown) and in human 293 cells (Figure 3B, 3D). Overall, the performance of the T7-synthesized hairpin RNAs closely matched the performance of either produced by chemical synthesis, both with respect to the magnitude of inhibition and with respect to the relative efficiency of differing sequences. Since T7 polymerase prefers to initiate at twin guanosine residues in the primer sequence, however, it was critical to consider initiation context when designing *in vitro* transcribed siRNAs (Figure 3B). By contrast, shRNAs, which are processed by Dicer (see Figure 1C), tolerate the addition of these bases at the 5' end of the transcript.

Studies in Drosophila embryo extracts indicate that siRNAs possess 5' phosphorylated termini, consistent with their production by an RNAse III family nuclease (Bernstein et al. 2001a; Elbashir et al. 2001b). *In vitro*, this terminus is critical to the induction of RNAi by synthetic RNA oligonucleotides (Elbashir et al. 2001c; Nykanen et al. 2001). Chemically synthesized siRNAs are non-phosphorylated, and enzymatic addition of a 5' phosphate group *in vitro* prior to transfection does not increase the potency of the silencing effect (our unpublished results). This suggests either that the requirement for phosphorylated termini is less stringent in mammalian cells or that a kinase efficiently phosphorylates siRNAs *in vivo*. RNAs synthesized with T7 RNA polymerase possess 5' triphosphate termini; however, monophosphate termini can be generated either by treatment *in vitro* with pyrophosphatase or by priming T7 transcription with GMP. We therefore explored the possibility of synthesizing siRNAs using T7 polymerase and modifying the termini to resemble those of siRNAs.

Transcription templates derived from firefly luciferase were designed to produce separately 21 nt. RNAs, which would produce an siRNA duplex upon hybridization. These RNAs were prepared with triphosphate termini and with monophosphate termini, hybridized and transfected into 293 cells. Surprisingly, not only were monophosphorylated siRNAs potent inducers of gene silencing but also transcription products bearing triphosphate termini were effective as siRNAs (Figure 3B).

Considered together, our data suggest that both shRNAs and siRNA duplexes can be prepared by synthesis with T7 RNA polymerase *in vitro*. This significantly reduces the cost of RNAi in mammalian cells and paves the way for application of RNAi on a whole-genome scale.

Transcription of shRNAs in vivo by RNA polymerase III

While siRNAs are an undeniably effective tool for probing gene function in mammalian cells, their suppressive effects are by definition of limited duration. Delivery of siRNAs can be accomplished by any of a number of transient transfection methodologies and both the timing of peak suppression and the recovery of protein levels as silencing decays can vary with both the cell type and the target gene (Y. Seger and E.B., unpublished). Thus, one limitation on siRNAs is the development of continuous cell lines in which the expression of a desired target is stably silenced.

Hairpin RNAs, consisting of long duplex structures, have been proven as effective triggers of stable gene silencing in plants, in C. elegans and in Drosophila (Kennerdell and Carthew 2000; Smith et al. 2000; Tavernarakis et al. 2000). We have recently demonstrated stable suppression of gene expression in cultured mammalian cells by continuous expression of a long hairpin RNA (Paddison et al. 2002). However, the scope of this approach was limited by the

necessity of expressing such hairpins only in cells that lack a detectable PKR response. In principle, shRNAs could bypass such limitations and provide a tool for evoking stable suppression by RNA in mammalian somatic cells.

To test this possibility, we initially cloned sequences encoding a firefly luciferase shRNA into a CMV-based expression plasmid. This was predicted to generate a capped, polyadenylated RNA polymerase II transcript in which the hairpin was extended on both the 5' and 3' ends by vector sequences and poly A. This construct was completely inert in silencing assays in 293T cells.

During our studies on chemically- and T7- synthesized shRNAs, we noted that the presence of significant single stranded extensions (either 5' or 3' of the duplex) reduced the efficacy of shRNAs (not shown). We therefore explored the use of alternative promoter strategies in an effort to produce more defined hairpin RNAs. In particular, RNA polymerase III promoters have well defined initiation and termination sites and naturally produce a variety of small, stable RNA species. Although many polIII promoters contain essential elements within the transcribed region, limiting their utility for our purposes; class III promoters use exclusively non-transcribed promoter sequences. Of these, the U6 snRNA promoter and the H1 RNA promoter have been well studied (Lobo et al. 1990; Hannon et al. 1991; Chong et al. 2001).

By placing a convenient cloning site immediately behind the U6 snRNA promoter we have constructed pShh-1, an expression vector in which short hairpins are harnessed for gene silencing. Into this vector either of two shRNA sequences derived from firefly luciferase were cloned from synthetic oligonucleotides. These were co-transfected with firefly and Renilla luciferase expression plasmids into 293T cells. One of the two encoded shRNAs provoked effective silencing of firefly luciferase without altering the expression of the internal control (Figure 4B). The second encoded shRNA also produced detectable, albeit weak, repression. In both cases, silencing was dependent upon insertion of the shRNA in the correct orientation with respect to the promoter (Figure 4B and not shown). Although the shRNA itself is bi-laterally symmetric, insertion in the incorrect orientation would affect pol III termination and is predicted to produce a hairpin with both 5' and 3' single-stranded extensions. Similar results were also obtained in a number of other mammalian cell lines including HeLa, COS-1, NIH 3T3, and IMR90 (Figure 4 and not shown). pShh1-Ff1 was, however, incapable of effecting suppression of the luciferase reporter in Drosophila cells, in which the human U6 promoter is inactive (data not shown).

As a definitive test of whether the plasmid-encoded shRNAs brought about gene silencing via the mammalian RNAi pathway, we assessed the dependence of suppression on an essential compent of the RNAi pathway. We transfected pShh1-Ff1 along with an siRNA homologous to human Dicer. Figure

5 shows that treatment of cells with Dicer siRNAs is able to completely depress the silencing induced by pShh1-Ff1. Addition of an unrelated siRNA had no effect on the magnitude of suppression by pShh1-Ff1 (data not shown). Importantly, Dicer siRNAs had no effect on siRNA-induced silencing of firefly luciferase (data not shown). These results are consistent with shRNAs operating via an RNAi pathway similar to those provoked by stRNAs and long dsRNAs. Furthermore, it suggests that siRNA-mediated silencing can operate independently of the Dicer enzyme.

Here, we have shown that small hairpin RNAs, modeled conceptually on miRNAs, are potent inducers of gene silencing in mammalian somatic cells. These shRNAs can be provided exogenously or can be synthesized *in vivo* from RNA polymerase III promoters. Not only does this enable the creation of continuous cell lines in which suppression of a target gene is stably maintained by RNAi but similar strategies may also be used in the construction of transgenic animals. Thus, short-hairpin activated gene silencing provides a complement to the use of siRNAs to rapidly investigate gene function in mammalian cells. Finally, the ability to encode a constitutive silencing signal may permit the marriage of shRNA-induced silencing with *in vivo* and *ex vivo* gene delivery methods for therapeutic approaches based upon stable RNAi in humans.

Materials and Methods

Cell Culture

HEK 293T, HeLa, COS-1, and IMR90 cells were cultured in DMEM (Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution (Gibco BRL). NIH 3T3 cells were cultured in DMEM supplemented with 10% heat-inactivated calf serum and 1% antibiotic/antimycotic solution.

RNA Preparation

Both shRNAs and siRNAs were produced *in vitro* using chemically synthesized DNA oligonucleotide templates (Sigma) and the T7 Megashortscript kit (Ambion). Transcription templates were designed such that they contained T7 promoter sequences at the 5' end. ShRNA transcripts subjected to *in vitro* Dicer processing were synthesized using a Riboprobe kit (Promega). Chemically synthesized RNAs were obtained from Dharmacon, Inc.

Transfection and Gene Silencing Assays

Cells were transfected with indicated amounts of siRNA, shRNA and plasmid DNA using standard calcium phosphate procedures at 50-70% confluence in 6-well plates. Dual luciferase assays (Promega) were carried out by co-transfecting cells with plasmids containing firefly luciferase under the control of SV40 promoter (pGL3-Control, Promega) and Renilla luciferase under the control

of the SV40 early enhancer/promoter region (pSV40, Promega). Plasmids were co-transfected using a 1:1 ratio of pGL3-Control (250ng/well) to pRL-SV40. RNAi in S2 cells was performed as previously described (Hammond et al. 2000).

Plasmids expressing hairpin RNAs

The U6 promoter region from -265 to +1 was amplified by PCR, adding 5' KpnI and 3' EcoRV sites for cloning into pBSSK+. A linker/terminator oligonucleotide set bearing the U6 terminator sequence and linker ends of 5' EcoRV and 3' NotI was cloned into the promoter construct, resulting in a U6 cassette with an EcoRV site for insertion of new sequences. This vector has been named pShh1. Blunt-ended, double stranded, DNA oligonucleotides encoding shRNAs with between 19 and 29 bases of homology to the targeted gene were ligated into the EcoRV site to produce expression constructs.

In vitro translation and in vitro Dicer assays

Logarithmically growing cells were harvested in PBS containing 5 mM EGTA washed twice in PBS and once in hypotonic buffer (10 mM HEPES pH 7.3, 6 mM β-mercaptoethanol). Cells were suspended in 0.7 packed-cell volumes of hypotonic buffer containing *Complete* protease inhibitors (Boehringer) and 0.5 units/ml of RNasin (Promega). Cells were disrupted in a dounce homogenizer with a type B pestle, and lysates were centrifuged at 30,000g for 20 min. Supernatants were used in an *in vitro* translation assay containing capped m7G(5')pppG Firefly and Renilla luciferase mRNA or in *in vitro* Dicer assays

containing 32P-labeled dsRNA. Reactions were carried out for one hour at 30 degrees and quenched by adding 1x passive lysis buffer (Promega). Extracts were then assayed for luciferase activity. *In vitro* assays for Dicer activity were performed as described (Bernstein et al. 2001a).

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Figure Legends

Figure 1. Short hairpins suppress gene expression in Drosophila S2 cells. **A.** Sequences and predicted secondary structure of representative chemically synthesized RNAs. Sequences correspond to positions 112-134 (siRNA) and 463-491 (shRNAs) of Firefly luciferase carried on pGL3-Control. **B.** Exogenously supplied short hairpins suppress expression of the targeted Firefly luciferase gene *in vivo*. 6 well plates of S2 cells were transfected with 250 ng/ well of plasmids that direct the expression of firefly and *Renilla* luciferases and 500ng/ well of the indicated RNA. Luciferase activities were assayed 48 hrs after transfection. Ratios of firefly to *Renilla* luciferase activity were normalized to a control transfected with an siRNA directed at the Green Fluorescent Protein. The average of three independent experiments is shown; error bars indicate standard deviation. **C.** Short hairpins are processed by the Drosophila Dicer enzyme. T7 transcribed hairpins shFfL22, shFfL29, and shFfS29 were incubated with (+) and without (-) 0-2 hour *Drosophila* embryo extracts. Those incubated with extract produced ~22 nt siRNAs, consistent with ability of these hairpins to induce RNA interference. A long dsRNA input (cyclin E 500mer) was used as a control. Cleavage reactions were performed as described in Bernstein et al. (Bernstein et al. 2001a).

Figure 2. Short hairpins function in mammalian cells.

A. HEK 293T, HeLa, Cos-1, and NIH 3T3 cells were transfected with plasmids and RNAs as in Figure 1 and subjected to Dual luciferase assays 48 hours post-

transfection. Ratios of Firefly to *Renilla* luciferase activity are normalized to a control transfected with an siRNA directed at the Green Fluorescent Protein. The average of three independent experiments is shown; error bars indicate standard deviation.

Figure 3. siRNAs and short hairpins transcribed *in vitro* suppress gene expression in mammalian cells.

A. Sequences and predicted secondary structure of representative *in vitro* transcribed siRNAs. Sequences correspond to positions 112-134 (siRNA) and 463-491 (shRNAs) of Firefly luciferase carried on pGL3-Control. **B.** *In vitro* transcribed siRNAs suppress expression of the targeted Firefly luciferase gene *in vivo*. HEK 293T cells were transfected with plasmids as in Figure 2. The presence of guanosine residues at the 5' end of siRNAs significantly reduce siRNA activity. **C.** Sequences and predicted secondary structure of representative *in vitro* transcribed shRNAs. Sequences correspond to positions 112-141 of Firefly luciferase carried on pGL3-Control. **D.** Short hairpins transcribed *in vitro* suppress expression of the targeted Firefly luciferase gene *in vivo*. HEK 293T cells were transfected with plasmids as in Figure 2.

Figure 4. Transcription of functional shRNAs *in vivo*. A. Schematic of the pShh1 vector. Sequences encoding shRNAs with between 19 and 29 bases of homology to the targeted gene are synthesized as 60-75 bp double stranded DNA oligonucleotides and ligated into an EcoRV site immediately downstream of

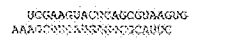
the U6 promoter. **B.** An shRNA expressed from the pShh1 vector suppresses luciferase expression in mammalian cells. HEK 293T, HeLa, Cos-1, and NIH 3T3 cells were transfected with reporter plasmids as in Figure 1, and either pShh1 vector, firefly siRNA, or pShh1 Firefly shRNA constructs as indicated. Ratios of firefly to *Renilla* luciferase activity were determined 48 hrs after transfection and represent the average of three independent experiments is shown; error bars indicate standard deviation.

Figure 5. Dicer is required for shRNA-mediated gene silencing. HEK 293T cells were transfected with luciferase reporter plasmids as well as pShh1-Ff1 and an siRNA targeting human Dicer either alone or in combination, as indicated. The dicer siRNA sequence (TCA ACC AGC CAC TGC TGG A) corresponds to coordinates 3137-3155 of the human Dicer sequence. Ratios of firefly to *Renilla* luciferase activity were determined 26 hrs after transfection and represent the average of three independent experiments is shown; error bars indicate standard deviation.

Fig. 1

A.

siRNA



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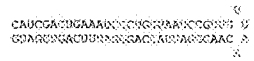

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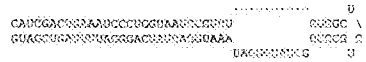

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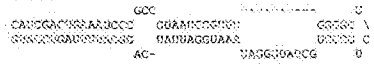


Fig. 1

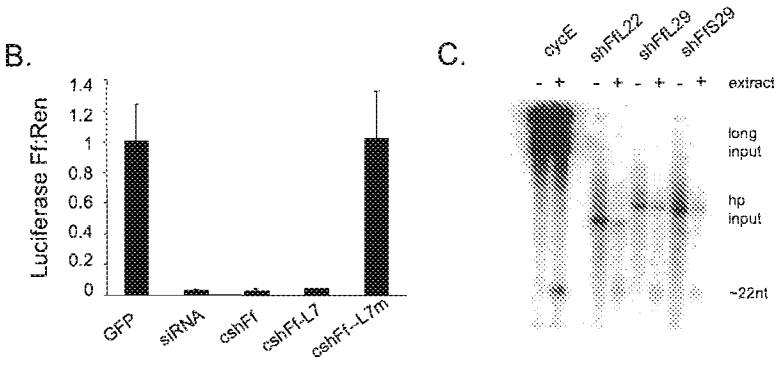
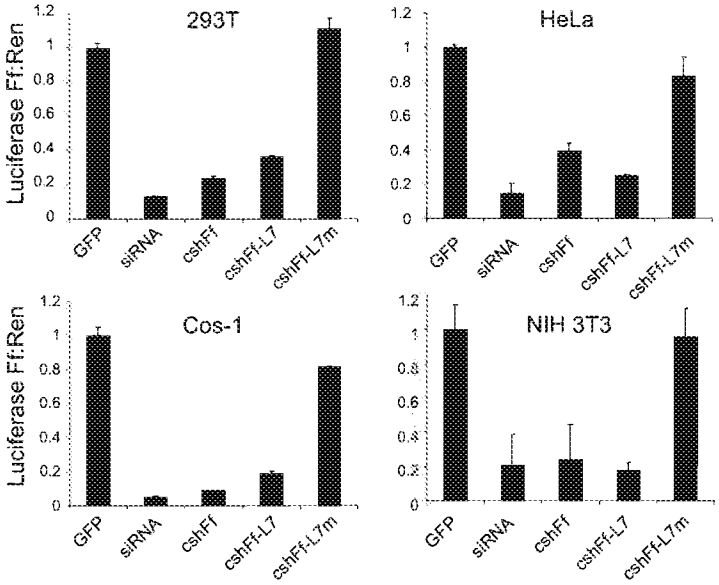


Fig. 2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HeLa
Cos-1
NIH 3T3
Luciferase Ff:Ren
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0.8
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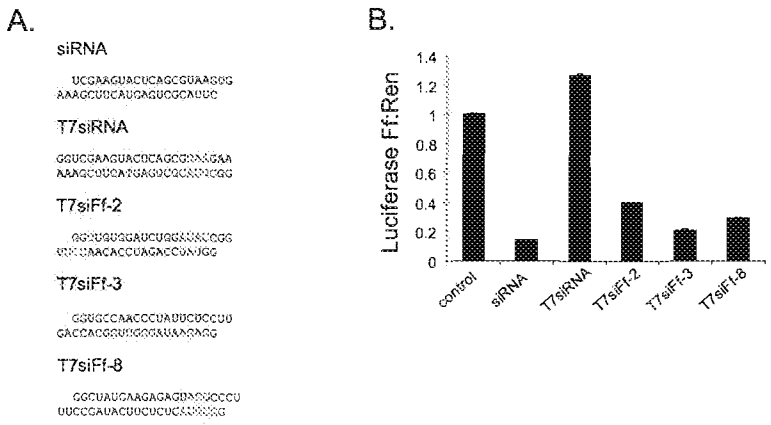
0.4
0.2
0
GFP
siRNA
cshFf
cshFf-L7
cshFf-L7m

Fig. 3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T7siRNA
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T7siFf-2
GGUGUGGAUCUGGAUACGG
CCCAACACCUAGACCUAUGG
T7siFf-3
GGUGCCAACCCUAUUCCCUU
GACCACGGUUGGGAUAAGAG
T7siFf-8
GGCUAUGAAGAGAUACGCCU
UUCCGAUACUUCUCUAUGCG
B.
Luciferase Ff:Ren
1.4
1.2
1
0.8
0.6
0.4
0.2
0
control
siRNA
T7siRNA
T7siFf-2
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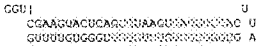
T7siFf-8

C.

T7shFf29



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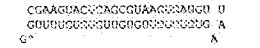
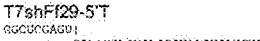
T7shFf25

T7shFf22



T7shFf29-3'T

T7shFf29-5'T



D.

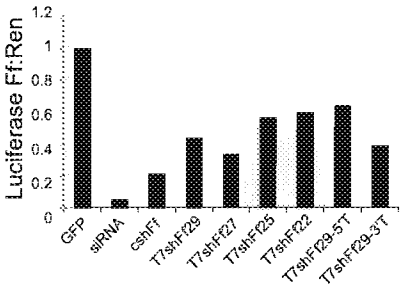


Fig. 4

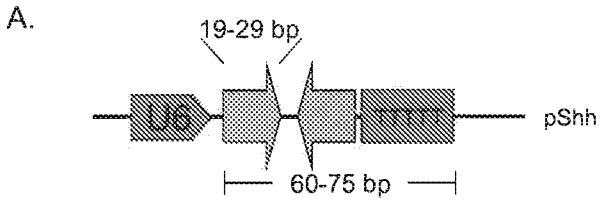


Fig. 4

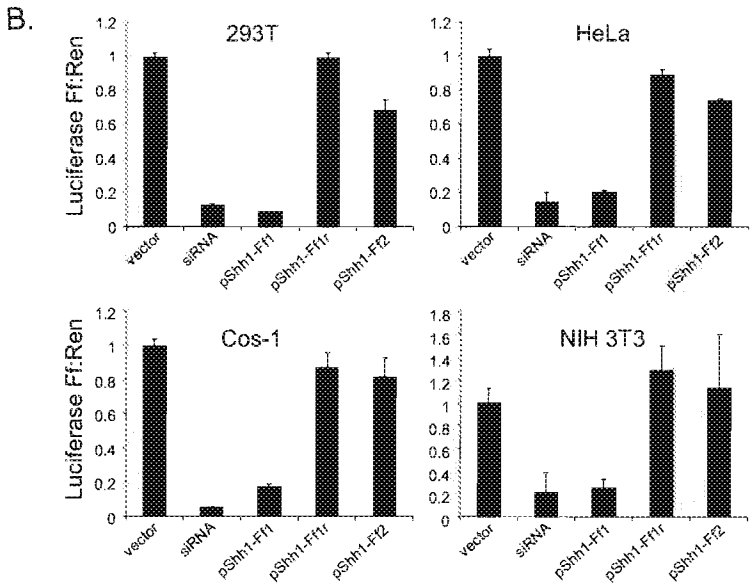


Fig. 5

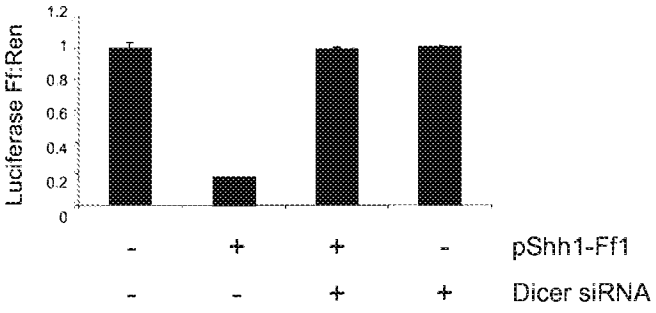


Exhibit M

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Analyses : Featured Analyses : Sequencing Biology's Hottest, 2002-06

FEATURED ANALYSIS, JAN./FEB. 2008

Sequencing Biology's Hottest, 2002-06

by Christopher King

To assess high-impact research in molecular biology & genetics over the last five years, *ScienceWatch.com* turned to an elite selection of papers: those ranking among the top 1% most cited in the field for their respective years of publication, among papers published and cited between 2002 and 2006. This benchmark, applied to a special five-year subset of reports collected in the "Highly Cited Papers" area of Thomson Scientific's *Essential Science Indicators*℠ database, produced a file of some 1,300 papers published in Thomson-indexed journals of molecular biology & genetics.

(The analysis included pertinent papers published in the multidisciplinary journals *Nature*, *Science*, and *PNAS*.)

From this population of high-impact reports, *ScienceWatch.com* identified the institutions, authors, and journals most heavily represented.

#Table 1 (below) features institutions that fielded at least 10 high-impact reports over the five-year period. In the left-hand column, institutions are ranked by total citations, while the right column ranks institutions by impact, or cites per paper. Listings in table 2 (below) specify individual researchers who each contributed to at least eight high-impact reports, along with journals (table to the right) that published 20 or more such papers.

As was the case when *Science Watch* last surveyed high-impact research in this field (11[5]: 1-2, September-October 2000), the Howard Hughes Medical Institute garnered the highest citation total of any institution, with nearly 38,000 collective cites. HHMI-affiliated authors, in fact, contributed to 199 high-impact reports, a total approached only by Harvard's 165 papers.

(As the previous survey noted, HHMI employs and supports researchers who are based at numerous universities and institutions. In tallying citations to papers by Hughes investigators, *Science Watch* credited both HHMI and the investigators' home-base institutions, since Hughes investigators usually list both affiliations in their papers, and since such papers often include the contributions of non-Hughes-supported coauthors at each institution.)

Journals Publishing High-Impact Research in Molecular Biology & Genetics, 2002-06
(Ranked by number of high-impact papers, among those that published ≥20)

Rank	Journal	# of high-impact papers
1	Cell	258
2	Nature	154
3	Science	149
4	Nature Genetics	104
5	Nature Reviews Molecular Cell Biology	93
6	Genes & Development	61
7	Molecular Cell	58
8	Nature Reviews Genetics	35
	Nature Cell Biology	35
9	PNAS	29
10	American J. Human Genetics	24
11	Ann. Rev. Cell/Devel. Biology	20
	Ann. Rev. Genetics	20
	Journal of Cell Biology	20

SOURCE: *Essential Science Indicators*℠ from Thomson Scientific

In the cites-per-paper column, no institution surpassed the University of California, Santa Cruz, even though UCSC fielded only 13 high-impact molecular biology & genetics reports during the five-year period. One of these, however, was the most-cited paper in the survey, a 2002 *Nature* report on the mouse genome (Mouse Genome Sequencing Consortium, *Nature* 420[6915]: 520-62, 2002). This paper has now been cited more than 1,700 times.

Among the 200-plus coauthors on this blockbuster mouse-genome report was MIT's Eric S. Lander, who topped the researcher list with 22 high-impact reports. (The list is ranked by number of high-impact papers, with the subsequent order determined by total citations). Lander also contributed to highly cited genomic studies centered on yeast, the chimpanzee, and the domestic dog.

Christopher King is the Editor of the *Science Watch*® Newsletter.

Table 1

Molecular Biology & Genetics Research:
Institutions Ranked by Citations and Citation Impact
(among those that published ≥10 high-impact papers, 2002-06)

Rank	Institution	#Citations 2002-06	Rank	Institution	Impact 2002-06
1	Howard Hughes Medical Inst.	37,810	1	Univ. Calif., Santa Cruz	414.5
2	Harvard University	31,725	2	Natl. Human Genome Res. Inst., NIH	337.5
3	MIT	24,868	3	Cold Spring Harbor Lab	336.5
4	Whitehead Institute	11,326	4	University of Utah	335.6
5	Univ. Calif., San Diego	11,120	5	Medical Research Council (U.K.)	333.1
6	Cold Spring Harbor Lab	10,767	6	Inst. for Systems Biology, Seattle	329.8
7	Univ. Calif., Berkeley	9,756	7	European Molec. Biology Lab	326.9
8	Baylor College of Medicine	9,754	8	University of Oxford	324.9
9	University of Oxford	9,421	9	Wellcome Trust Sanger Inst.	311.6
10	Max Planck Society	9,354	10	Pennsylvania State Univ.	304.4
11	Johns Hopkins University	9,076	11	Whitehead Institute	290.4
12	Stanford University	8,850	12	University of Edinburgh	272.2
13	European Molec. Biology Lab	8,826	13	Washington University	269.6
14	Yale University	8,808	14	MIT	267.4
15	Univ. Calif., San Francisco	8,692	15	Univ. Calif., Berkeley	256.7
16	Wellcome Trust Sanger Inst	8,412	16	Univ. Calif., San Diego	252.7
17	Washington University	8,358	17	Massachusetts General Hosp.	251.0
18	Massachusetts General Hosp.	7,029	18	Univ. Southern California	250.8
19	National Cancer Institute, NIH	6,408	19	CNRS (France)	247.5
20	University of Cambridge	6,080	20	Mt. Sinai Hospital, Toronto	246.5
21	University of Washington	5,878	21	Max Planck Society	246.2
22	Rockefeller University	5,850	22	Baylor College of Medicine	243.9
23	Scripps Research Institute	5,809	23	Scripps Research Institute	241.8
24	Natl. Human Genome Res. Inst., NIH	5,737	24	Fred Hutchinson Cancer Res. Ctr	232.8
25	Medical Research Council (U.K.)	5,662	25	Yale University	231.8

SOURCE: *Essential Science Indicators*℠ from Thomson Scientific.

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Table 2

Authors of High-Impact Papers in Molecular Biology & Genetics, 2002-06

(Ranked by number of high-impact papers)

Rank	Name	Affiliation	Number of high-impact papers	Citations	Citations per high-impact paper
1	Eric S. Lander	MIT Broad Institute	22	9,710	441.4
2	David P. Bartel	HHMI, MIT, Whitehead Institute	19	4,542	239.1
3	C. David Allis	Rockefeller University	13	1,986	152.8
4	W. James Kent	Univ. Calif., Santa Cruz	12	5,158	429.8
5	Gregory J. Hannon	HHMI, Cold Spring Harbor Lab	12	3,542	295.2
6	Thomas Jenuwein	Res. Inst. Molec. Pathology, Vienna	11	1,762	160.2
7	Yi Zhang	HHMI, University of North Carolina	11	1,249	113.6
8	Mark J. Daly	Harvard University	10	5,428	542.8
9	David Haussler	HHMI, Univ. Calif., Santa Cruz	10	4,196	419.6
10	David Altshuler	Harvard University	10	3,868	386.8
11	Matthias Mann	Max Planck Inst. Biochemistry	10	2,960	296.0
12	Richard A. Young	MIT, Whitehead Institute	10	2,579	257.9
13	V. Narry Kim	Seoul National University	9	1,493	165.9
14	Peer Bork	European Molecular Biology Lab	8	5,068	633.5
15	Ewan Birney	European Molecular Biology Lab	8	4,141	517.6
16	Richard A. Gibbs	Baylor College of Medicine	8	3,533	441.6
17	Mark Gerstein	Yale University	8	2,037	254.6
18	Phillip Zamore	University of Massachusetts	8	1,802	225.3
19	Douglas R. Green	Univ. Calif., San Diego	8	1,754	219.3
20	Kari Stefansson	deCODE genetics, Iceland	8	1,650	206.3
	Jeffrey R. Gulcher	deCODE genetics, Iceland	8	1,650	206.3
	Augustine Kong	deCODE genetics, Iceland	8	1,650	206.3
21	Thomas Tuschl	HHMI, Rockefeller University	8	1,619	202.4
22	Jack F. Greenblatt	University of Toronto	8	1,236	154.5
	Nevan J. Krogan	University of Toronto	8	1,236	154.5
23	Paul Tempst	Memorial Sloan-Kettering Cancer Ctr.	8	1,391	173.9
	Hediye Erdjument-Bromage	Memorial Sloan-Kettering Cancer Ctr.	8	1,391	173.9
24	Steven P. Gygi	Harvard University	8	1,350	168.8
25	Stephen P. Jackson	University of Cambridge	8	1,052	131.5

SOURCE: *Essential Science Indicators*SM from Thomson Scientific.

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Analyses : Featured Analyses : Sequencing Biology's Hottest, 2002-06

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Exhibit N

Analyze Results

765 records. Paddison, PJ. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells

Rank the records by this field: Authorsss / Country/Territory / Funding Agency / Grant Number

Analyze: Up to 1000 Records.

Set display options: Show the top 500 Results. Minimum record count (Threshold): 2

Sort by: Record count / Selected field

Analyze

Use the checkboxes below to view the records. You can choose to view those selected records, or you can exclude them (and view the others)..
Note: The number of records displayed may be greater than the listed Record Count if the original set contained more records than the number of records analyzed.

View Records / Exclude Records — Save Analysis Data to File

Field: Publication Year	Record Count	% of 765	Bar Chart
2002	27	3.5294 %	
2003	122	15.9477 %	
2004	142	18.5621 %	
2005	115	15.0327 %	
2006	105	13.7255 %	
2007	82	10.7190 %	
2008	74	9.6732 %	
2009	59	7.7124 %	
2010	39	5.0980 %	

View Records / Exclude Records — Field: Publication Year — Record Count — % of 765 — Bar Chart — Save Analysis Data to File

This Year's AACR Awards

Exhibit O

Among this year's American Association for Cancer Research awards, presented at the Annual Meeting, were the following:

■ **Alfred G. Knudson Jr.**, MD, PhD, a Fox Chase Cancer Center Distinguished Scientist, received AACR's Lifetime Achievement in Cancer Research, given to an individual who has made significant, fundamental contributions to cancer research. He was honored for developing the well-known "two-hit" model that explained both the hereditary and sporadic forms of retinoblastoma, and later of neuroblastoma and Wilms' tumor. The research also inspired the discovery and study of tumor-suppressor genes.

■ **Lewis C. Cantley**, PhD, Professor of Systems Biology at Harvard Medical School and Chief of the Division of Signal Transduction at Beth Israel Deaconess Medical Center, received the Pezcoller Foundation-AACR International Award for Cancer Research, which recognizes an individual who has made a major scientific discovery in basic or translational cancer research. Dr. Cantley was recognized for his work in signal transduction, including the discovery of phosphoinositide 3-kinase.

■ **David M. Livingston**, MD, Deputy Director of Dana-Farber/Harvard Cancer Center and the Emil Frei Professor of Genetics and Medicine at Harvard Medical School, received the AACR-G.H.A. Clowes Memorial Award for groundbreaking contributions to the understanding of the molecular basis of cancer. The AACR and Eli Lilly and Company established this award in 1961 to honor Dr. Clowes, a founding member of AACR and a research director at Eli Lilly.

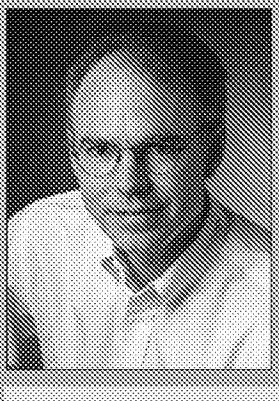
■ **Charles L. Sawyers**, MD, a Howard Hughes Medical Institute investigator and the Peter Bing Professor of Medicine and Director of the Prostate Cancer Program at UCLA Jonsson Comprehensive Cancer Center, received the AACR-Richard and Hinda Rosenthal Foundation Award, which recognizes notable contributions to improved clinical care by young investigators relatively early in their careers. Dr. Sawyers was honored for his outstanding research in molecularly targeted therapy, specifically signaling pathway abnormalities in cancer cells as targets for drug therapy.

■ **Napoleone Ferrara**, MD, a fellow at Genentech, was the recipient of the AACR-Bruce F. Cain Memorial Award, honored for his discovery of vascular endothelial cell growth factor and his research leading to the development of the anti-VEGF antibody.

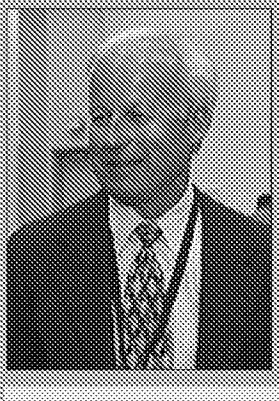
■ **Ross L. Prentice**, PhD, a member of the Division of Public Health Sciences at Fred Hutchinson Cancer Research Center and Professor of Biostatistics of the University of Washington, received the AACR-American Cancer Society Award for Research Excellence in Cancer Epidemiology and Prevention, honored especially for his role in conceiving, designing, and organizing the clinical trial arm of the Women's Health Initiative.

Alfred G. Knudson Jr., MD, PhD

Lewis C. Cantley, PhD



Charles L. Sawyers, MD



Daniel Q. Haney

■ **Jimmie C. Holland**, MD, the Wayne E. Chapman Chair in Psychiatric Oncology at Memorial Sloan-Kettering Cancer Center and Professor of Psychiatry at Weill Medical College of Cornell University, was honored as the recipient of the AACR-Joseph H. Burchenal Clinical Research Award, given for her central role in the establishment of psycho-oncology as a subspecialty.

■ **Gregory J. Hannon**, PhD, Professor in the Watson School of Biological Sciences of Cold Spring Harbor Laboratory, received the Award for Outstanding Achievement in Cancer Research, given to an accomplished young investigator in the field who is no more than 40 years old at the time the award is conferred. He was honored for his work uncovering the biochemical mechanism of RNA interference of gene expression (RNAi) and his contributions to the discovery and development of short hairpin RNAs as tools for genetic manipulation of mammalian cells.

■ **Edward Giovannucci**, MD, ScD, Professor of Nutrition and Professor of Epidemiology at Harvard School of Public Health, was chosen to deliver the AACR-DeWitt S. Goodman Memorial Lectureship. He spoke on "The Role of Vitamin D in Cancer Incidence and Mortality."

■ **Joan S. Brugge**, PhD, Chair of the Department of Cell Biology at Harvard Medical School, was chosen to give the AACR-Women in Cancer Research-Charlotte Friend Memorial Lectureship. She was honored for her research accomplishments that have provided critical insights into an understanding of the processes involved in oncogenesis and the normal functions of proto-oncogenes and for being a role model for women in cancer and biomedical science.

■ Public Service Awards were given to **Daniel Q. Haney**, former medical editor with the Associated Press, who retired last year after 34 years; US Rep. **Randy "Duke" Cunningham (R-CA)**; and **Miss America 2005 Deidre Downs**. Rep. Cunningham has been a strong and long-time supporter of funding for cancer research through his committee work in the House and related efforts on behalf of the biomedical research community, and his leadership was instrumental in achieving the five-year doubling of the budget for the NIH.

As Miss American, Ms. Downs made funding for childhood cancer research a central theme of her reign. Ms. Downs, an aspiring pediatrician who has been accepted as a medical student at the University of Alabama School of Medicine, is serving as a national spokesperson for childhood cancers and their families through CureSearch, a partnership with the Children's Oncology Group. Prior to her reign as Miss America, she launched outreach programs for cancer patients and research in her home state of Alabama. She created "Making Miracles," a program where high school students volunteer in the cancer unit at Children's Hospital in Birmingham, and spearheaded an initiative to persuade the state of Alabama to offer a "Curing Childhood Cancer" automobile license plate, the purchase of which helps to fund cancer research. ■

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Exhibit P

NEWS

FROM THE NATIONAL ACADEMIES

NATIONAL ACADEMY OF SCIENCES
NATIONAL ACADEMY OF ENGINEERING
INSTITUTE OF MEDICINE
NATIONAL RESEARCH COUNCIL

Date: Jan. 17, 2007
Contacts: Maureen O'Leary, Director of Public Information
Sarah Morocco, Media Assistant
Office of News and Public Information
202-334-2138; e-mail <news@nas.edu>

ACADEMY HONORS 18 FOR MAJOR CONTRIBUTIONS TO SCIENCE

WASHINGTON -- The National Academy of Sciences (NAS) will honor 18 individuals with awards recognizing extraordinary scientific achievements in the areas of astronomy, biology, medicine, chemistry, geology, oceanography, physics, and psychology. These outstanding scientists have made fundamental contributions to human knowledge, including a near-infrared survey of the entire sky, the discovery of the first statin for lowering cholesterol, and insights into how the human visual system learns to recognize objects.

The awards and recipients for 2007 are:

ALEXANDER AGASSIZ MEDAL – a medal and a prize of $15,000 awarded every three years for original contributions in the science of oceanography – goes to JAMES R. LEDWELL, senior scientist, department of applied ocean physics and engineering, Woods Hole Oceanographic Institution, Woods Hole, Mass., "for innovative and insightful tracer experiments using sulfur hexafluoride to understand vertical diffusivity and turbulent mixing in the open ocean." The medal was established by a gift of Sir John Murray and has been awarded since 1913.

JOHN J. CARTY AWARD FOR THE ADVANCEMENT OF SCIENCE – a medal and a prize of $25,000 awarded annually for noteworthy and distinguished accomplishment in any field of science (plant science in 2007) – goes to JOSEPH R. ECKER, professor, plant biology laboratory and genomic analysis laboratory, Salk Institute for Biological Studies, La Jolla, Calif., "for contributions in the areas of ethylene signal transduction and ARABIDOPSIS genomics that have paved the way for a revolution in modern agriculture." The award was established by the American Telephone & Telegraph Co. in honor of John J. Carty and has been awarded since 1932.

ALEXANDER HOLLAENDER AWARD IN BIOPHYSICS – a prize of $20,000 awarded every three years for outstanding contributions in the field of biophysics – goes to BARRY H. HONIG, investigator, Howard Hughes Medical Institute, and director, center for computational biology and bioinformatics, Columbia University, New York City, "for pioneering theoretical and computational studies of electrostatic interactions in biological macromolecules and of the energetics of protein folding." The award was established by the bequest of Henrietta W. Hollaender in honor of her husband, Alexander W. Hollaender, and has been presented since 1998.

JESSIE STEVENSON KOVALENKO MEDAL – a medal and a prize of $25,000 awarded every three years for important contributions to the medical sciences – goes to JEFFREY M. FRIEDMAN, investigator, Howard Hughes Medical Institute, and Marilyn M. Simpson

Professor, laboratory of molecular genetics, Rockefeller University, New York City, "for the discovery of leptin and its role in the regulation of appetite, energy expenditure, and the molecular mechanisms underlying obesity." The award was established by a gift of Michael S. Kovalenko in memory of his wife, Jessie Stevenson Kovalenko, and has been presented since 1952.

RICHARD LOUNSBERY AWARD – a medal and a prize of $50,000 awarded to French and American scientists in alternate years for extraordinary scientific achievement in biology and medicine – goes to XIAODONG WANG, investigator, Howard Hughes Medical Institute, and George L. MacGregor Distinguished Chair in Biomedical Science, department of biochemistry, University of Texas Southwestern Medical Center, Dallas, "for pioneering biochemical studies on apoptosis, which have elucidated a molecular pathway leading into and out of the mitochondrion and to the nucleus." The award was established by Vera Lounsbery in memory of her husband and has been presented since 1979.

NAS AWARD IN CHEMICAL SCIENCES – a medal and prize of $15,000 awarded annually for innovative research in the chemical sciences that, in the broadest sense, contributes to the better understanding of the natural sciences and to the benefit of humanity – goes to ROBERT G. BERGMAN, Gerald E.K. Branch Distinguished Professor, department of chemistry, University of California, Berkeley, "for numerous innovative contributions at the interfaces of physical, organic, and inorganic chemistry, including the discoveries of alkane carbon-hydrogen bond oxidative addition and 1,4-benzene diradicals." The award, supported by the Merck Company Foundation, has been presented since 1979.

NAS AWARD FOR CHEMISTRY IN SERVICE TO SOCIETY – a prize of $20,000 awarded biennially for contributions to chemistry, either in fundamental science or its application, that clearly satisfy a societal need. The award, given in alternate years to chemists working in industry and to those in academia, government, and nonprofit organizations (presented to a chemist working in industry in 2007) – goes to ARTHUR A. PATCHETT, retired vice president, medicinal chemistry, Merck Research Laboratories, Rahway, N.J., "for innovative contributions in discoveries of Mevacor, the first statin that lowers cholesterol levels, and of Vasotec and Prinivil for treating hypertension and congestive heart failure." The award, established by E.I. du Pont de Nemours & Co., has been presented since 1991.

NAS AWARD FOR INITIATIVES IN RESEARCH – a prize of $15,000 awarded annually to recognize innovative young scientists and to encourage research likely to lead toward new capabilities for human benefit (the 2007 field is optical science) – goes to SHANHUI FAN, assistant professor, department of electrical engineering, Stanford University, Stanford, Calif., "for innovative research on the theory and applications of photonic crystal devices." The award, presented since 1981, was established by AT&T Bell Laboratories in honor of William O. Baker, and is supported by Alcatel-Lucent.

NAS AWARD IN MOLECULAR BIOLOGY – a medal and a prize of $25,000 awarded annually for a recent notable discovery in molecular biology by a young scientist – goes to GREGORY J. HANNON, investigator, Howard Hughes Medical Institute, and professor, Watson School, Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y., "for elucidation of the enzymatic engine for RNA interference." The award is supported by Pfizer Inc and has been presented since 1962.

NAS AWARD IN THE NEUROSCIENCES – a prize of $25,000 awarded every three years for extraordinary contributions to progress in the fields of neuroscience – goes to JEAN-PIERRE CHANGEUX, emeritus professor, Institut Pasteur and Collège de France, Paris, "for the pioneering discovery that fast-acting neurotransmitters mediate their effects through allosteric regulation of the neurotransmitter protein." The award was established by the Fidia Research Foundation and has been presented since 1988.

NAS AWARD FOR SCIENTIFIC REVIEWING – a prize of $10,000 awarded annually for excellence in scientific reviewing within the past 10 years (the 2007 field is astronomy) – goes to GEOFFREY R. BURBIDGE, professor, department of physics, University of California, San Diego, "for contributions as editor of THE ANNUAL REVIEW OF ASTRONOMY from 1974 to 2004, using his vast knowledge to make it the premier astronomy review journal worldwide." The award is supported by Annual Reviews Inc., the Institute for Scientific Information, and THE SCIENTIST in honor of J. Murray Luck and has been presented since 1979.

TROLAND RESEARCH AWARDS – a research award of $50,000 given annually to each of two recipients to recognize unusual achievement and to further their research within the broad spectrum of experimental psychology – goes to RANDY L. BUCKNER, investigator, Howard Hughes Medical Institute, and professor, FAS Department of Psychology and center for brain science, Harvard University, Cambridge, Mass., and to PAWAN SINHA, associate professor of computational neuroscience, department of brain and cognitive science, Massachusetts Institute of Technology, Cambridge. Buckner was chosen "for substantive contributions to understandings of the neural mechanisms of memory formation and retrieval." Sinha was chosen "for elucidating how humans learn to recognize visual objects, and for developing computational models of the mechanisms that mediate this learning." The Troland Research Awards were established by a bequest from Leonard T. Troland and have been presented since 1984.

SELMAN A. WAKSMAN AWARD IN MICROBIOLOGY – a prize of $5,000 given biennially to recognize excellence in the field of microbiology – goes to RICHARD M. LOSICK, professor, biological laboratories, Harvard University, Cambridge, Mass., for "discovering alternative bacterial sigma factors and his fundamental contributions to understanding the mechanism of bacterial sporulation." The award was established by a gift of the Foundation for Microbiology and has been presented since 1968.

CHARLES DOOLITTLE WALCOTT MEDAL – a medal and a prize of $10,000 given every five years to encourage and reward individual achievement in advancing our knowledge of Cambrian or Precambrian life and its history in any part of the world – goes to JOHN P. GROTZINGER Fletcher Jones Professor of Geology, department of geological and planetary sciences, California Institute of Technology, Pasadena, "for the insightful elucidation of ancient carbonates and the stromatolites they contain, and for meticulous field research that has established the timing of early animal evolution." The award was established by a gift of Mrs. Mary Vaux Walcott in memory of her husband and has been presented since 1934.

JAMES CRAIG WATSON MEDAL – a medal and a prize of $25,000 plus $25,000 to support the recipient's research, given every three years for contributions to the science of astronomy – goes to MICHAEL F. SKRUTSKIE, professor, department of astronomy, University of Virginia, Charlottesville, and ROC M. CUTRI, deputy executive director, infrared processing and analysis center, California Institute of Technology, Pasadena, "for their monumental work in developing and completing the Two Micron All-Sky Survey, thus enabling a thrilling variety of explorations in astronomy and astrophysics." The award was established by the will of James C. Watson and has been presented since 1887.

Also to be honored at the April 29 ceremony is MAXINE F. SINGER, president emeritus, Carnegie Institution of Washington, who was chosen to receive the Academy's PUBLIC WELFARE MEDAL. The Academy selected Singer "for providing inspired and effective leadership in matters of science and its relationship to education and public policy." The medal was established to recognize distinguished contributions in the application of science to the public welfare and has been presented since 1914.

The National Academy of Sciences is a private, nonprofit honorific society of distinguished scholars engaged in scientific and engineering research, dedicated to the furtherance of science and technology and to their use for the general welfare. Since 1863, the National Academy of Sciences has served to "investigate, examine, experiment, and report upon any subject of science or art" whenever called upon to do so by any department of the government.

# # #

[This news release is available at HTTP://NATIONAL-ACADEMIES.ORG]

Exhibit Q

Memorial Sloan-Kettering Cancer Center

Press Releases

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Paul Marks Prize Recognizes Three Young Cancer Researchers

Biennial Award Honors Investigators Who Have Made Important Contributions to the Biological Understanding of Cancer

September 26, 2007

NEW YORK, NY - Three young investigators who have made major accomplishments in cancer research will be the recipients of the 2007 Paul Marks Prize for Cancer Research and will share a $150,000 award, announced Memorial Sloan-Kettering Cancer Center (MSKCC).

The winners are Angelika Amon, PhD, of the Massachusetts Institute of Technology (MIT) and the Howard Hughes Medical Institute (HHMI), who studies how chromosomes segregate during cell division; Todd R. Golub, MD, of the Dana-Farber Cancer Institute, the Broad Institute of Harvard and MIT, and the HHMI, who employs genomic approaches to better classify subtypes of cancer; and Gregory J. Hannon, PhD, of Cold Spring Harbor Laboratory (CSHL) and the HHMI, who uses model systems to study the biochemistry and biology of the RNA interference mechanism.

The prize, named after Paul A. Marks, MD, President Emeritus of MSKCC, recognizes significant contributions to the basic understanding and treatment of cancer by scientists no more than 45 years old at the time they are nominated. The winners were selected by a committee chaired by Jeffrey M. Friedman, MD, PhD, a professor at The Rockefeller University.

"It is important to show appreciation for the work of younger scientists while they are still in the early stages of their careers," said Dr. Friedman. "The Paul Marks Prize pays tribute to the man for whom it was named by honoring some of the most promising researchers of the next generation."

> ***"Each investigator we are honoring is already a leader in his or her respective field. These scientists have made major contributions to the biological understanding of cancer, shedding light on what causes cancer and offering promising solutions that may someday provide benefits to patients everywhere."***
>
> **-- Harold Varmus, MD**
> **President, MSKCC**

Characterizing Key Proteins in the Cell Division Process

Dr. Amon combines genetic, biochemical, and cell biology techniques to study the regulation of cell division in the budding yeast *S. cerevisiae*, an important model organism for studying cellular behavior. Cell division encompasses the sequence of steps in which a cell copies its DNA and separates its chromosomes to form two new daughter cells. Because uncontrolled cell division causes tumors to form, understanding

the process has important applications for cancer research.


Angelika Amon, PhD

One focus of Dr. Amon's work has been to examine the last step of the cell division process, known as exit from mitosis. Her laboratory described the regulation of the phosphatase Cdc14, a protein that plays a key role in this final step. Her team also characterized two regulatory pathways, known as the FEAR network and the Mitotic Exit Network (MEN), which promote the release of Cdc14 from the inhibitor that it binds.

Dr. Amon also has studied chromosome segregation during meiosis, the specialized form of cell division needed to create egg and sperm. Improper separation of chromosomes during meiosis is a major cause of miscarriages and birth defects. Again, using budding yeast as a model system her team was able to characterize several proteins that regulate proper meiotic division.

A current focus of Dr. Amon's laboratory is studying the effects of aneuploidy on the way that cells proliferate. Aneuploidy, in which a cell has an abnormal number of chromosomes, occurs if chromosomes do not separate properly. "Our recent work has dealt with the question of how aneuploidy affects the yeast cell's physiology," Dr. Amon explained. "We are now eager to investigate how aneuploidy affects mammalian cells."

"There is no doubt that Angelika Amon is a true star in basic cancer research," said Tyler E. Jacks, PhD, director of the MIT Center for Cancer Research. "Her work has had a deep and lasting impact on our understanding of mechanisms crucial to proper cell division, which has helped to shape our insight into how defects in these processes contribute to cancer and other disorders."

Using Genomics To Better Classify Cancer Types

Dr. Golub is an expert in cancer genomics, a field that is using information from the Human Genome Project to classify cancers based on their genes and the way that those genes function. He has made major contributions to the understanding of how genes can be used to classify cancers, which is important for the diagnosis and prognosis of disease, as well as for developing better targeted therapies.


Todd R. Golub, MD

Dr. Golub's group has made important discoveries on the molecular basis of several types of leukemia, some of which have resulted in genetic tests that are now standard at most major medical centers worldwide. His team was among the first to use microarrays (also known as DNA chips) for the classification of cancer. His work in using genomics to diagnose and prognose disease also has been applied to pediatric brain cancer, lymphoma, prostate cancer, and lung cancer.

"The goal of our work is to develop a new molecular taxonomy of cancer," Dr. Golub explained. "It is not enough to say someone has breast cancer or lung cancer, for example. There is a real need to subclassify patients, so that we can match patients very closely with targeted drugs and conduct smarter clinical trials."

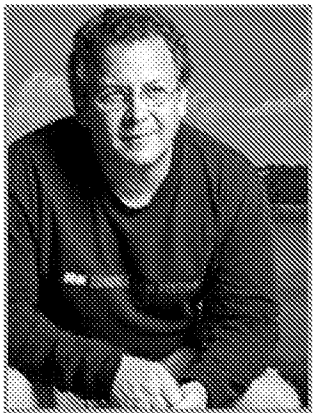
In the area of drug discovery, Dr. Golub has focused on ways in which an understanding of cancer genomics can lead to the development of better targeted therapies. His team's work with a technique called gene expression-based high-throughput screening already has led to the

discovery of two drugs that are now in clinical trials -- one for acute myeloid leukemia and one for Ewing's sarcoma. An approach that his team developed called the Connectivity Map uses analytical tools to match the gene expression profiles of certain diseases with the mechanism of action of drugs -- both existing drugs and new ones -- that may have the potential to treat those diseases.

"Todd Golub has made important contributions -- both conceptual and technical -- that have had wide-ranging impact on cancer research," said Eric Lander, PhD, founding director of the Broad Institute. "In my opinion, he is one of the most creative and accomplished cancer scientists of his generation."

Harnessing a Natural Process for Cancer Therapy

Dr. Hannon is a leader in the relatively new field of RNA interference (RNAi). RNAi is a naturally occurring mechanism for regulating the expression of genes (controlling which genes are turned on and turned off in cells). In the laboratory, it is used as a tool to study the function of specific genes, and it's being investigated as a therapeutic approach for treating many different diseases, including cancer.


Gregory J. Hannon, PhD

Dr. Hannon's laboratory has elucidated key biochemical details of the components of the pathways involved in RNAi and is using these findings to develop molecular tools that can be used for gene discovery, the evaluation of gene function, and the generation of animal models. He has developed new techniques for using RNAi to study cancer development and is investigating possible cancer therapies that make use of small interfering RNAs (siRNAs).

Dr. Hannon discovered several proteins and enzymes that are an essential part of the RNAi mechanism, including Dicer, which cleaves double-stranded RNA into siRNAs; the RISC complex, which helps regulate protein translation and is involved in the body's defense against viral infections; and Argonaute2, which cleaves messenger RNA.

He also has been at the forefront of adapting RNAi techniques to study genes in mammals, and using these techniques to understand the variety of pathways that can lead to the formation of tumors.

"We believe that engaging the RNAi pathway will provide a new route to cancer therapies," Dr. Hannon said. "Our tools enable researchers everywhere to conduct genomewide, RNA-based screens for new drug targets. The current approaches for developing targeted therapies has limits, but with RNAi you can target any pathway that leads to tumor formation and drug even the 'undruggable.'"

"Greg Hannon's discoveries have had a broad impact on research related to the field of small RNA biology," said Bruce Stillman, PhD, president of CSHL. "I would venture to say that no person has contributed more to our understanding of the biochemistry of RNAi than has Greg."

The Paul Marks Prize was established in 2001 and is awarded biennially. This year's winners will speak about their work at a public symposium held at Memorial Sloan-Kettering Cancer Center on December 6, 2007.

Dr. Amon is a professor in the Department of Biology and the Center for Cancer Research at

MIT, as well as an HHMI investigator. She received her PhD degree in biology from the University of Vienna.

Dr. Golub is Charles A. Dana Investigator of Human Cancer Genetics at the Dana-Farber Cancer Institute, an associate professor of Pediatrics at Harvard Medical School, and founding director of the Cancer Program at the Broad Institute of MIT and Harvard. He is also an HHMI investigator. He received his MD degree from the University of Chicago Pritzker School of Medicine.

Dr. Hannon is a professor at Cold Spring Harbor Laboratory and an HHMI investigator. He earned his PhD degree in molecular biology from Case Western Reserve University.

In addition to Dr. Friedman, other members of the selection committee were Joan S. Brugge, PhD, of the Department of Cell Biology at Harvard Medical School; Titia de Lange, PhD, of The Rockefeller University; Stephen J. Elledge, PhD, of the Department of Genetics at Harvard University; Stephen P. Goff, PhD, of the Department of Microbiology at Columbia University; Alan Hall, PhD, of the Cell Biology Program in the Sloan-Kettering Institute; Scott W. Lowe, PhD, of the Cold Spring Harbor Cancer Center; and William G. Kaelin Jr., MD, of the Dana-Farber Cancer Institute.

Journalists may contact the Department of Public Affairs for more information.

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Paul Marks Prize for Cancer Research

Paul Marks Prize for Cancer Research: 2007 Prize Winners

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Hannon et al.	Confirmation No.:	8161
Application No:	11/894,676	Art Unit:	1635
Filed:	August 20, 2007	Examiner:	K. CHONG
Title:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Docket No.: 287000-130-US3

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Declaration Under 37 C.F.R. §1.131

We, Gregory J. Hannon, Patrick J. Paddison, Scott Hammond, Amy Caudy and Emily Bernstein, Douglas Conklin hereby declare as follows:

1. We are the inventors of the above-referenced patent application.

2. All the work described within this declaration was performed in the United States.

3. All of the work described within this declaration was performed by us, or on our behalf and under our direction.

4. We have reviewed our records, including the slides documents submitted herewith, and declare that the claimed invention, which is

> a method for attenuating expression of a target gene in a mammalian cell, the method comprising introducing into a mammalian cell a library of RNA expression constructs, each expression construct comprising:
>
> (i) an RNA polymerase promoter, and

> (ii) a sequence encoding a short hairpin RNA molecule comprising a double-stranded region wherein the double-stranded region consists of at least 20 nucleotides but not more than 29 nucleotides,
>
> wherein the short hairpin RNA molecule is a substrate for Dicer-dependent cleavage and does not trigger a protein kinase RNA-activated (PKR) response in the mammalian cells,
>
> wherein the double-stranded region of the short hairpin RNA molecule comprises a sequence that is complementary to a portion of the target gene, and
>
> wherein the short hairpin RNA molecule is stably expressed in the mammalian cell in an amount sufficient to attenuate expression of the target gene in a sequence specific manner, and is expressed in the cell without use of a PK inhibitor,
>
> whereby expression of the target gene is inhibited

including original (and amended) claims 50, 52, 54-63 was conceived and reduced to practice at least prior to August 14, 2001, the publication date of Caplen et al., *PNAS*, Vol. 98, No. 17, pp. 9742-9747, which is also prior to December 28, 2001, *i.e.*, the filing date of U.S. Publication No. US 2002/0160393, Symonds et al., U.S. Serial No. 10/035,098 and which is also prior to the date of filing of the parent application of Kreutzer et al. (U.S. Serial No. 09/889,802, filed September 17, 2001).

A. Hannon Draft Grant Application

5. We attach a copy of a draft grant application (**Exhibit A**) which was prepared prior to August 14, 2001. A review of email indicates that this draft grant application was prepared at least by sometime in January 2000. The specific aims, as indicated on the first page of the draft grant application (**Exhibit A**, page 12), were directed to identifying and characterizing the critical components of the RNA interference (RNAi) machinery. The "Preliminary Results" this page refers to (see 4th paragraph on page 12) were reported in Hammond et al., *Nature* 404:293-

296 (2000) (**Exhibit B**) in a paper entitled "An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophia* cells."

6. In particular, one aspect of the proposed work was directed to isolating and cloning the protein and RNA components of the RISC complex (RNA-induced silencing complex), the nuclease complex responsible for degradation of target mRNAs, and characterizing it's function, both *in vitro* and *in vivo*. To allow us to carry out such studies, we established a model system using cultured *Drosophila* cells that provided a readily available source of material in sufficient quantities for the necessary biochemical studies.

7. The Summary on page 15 provides the rationale for the proposed work:

> My laboratory has devoted a number of years to creating improved tools for probing gene function in cultured mammalian cells; however, our experience indicates that a facile loss-of-function tool is lacking. Unfortunately, dsRNA induces somewhat generic responses in mammalian cells. It is our hope that by understanding the mechanistic basis of dsRNA-induced silencing, we may not only unravel a mysterious and important piece of biology but also provide the means to create improved tools for analyzing gene function in diverse organisms in which traditional genetic methods are either cumbersome or unavailable. This notion that has contributed to the decision to focus substantial effort in my laboratory toward elucidating the mechanism of RNA interference.

The final paragraph on page 36 further elaborates on this rationale:

> In this application, we propose a biochemical approach to deciphering the mechanisms that underlie dsRNA-induced gene silencing. RNA-interference allows an adaptive defense against both exogenous and endogenous dsRNAs, providing something akin to a dsRNA immune response. The primary goal of the work proposed in this application is to understand the mechanisms by which a cell can raise this response. We have presented evidence that RNA interference is accomplished, at least in part, through the action of a sequence-specific nuclease that is generated in response to dsRNA. Our data, and that of others (Hamilton and Baulcombe, 1999), is consistent with a model in which dsRNAs present in a cell are converted, in a manner analogous to antigen processing, into discrete, small RNAs that guide the nuclease in the choice of substrate.

> We propose to purify and characterize the nuclease and to clone the protein and RNA components of the enzyme. In addition, we propose to develop approaches that may allow the use of cultured *Drosophila* cells as a general tool for probing gene function. The combination of these studies may lead eventually to an ability to harness RNA interference as a genetic tool in other organisms, particularly mammals, in which analogous tools are presently lacking.

8. At that time, there was a lack of available practical loss-of-function tools for probing gene function in mammalian cells. The work proposed in this draft application to elucidate the mechanism of RNA interference was intended to develop such tools. In other words, by understanding the mechanistic basis of RNA interference, we could use that understanding to exploit the RNAi pathway and create new tools to study gene function and the lack of certain gene function in mammalian cells.

9. The work proposed in this application to identify and characterize components of the RNAi cellular machinery was carried out by us prior to August 14, 2001. Certain aspects of this work were reported in Bernstein et al. *Nature* 409: 363-366 (2001) (**Exhibit C**) in a paper entitled "Role for a bidentate ribonuclease in the initiation step of RNA interference." This paper describes the identification and cloning of the enzyme, which we named "Dicer." The paper describes how this enzyme is evolutionarily conserved in worms, flies, plants, fungi and mammals, and the paper reports the role of this newly discovered enzyme in the RNAi pathway in cells. In particular, these results indicated that the process of gene silencing through the RNAi pathway could be divided into at least two distinct steps. In the first step, long dsRNA (double-stranded RNA) is processed by Dicer into approximately 22 nt (nucleotide) "guide" sequences. In the second step, these guide RNAs are incorporated into a distinct nuclease complex we first called the "RNA-induced silencing complex" or RISC. The RISC complex uses the guide sequences to specifically identify and destroy homologous mRNAs. We named the RNAs that

were processed by Dicer "guide sequences" or "guide RNAs" based on their role in targeting RISC to specific mRNAs based on sequence. The results and work described in Bernstein et al. (2001) were included in this patent application, U.S. Serial No. 11/894,676, and also in the related application U.S. Serial No. 10/055,797, such as in Example 2.

B. Draft SBIR Grant Application

10. We attach as **Exhibit D** a copy of a draft grant application to SBIR (Small Business Innovation Research) which was prepared prior to August 14, 2001.[1]

11. The first page of this draft grant lists three Aims directed toward achieving stable gene silencing in mammalian cells. Aim 1 is the "creation of stable, loss-of-function mutations in embryonic cells using RNAi." Aim 2 is the "creation of stable loss-of-function mutations in non-embryonic cell types," which proposes "numerous strategies for bypassing [the] problem" that "long dsRNAs provoke a PKR response in differentiated cell types."

12. Attached pages 13-25 of **Exhibit D** provide more detail regarding each of these Aims. Starting on page 13, the grant application describes the Experimental Procedures for Aim 1. Aim 1 is defined as "Creation of stable, loss-of-function mutations in embryonic cells using RNAi." On page 14, the grant states that "[w]e have chosen to approach this goal by encoding dsRNA in the form of an inverted repeat or hairpin that can be expressed from a promoter of choice." Regarding this objective, on page 14 the draft grant states that "[w]e have achieved the goal of simplified hairpin construction by dividing the process into two steps (Fig. 6)." Figure 6 is on page 15 and depicts a "strategy for the creation of hairpin RNAs for stable expression of dsRNA" and illustrates that "expression of a GFP hairpin RNA induced stable silencing of an

[1] For convenience, we have added page numbers to this document.

exogenous GFP reporter in [mouse embryonic] P19 cells." The use of the strategy and also the results described in Aim 1 are described in Example 3 (entitled "A Simplified Method for the Creation of Hairpin Constructs for RNA Interference") and Fig. 27, and in Example 4 (entitled "Long dsRNAs Suppress Gene Expression in Mammalian Cells") and Figs. 28-34 of the parent application, U.S. Serial No. 10/055,797. Aim 1 also describes silencing mammalian genes for which assays are available to allow "positive selection for loss-or function" in mammalian cells, *e.g.*, HPRT and TK. (See 2nd paragraph on page 16 of **Exhibit D**.)

13. The grant application states the goals for Aim 2 on the top of page 18 of **Exhibit D**: "our goal is to devise strategies for presentation of the dsRNA trigger that allow it to elude PKR surveillance." The "Expression Strategies" provided in the grant state that "PKR requires approximately 30 bp of contiguous double-stranded sequence to trigger dimerization and activation of the enzyme." (See first paragraph under "Expression Strategies" on page 18. The third paragraph in that section on page 18 describes expression of hairpin RNAs in various mammalian cells: "NIH 3T3, 293, HeLa, U2OS, Rat 1 and C2C12" and various expression vectors incorporating various promoters, including U1, U6 and CMV.

14. In the section entitled "Short RNA hairpins" on page 19 of **Exhibit D**, the grant application describes use of short RNA hairpins that are "below the cut-off for triggering RNA for investigating "whether the expression of short RNA hairpins can be used to induce efficient silencing." The research plan here also refers to "short synthetic RNAs that mimic our Dicer products." In other words, this refers to RNAs that have a double-stranded region of 20 to 22 base pairs. It further states that "short synthetic hairpins directed against GFP, TK and HPRT will be expressed from CMV, U1 and U6 promoter vectors in the cell types noted above." (See page 19.)

15. The grant application on page 19 describes two methods for modifying the approach described in Aim 1 to "create hairpins with significantly shorter loops." The first is "to simply clone short hairpin sequences [either] as single, synthetic DNA fragments, and the second is to clone "in two steps if hairpin formation in such synthetic oligonucleotides competes too vigorously with intermolecular hybridization to produce clonable fragments." Furthermore, Figure 7 on page 21 of **Exhibit D** depicts the use of libraries of expression vectors expressing an encoded "dsRNA cassette" to carry out functional screens in cultured cells.

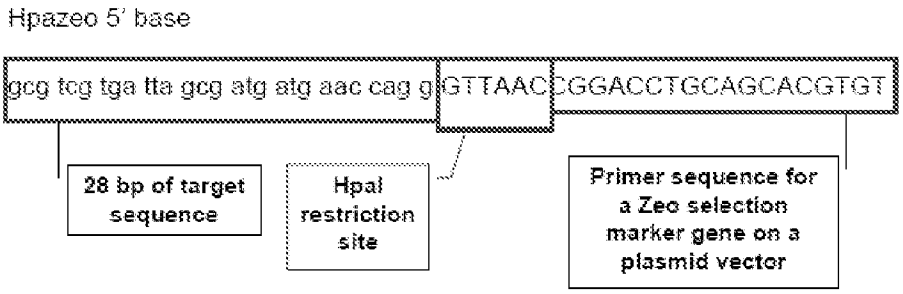
C. Primer Order to Invitrogen

16. Attached at **Exhibit E** is a copy of an email that was sent to Invitrogen to order oligonucleotide primers. The email was sent prior to August 14, 2001.

17. The email lists a number of pairs of oligonucleotide primers which were to be synthesized for use in cloning a sequence encoding a short hairpin RNA into a plasmid expression vector in order to obtain a short hairpin expression product as shown in Figure 37 of the parent application, U.S. Serial No. 10/055,797. These oligonucleotides requested through this e-mail order are examples of oligonucleotide primers designed for cloning such an expression vector using a two step cloning method, as referred to at paragraph 15 above and described in the grant application (**Exhibit D**) on the bottom of page 19. Note that the nucleic acids are synthesized in pairs (5' and 3') for use as 5' and 3' primers in a PCR amplification. For example, this is indicated by a "5" or a "3" at the end of each label, e.g., as in the first primer pair listed in the e-mail, "HPRTHpaZeo1 – 5" and "HPRThpazeo 1 – 3.".

18. Each primer consists of (a) a 28 nucleotide region of the target gene, followed by (b) a Hpa I restriction site (GTTAAC), followed by (c) a primer sequence for a Zeomycin selection

marker gene (Zeo) on a plasmid. Both a 5' nucleic acid and a 3' nucleic acid with these elements were to be synthesized as shown by the pairs of nucleic acids listed in **Exhibit E**. The elements of the first-listed nucleic acid in **Exhibit E** are labeled below:



19. The sequence of these nucleic acids reflects a two step cloning strategy for generating a DNA expression vector capable of expressing a short hairpin RNA having a double-stranded region of 28 base pairs. The nucleic acid pairs as indicated in **Exhibit E** are used as primers for a PCR reaction, using a Zeo selection marker gene as the PCR template. The amplified PCR product resulting from that PCR reaction is a double-stranded nucleic acid product that has a 28 nucleotide region of the target gene sequence, followed by a Hpa I restriction enzyme cleavage site, followed by the Zeomycin gene, followed by another Hpa I cleavage site, followed by the reverse complement of the 28 nucleotide region of the target gene.

20. In the first cloning step, the PCR product is cloned into an expression vector using Zeomycin selection. In the second cloning step, the vector is then digested using the HpaI restriction enzyme, resulting in a vector encoding a short hairpin consisting of (a) the target gene sequence, (b) a loop consisting of a HpaI restriction enzyme cleavage site and (c) the reverse complement of the target gene sequence. When transformed into bacterial cells, the HpaI site

facilitates selection of positive bacterial clones, i.e., those transformed with the expression vector. The draft SBIR Grant Application (**Exhibit D**) refers to such a two step cloning strategy at the bottom of page 19. s

21. The resulting expression vector constructed through this two step strategy encodes a short hairpin having a 28 base pair double-stranded region and an intervening loop consisting of an HpaI site. The short RNA hairpin encoded by an expression vector constructed using the primers listed in the Primer Order to Invitrogen (**Exhibit E**) has the same hairpin structure as shown in Figure 37 of the '797 application (see also **Exhibit F**).

22. The target genes referred to in **Exhibit E** and in **Exhibit D** include: human hypoxanthine—guanine phosphoribosyl transferase (HGPRT1 and HGPRT2 primers) and the mouse tyrosinase gene (tyro1 and tyro2 primers). The indicated target genes therefore indicate the resulting encoded short RNA hairpins (and expression constructs) are directed to silencing their corresponding target gene in mammalian cells, in particular, human cells and mouse cells. Additionally, as indicated in the Draft SBIR Grant Application (**Exhibit D**) on page 16 (second paragraph), HGRPT gene is directed to a gene target "for which exists a positive selection for loss-of-function" upon stable expression of the hairpin RNA in the cell.

D. Luciferase Simple Hairpin

23. Attached at **Exhibit F** is a copy of a slide dated at least by December 28, 2001. Information in this slide is also shown in Figure 37 in the parent application U.S. Serial No. 10/055,797. The slide illustrates two short hairpin RNA molecules. The second hairpin, the "Luciferase simple hairpin" has a double-stranded region consisting of 28 base pairs in length. .

The double-stranded region is highlighted. The double-stranded region of the short hairpin RNA molecule has a sequence that is complementary to a portion of the target gene, firefly luciferase.

24. The loop region of the hairpin on **Exhibit F** contains the sequence GUUAAC which is a HpaI restriction site. This is an example of a cloned simple hairpin that would be obtained using the methods described above in **Exhibit D** (specifically, the two-step method of hairpin cloning referred to here at paragraph 15) and using the PCR primers listed in **Exhibit E**.

E. Short Hairpin RNA Experiment in Human 293 T Cells

25. Attached at **Exhibit G** is a copy of a slide dated at least as early as October 2001. The title of the slide is "SHP 293T" indicating that this data is from an experiment using short hairpin RNA in 293T cells, a line of human embryonic kidney cells. This experiment assessed the ability of various short hairpin RNAs to specifically suppress gene expression in these cells, without provoking a PKR response. The 293T cells were co-transfected with a plasmid expressing the target gene, firefly luciferase, a plasmid expressing Renilla luciferase and one of various test hairpin RNAs. Subsequent to transfection, the level of expression of both luciferase proteins was measured. In the slide, the different test hairpin RNAs are indicated on the X axis of the slide underneath each of the bars. The respective bars indicate the degree to which the various introduced RNAs, including short hairpin RNAs, suppressed expression of the target firefly luciferase gene, as assayed by the ratio of firely luciferase to Renilla luciferase expression. As indicated in the slide, these results demonstrated that short RNA hairpins specifically suppressed expression of their target gene without provoking a PKR response in the cells.

26. For example, the nomenclature "SHP 25 luc hp" indicates a short hairpin RNA that has a double stranded region of 25 nucleotides in length. As the nomenclature indicates, the double-

stranded region of this short hairpin RNA molecule has a sequence that is complementary to a portion of the target gene, firefly luciferase. The bar graph shows, as a result of the experiment, a specific suppression of firefly luciferase gene expression in the 293T cells. See the bar labeled "SHP 25 luc hp" on the graph.

27. In the slide, the nomenclature "SHP 33 luc hp mism ngl3" indicates a short hairpin RNA that has a double stranded region of 33 nucleotides in length and has a mismatch in the sequence so that the sequence is not fully complementary to the sequence of the luciferase target gene. This bar of the bar graph shows, as a result of the experiment using a mismatched hairpin sequence, no specific suppression of firefly luciferase gene expression. The slide shows that short hairpin constructs with double-stranded regions of 32 nucleotides, 33 nucleotides, 34 nucleotides and 35 nucleotides did not exhibit attenuation of luciferase gene expression.

28. This slide shows an example of a short hairpin with a double-stranded region of 25 nucleotides in length, which did not trigger a protein kinase RNA-activated (PKR) response in the mammalian cell, and which did attenuate expression of the target gene, luciferase, in a sequence specific manner in the mammalian cells, 293T. The information in this slide was also included as Figure 39 of the parent application U.S. Serial No. 10/055,797.

F. Short Hairpin RNA Experiment in Human HeLa Cells

29. Attached at **Exhibit H** is a copy of a slide dated at least as early as October 2001. The slide shows data from an experiment using human HeLa cells (a cell line derived from human cervical cancer cells). We knew at the time of this experiment that long dsRNA initiates a PKR response in these cells. Using the same protocol as the experiment discussed above (**E**), this

experiment similarly assessed the ability of various short hairpin RNAs to specifically suppress gene expression in HeLa cells, without provoking a PKR response.

30. As indicated in the slide, these results demonstrated that short RNA hairpins specifically suppressed expression of their target gene without provoking a PKR response in the cells. For example, introducing a short hairpin RNA having a double-stranded region of 25 base pairs ("SHP 25 Luc hp") into the cells specifically suppressed expression of the firefly luciferase target gene. Longer double-stranded regions or mismatched target sequences did not result in suppression of target gene expression. The information in this slide was also included as Figure 40 of the parent application U.S. Serial No. 10/055,797.

G. Short Hairpin RNA Experiment in *Drosophila* S2 Cells

31. Attached at **Exhibit I** is a copy of a slide dated at least as early as October 2001. The data in this slide was generated using the same type of experimental procedure as discussed above in **Exhibits G and H**. The data in this slide indicates that short hairpin with a double-stranded region of 25 nucleotides ("SHP 25 luc hp") functioned to specifically inhibit expression of the target gene in the cells. The information in this slide was also included as Figure 38 of the parent application U.S. Serial No. 10/055,797.

H. Expression of Encoded Short Hairpins Specifically Suppress Gene Expression in Mammalian Cells

32. Attached at **Exhibit J** is a copy of a slide dated as least by January 2002 which shows results from an experiment which was included as Figure 42 (bottom) of the parent application U.S. Serial No. 10/055,797. The description of this experiment and the data can be found on page 17 of the '797 application. The results of this experiment demonstrate that expression of

encoded short hairpin RNAs effectively and specifically suppressed expression of a target gene in 293T cells, without provoking a PKR response. .

I. Paddison et al., *Genes Dev.* 2002, 16:948-958

33. The work described above culminated in several publications. One paper was published in *Genes and Development* in March 2002 entitled "Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells." (See **Exhibit K**.) This paper reports that "short hairpin RNAs (shRNAs) can be engineered to suppress the expression of desired genes in culture *Drosophila* and mammalian cells. shRNA can be synthesized exogenously or can be transcribed from RNA polymerase III promoters *in vivo*, thus permitting the construction of continuous cell lines or transgenic animals in which RNAi enforces stable and heritable gene silencing." (See Abstract of **Exhibit K**.)

34. A copy of a manuscript of the Paddison et al. paper (**Exhibit K**) that was prepared prior to publication and no later than January 31, 2002, as indicated by e-mails to which the manuscript was attached, is attached at **Exhibit L**.

35. Results of additional representative experiments, conducted similarly to the experiment referred to here in part **H**, "Expression of Encoded Short Hairpins Specifically Suppress Gene Expression in Mammalian Cells," are also reported in Paddison et al., among other places, at Fig. 4. (**Exhibit K**). Results of additional representative experiments conducted similarly to the Short Hairpin RNA Experiment in *Drosophila* S2 Cells (**G**), the Short Hairpin RNA Experiment in Human 293 T Cells (**E**) and the Short Hairpin RNA Experiment in Human HeLa Cells (**F**) are reported in Paddison et al., among other places, at Figs. 1 through 3. (**Exhibit K**). Figures 44A and 44B of the '676 application correspond to Figure 6A and 6B of Paddison et al. (**Exhibit K**).

J. Industry Awards

36. During 2002-2006, Paddison et al. (**Exhibit K**), having been cited by more than 500 subsequently published scientific papers, was therefore among the most highly cited "high impact" papers in the fields of molecular biology and genetics, as indicated by an analysis published by ScienceWatch.com (**Exhibit M**, see Table 2). A citation history summary for Paddison et al. (**Exhibit K)** is shown in **Exhibit N**.

37. Since we made the claimed invention and published Paddison et al., (**Exhibit K**), the invention of using stably expressed short hairpin RNAs to inhibit gene expression in mammalian cells has been recognized by industry organizations. For example, in 2005, Dr. Hannon received the Award for Outstanding Achievement in Cancer Research from the American Association for Cancer Research (AACR), which honored Dr. Hannon "…for his work uncovering the biochemical mechanism of RNA interference of gene expression (RNAi) and his contributions to the discovery and development of short hairpin RNAs as tools for genetic manipulation of mammalian cells." (**Exhibit O**).

38. In 2007, Dr. Hannon received two more prestigious awards, the Award in Molecular Biology from the National Academy of Sciences (**Exhibit P**), and the Paul Marks prize for the valuable contribution his RNAi work to cancer research from Memorial Sloan-Kettering Cancer Center (**Exhibit Q**). In granting that award, MSKCC noted how Dr. Hannon had applied his research in understanding the RNAi pathway to develop this valuable new technology, and his recognition as a leader in the field:

> Dr. Hannon is a leader in the relatively new field of RNA interference (RNAi). RNAi is a naturally occurring mechanism for regulating the expression of genes (controlling which genes are turned on and turned off in cells). In the laboratory, it is used as a

tool to study the function of specific genes, and it's being investigated as a therapeutic approach for treating many different diseases, including cancer.

Dr. Hannon's laboratory has elucidated key biochemical details of the components of the pathways involved in RNAi and is using these findings to develop molecular tools that can be used for gene discovery, the evaluation of gene function, and the generation of animal models. He has developed new techniques for using RNAi to study cancer development and is investigating possible cancer therapies that make use of small interfering RNAs (siRNAs).

Dr. Hannon discovered several proteins and enzymes that are an essential part of the RNAi mechanism, including Dicer, which cleaves double-stranded RNA into siRNAs; the RISC complex, which helps regulate protein translation and is involved in the body's defense against viral infections; and Argonaute2, which cleaves messenger RNA.

He also has been at the forefront of adapting RNAi techniques to study genes in mammals, and using these techniques to understand the variety of pathways that can lead to the formation of tumors.

K. Conclusion

39. The documents attached hereto as **Exhibits A - M** demonstrate that that the invention claimed, including claims 50, 52, 54-60, 62 and 63, was conceived at least as early as August 14, 2001, which is prior to the effective filing date of Caplen et al., Symonds et al., and Kreutzer et al. These documents and our declaration also show diligence and reduction(s) to practice.

40. We further declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that the making of willfully false statements and the like is punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United

States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed: _____
Gregory J. Hannon

Dated: _____

Signed: _____
Patrick J. Paddison

Dated: _____

Signed: _____
Scott Hammond

Dated: _____

Signed: _____
Amy Caudy

Dated: _____

Signed: _____
Emily Bernstein

Dated: 1/21/2011

Signed: _____
Douglas Conklin

Dated: _____

Exhibits to Declaration Under 37 C.F.R. §1.131

Exhibit	Title
A	Hannon Draft Grant Application
B	Hammond et al., *Nature* 404:293-296 (2000)
C	Bernstein et al. *Nature* 409: 363-366 (2001)
D	Draft grant application to SBIR (Small Business Innovation Research)
E	Email of Primer Order to Invitrogen
F	Luciferase Simple Hairpin Slide
G	Short Hairpin RNA Experiment in Human 293 T Cells Slide
H	Short Hairpin RNA Experiment in Human HeLa Cells Slide
I	Short Hairpin RNA Experiment in *Drosophila* S2 Cells Slide
J	Short Hairpins Specifically Suppress Gene Expression Slide
K	Paddison et al., *Genes Dev.* 2002, 16:948-958
L	Manuscript of Paddison et al.
M	ScienceWatch Biology's Hottest 2002-2006
N	Paddison et al. Citations
O	2005 Award for Outstanding Achievement in Cancer Research from AACR
P	2007 Award in Molecular Biology from the National Academy of Sciences
Q	2007 Paul Marks Prize from Memorial Sloan-Kettering Cancer Center

Electronic Patent Application Fee Transmittal

Application Number:	11894676
Filing Date:	20-Aug-2007
Title of Invention:	Methods and compositions for RNA interference
First Named Inventor/Applicant Name:	Gregory J. Hannon
Filer:	Jane Maureen Love/sophie murray
Attorney Docket Number:	287000.130US3

Filed as Small Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 2 months with $0 paid	2252	1	245	245

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
			Total in USD ($)	**245**

Electronic Acknowledgement Receipt

EFS ID:	9345320
Application Number:	11894676
International Application Number:	
Confirmation Number:	8161
Title of Invention:	Methods and compositions for RNA interference
First Named Inventor/Applicant Name:	Gregory J. Hannon
Customer Number:	84834
Filer:	Jane Maureen Love/sophie murray
Filer Authorized By:	Jane Maureen Love
Attorney Docket Number:	287000.130US3
Receipt Date:	31-JAN-2011
Filing Date:	20-AUG-2007
Time Stamp:	19:25:10
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Credit Card
Payment was successfully received in RAM	$245
RAM confirmation Number	6981
Deposit Account	080219
Authorized User	LADD,CATHLEEN

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

Charge any Additional Fees required under 37 C.F.R. Section 1.16 (National application filing, search, and examination fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.17 (Patent application and reexamination processing fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.19 (Document supply fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.20 (Post Issuance fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		287000_130US3_Response_OA.pdf	418783 bbe42a39cad47e961fcc9b70a18f2c13fac0455c	yes	34

Multipart Description/PDF files in .zip description

Document Description	Start	End
Amendment/Req. Reconsideration-After Non-Final Reject	1	1
Claims	2	3
Applicant Arguments/Remarks Made in an Amendment	4	34

Warnings:					
Information:					
2	Rule 130, 131 or 132 Affidavits	287000_130US3_131_Declaration_Caudy.PDF	831739 dffc4a1a24b89dff5642d4d099c80212b553a275	no	17
Warnings:					
Information:					
3	Rule 130, 131 or 132 Affidavits	287000_130US3_131_Declaration_Conklin.pdf	85276 698ca1a5e45c9da5be10849a5e2a66a9f323e36e	no	17
Warnings:					
Information:					
4	Rule 130, 131 or 132 Affidavits	287000_130US3_131_Declaration_Hammond.pdf	279440 cabca309ef8254039b2845a06912b71a99135877	no	17
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Information:					
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Information:					
6	Rule 130, 131 or 132 Affidavits	287000_130US_131_declaration_Paddison.pdf	1334293 6fcd98e08d2236dff51bf0e1f82f62d50f2c15d8	no	17
Warnings:					

Information:					
7	Rule 130, 131 or 132 Affidavits	287000_130US3_132_second_dec_Dr_Hernandez.pdf	704383 12ece501cc97611d106637e583f5cfa274efb93f	no	18
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Information:					
8	Miscellaneous Incoming Letter	Exhibit_B_132_declaration.pdf	72346 0049cb43d2378bf514e12c9916e3bc52e6a42b8f	no	2
Warnings:					
Information:					
9	Extension of Time	287000_130Us3_EOT_01312011.pdf	92788 f3267d74ce987e91d57802255ebcb13c83ebc7bd	no	1
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Information:					
10	Miscellaneous Incoming Letter	Exhibit_A_131_Dec_Hannon_Draft_grant.pdf	752180 8f232a192e807617ce7b6b847c3d819a14e55081	no	26
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Information:					
11	Miscellaneous Incoming Letter	Exhibit_A_132_Declaration.pdf	64924 90384f3c301ea73753f0d442b2f4535d8b3eb5af	no	9
Warnings:					
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12	Miscellaneous Incoming Letter	Exhibit_B_131_Dec_Hammond_Nature.pdf	194145 8c059074193f173977d877440f832aa150bce3f8	no	4
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Information:					
13	Miscellaneous Incoming Letter	Exhibit_c_131_Dec_Bernstein.pdf	199765 fe4a6fadc17ebcd09921477555eb1a824faf1c98	no	4
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Information:					
14	Miscellaneous Incoming Letter	Exhibit_D_131_declaration_SBIR_Draft_Grant.pdf	829686 b551ea13f06c7d82d8d8287b97d6db1344bcc094	no	25
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15	Miscellaneous Incoming Letter	Exhibit_E_Email_of_Primer_Order_to_Invitrogen.pdf	86369 a1b48bee0325429b05ab8f0f2bb15dfb0e0dc0c4	no	3
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Information:					
16	Miscellaneous Incoming Letter	Exhibit_F_luciferase_simple_hairpin.pdf	20031 b25a998e9364433ea05cc4092926d085713 8a8c4	no	1
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17	Miscellaneous Incoming Letter	Exhibit_G_293T_Cells.pdf	23476 04211938e02f7a32a05d9069de967bc489d 83eaa	no	1
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Information:					
18	Miscellaneous Incoming Letter	Exhibit_H_HeLa_Cells.pdf	23275 2d0d40c32d828f80fb380b76ee9c6605bb3 8a4be	no	1
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21	Miscellaneous Incoming Letter	Exhibit_K_Paddison_Genes.pdf	327083 2e2c09dc7687c3e3381752e9fab2223365c bd742	no	12
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Information:					
22	Miscellaneous Incoming Letter	Exhibit_L_Manuscript_Paddison.pdf	1001478 4546d63ea5f68352c1c2c1e7664b6930367 8a369	no	39
Warnings:					
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23	Miscellaneous Incoming Letter	Exhibit_M_Science_watch.pdf	92306 5970f6a5875520f5158a93b4199cd653a89e fb9e	no	3
Warnings:					
Information:					
24	Miscellaneous Incoming Letter	Exhibit_N_Paddison_Et_al_Citations.PDF	54459 4429e06f43fea0de64c2a6a85ff7f18ae90df b07	no	1
Warnings:					

Information:					
25	Miscellaneous Incoming Letter	Exhibit_O_2005_AACR_Award.pdf	206462 2eb1b915f46dffc54abfaa8f7c0fb34bb07ab177	no	1
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Information:					
26	Miscellaneous Incoming Letter	Exhibit_P_NAS_Molecular_Biology_Award.pdf	118288 71da10cc900cfa97d6b8e022f29f35bb3bb56611	no	3
Warnings:					
Information:					
27	Miscellaneous Incoming Letter	Exhibit_Q_Paul_Marks_Prize_for_Cancer_Research.pdf	103504 81657ec9b11aecb6f9abcc754bb25490a83e74bb	no	4
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Information:					
28	Rule 130, 131 or 132 Affidavits	287000_130US3_131_Declaration_Bernstein.pdf	99857 8632f69ec5900ce9317890d7a82dc775ca756503	no	17
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Information:					
29	Fee Worksheet (PTO-875)	fee-info.pdf	30226 38ad2c3978b20527c2dacb9da059383f443896e8	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	8648809		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

<u>New Applications Under 35 U.S.C. 111</u>
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

<u>National Stage of an International Application under 35 U.S.C. 371</u>
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

<u>New International Application Filed with the USPTO as a Receiving Office</u>
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/06 (07-06)
Approved for use through 1/31/2007. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number	Filing Date	☐ To be Mailed
11/894,676	08/20/2007	

APPLICATION AS FILED – PART I

	(Column 1)	(Column 2)	SMALL ENTITY ☒		OR	OTHER THAN SMALL ENTITY	
FOR	NUMBER FILED	NUMBER EXTRA	RATE ($)	FEE ($)		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT	01/31/2011	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 12	Minus	** 20	= 0	X $26 =	0	OR	X $ =	
	Independent (37 CFR 1.16(h))	* 1	Minus	***3	= 0	X $110 =	0	OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE	0	OR	TOTAL ADD'L FEE	

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/TYWANA P. LOVELACE/

PTO/SB/08a (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 3

Complete if Known	
Application Number	11/894,676-Conf. #8161
Filing Date	August 20, 2007
First Named Inventor	Gregory J. HANNON
Art Unit	1635
Examiner Name	K. Chong
Attorney Docket Number	0287000.00130US3

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	AA*	US-20040001811	01-01-2004	Kreutzer et al.	
	AB*	US-20040086884	05-06-2004	Beach et al.	
	AC*	US-5,814,500	09-29-1998	Dietz	
	AD*	US-6,541,248	04-01-2003	Kingsman et al.	

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]
	BA**	WO-01/68836	09-20-2001	David Beach et al.		
	BB**	WO-03/020931	03-13-2003	Galapagos Genomics NV		

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. * CITE NO.: Those application(s) which are marked with an single asterisk (*) next to the Cite No. are not supplied (under 37 CFR 1.98(a)(2)(iii)) because that application was filed after June 30, 2003 or is available in the IFW. ** CITE NO.: Those document(s) which are marked with an double asterisk (**) next to the Cite No. are not supplied because they were previously cited by or submitted to the Office in a prior application relied upon in this application for an earlier filing date under 35 U.S.C. 120. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

PTO/SB/08b (07-09)
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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 2 of 3

Complete if Known	
Application Number	11/894,676-Conf. #8161
Filing Date	August 20, 2007
First Named Inventor	Gregory J. HANNON
Art Unit	1635
Examiner Name	K. Chong
Attorney Docket Number	0287000.00130US3

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
	CA**	Buchholz et al., "Enzymatically prepared RNAi libraries," Nature Methods, Vol 3, No 9, pp. 696-700 (September 2006)	
	CB**	Caplen et al., "Rescue of polyglutamine-mediated cytotoxicity by double-stranded RNA-mediated RNA interference," Human Molecular Genetics, Vol 11, pp. 175-184 (2002)	
	CC**	Chang et al., "Lessons from Nature: microRNA-based ShRNA libraries," Nature Methods, Vol 3, No 9, pp. 707-714 (September 2006)	
	CD**	Cullen, "Enhancing and confirming the specificity of RNAi experiments," Nature Methods, Vol 3, pp. 677-681 (September 2006)	
	CE**	Elbashir et al., "Duplexes of 21-nucleotide RNA's mediate RNA interference in cultured mammalian cells," Nature, Vol 411, pp. 494-498 (May 2001)	
	CF**	Elbashir et al., "RNA interference is mediated by 21- and 22-nucleotide RNA,s," Gene and Development, Vol 15, pp188-200 (2001)	
	CG**	Gil et al., "Induction of apoptosis by the DsRNA-dependent protein Kinase (PKR): mechanism of Action," Apoptosis, Vol 5, pp. 107-114 (2000)	
	CH**	Hutvagner et al., 'A Cellular Function for the RNA-Interference Enzyme Dicer i the maturation of the let-7 Small Temporal RNA," Science, Vol 293, pp. 834-838 (August 2001)	
	CI**	McManus et al., "Gene Silencing in mammals by small interfering RNA's," Nature Reviews, Vol 3, pp. 737-747 (October 2002)	
	CJ**	Pei et al., "On the art of identifying effective and specific siRNAs," Nature Methods, Vol 3, No 9, pp. 670-676 (September 2006)	

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. ** CITE NO.: Those document(s) which are marked with an double asterisk (**) next to the Cite No. are not supplied because they were previously cited by or submitted to the Office in a prior application relied upon in this application for an earlier filing date under 35 U.S.C. 120.

[1]Applicant's unique citation designation number (optional). [2]Applicant is to place a check mark here if English language Translation is attached.

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First Named Inventor	Gregory J. HANNON
Art Unit	1635
Examiner Name	K. Chong
Attorney Docket Number	0287000.00130US3

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
	CK**	Sen et al., "A brief history of RNAi: the silence of the genes," FASEB J., Vol 20, pp. 1293-1299 (2006)	
	CL**	Snove Jr et al., "Expressing short Hairpin RNAs in vivo," Nature Methods, Vol 3 No 9, pp. 689-695 (September 2006)	
	CM**	Svoboda et al., " RNAI in mouse Oocytes and Preimplantation Embryos: effectiveness of Hairpin dsRNA," Biochem. Biophys. Res. Commum. Vol 287, pp. 1099-1104 (2001)	
	CN**	Vermeulen et al., "the contributions of DsRNA structure to Dicer specificity and efficiency," RNA, Vol 11, pp. 674-682 (2005)	
	CO**	Brummelkamp et al., "A system for stable expression of short interfering RNAs in mammalian cells," Science, Vol 296, pp. 550-553 (April 2002)	

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Docket No.: 0287000.00130US3
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Gregory J. Hannon et al.	Confirmation No.:	8161
Application No.:	11/894,676	Art Unit:	1635
Filed:	August 20, 2007	Examiner:	K. Chong
Title:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT (IDS)

Dear Sir:

This Information Disclosure Statement is being filed after the mailing date of the first Office Action on the merits and before the mailing date of a final Office Action or a Notice of Allowance.

The $180.00 fee is included herewith. The Commissioner is authorized to charge any additional fees occasioned by this paper or to credit any overpayment in fees to Deposit Account No. 08-0219.

Those documents which are marked with a double asterisk (**) next to the Cite No. in the attached form PTO/SB/08 are not supplied because they were previously cited by or submitted to the Office in prior application number 10/997086 filed November 23, 2004 and relied upon in this application for an earlier filing date under 35 U.S.C. 120.

Applicants request that the Examiner initial and return a copy of the enclosed Form PTO SB-08 with the next communication.

Respectfully submitted,

Dated: February 11, 2011

/Anne-Marie C. Yvon/
Anne-Marie C. Yvon, Ph.D.
Registration No.: 52,390
Attorney for Applicant(s)

Wilmer Cutler Pickering Hale and Dorr LLP
399 Park Avenue
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Electronic Patent Application Fee Transmittal

Application Number:	11894676
Filing Date:	20-Aug-2007
Title of Invention:	Methods and compositions for RNA interference
First Named Inventor/Applicant Name:	Gregory J. Hannon
Filer:	Anne-Marie Yvon/sophie murray
Attorney Docket Number:	287000.130US3

Filed as Small Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
Submission- Information Disclosure Stmt	1806	1	180	180
		Total in USD ($)		**180**

Electronic Acknowledgement Receipt

EFS ID:	9424186
Application Number:	11894676
International Application Number:	
Confirmation Number:	8161
Title of Invention:	Methods and compositions for RNA interference
First Named Inventor/Applicant Name:	Gregory J. Hannon
Customer Number:	84834
Filer:	Anne-Marie Yvon/sophie murray
Filer Authorized By:	Anne-Marie Yvon
Attorney Docket Number:	287000.130US3
Receipt Date:	11-FEB-2011
Filing Date:	20-AUG-2007
Time Stamp:	12:32:29
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Credit Card
Payment was successfully received in RAM	$180
RAM confirmation Number	13043
Deposit Account	080219
Authorized User	LADD,CATHLEEN

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

Charge any Additional Fees required under 37 C.F.R. Section 1.16 (National application filing, search, and examination fees)

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Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement (IDS) Filed (SB/08)	287000_130US3_SB08_02112011.pdf	119880 e30b90acdecf9573db15d47c49691a2cb5d54827	no	3
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
2	Transmittal Letter	287000_130US3_IDS_02112011.pdf	75354 b0938db6c1e90f24e182e032524bcb9df216a471	no	2
Warnings:					
Information:					
3	Fee Worksheet (PTO-875)	fee-info.pdf	30155 9e5f7b8530c1368a2f452d5bcfa7ecfabe71942d	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	225389		

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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/08a (07-09)
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First Named Inventor	Gregory J. HANNON
Art Unit	1635
Examiner Name	K. Chong
Attorney Docket Number	0287000.00130US3

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]
	BA	EP-1462525	09-29-2004	Toudai TLO LTD		

Examiner Signature		Date Considered	

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Attorney Docket Number	0287000.00130US3

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
	CA	European Search Result mailed on February 17, 2010, for European Application No. EP 03732052 filed January 22, 2003	
	CB	European Search Result mailed on September 22, 2009 for European Application No. EP 03732052 filed January 22, 2003	
	CC	Miller et al., "Improved retroviral vectors for gene transfer and expression," Biotechniques, Vol 7(9), pp. 980-990 (1989)	
	CD	Non final office action mailed on February 9, 2005 for US Application No. 10/055,797 filed January 22, 2002	
	CE	Non final office action mailed on November 8, 2005 for US Application No. 10/055,797 filed January 22, 2002	
	CF	Non final office action mailed on June 23, 2010, for US Application No. 12/152,837 filed January 22, 2002	
	CG	Final office action mailed on April 17, 2007, for US Application No. 10/055,797 filed January 22, 2002	
	CH	Non final office action mailed on July 26, 2006, for US Application No. 10/055,797 filed January 22, 2002	
	CI	Final Office Action mailed on May 12, 2009, for US Application No 10/997,086 filed November 23, 2004	
	CJ	Final Office Action mailed on July 2, 2010, for US Application No 10/997,086 filed November 23, 2004	

Examiner Signature		Date Considered	

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Sheet 3 of 3

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Art Unit	1635
Examiner Name	K. Chong
Attorney Docket Number	0287000.00130US3

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
	CK	Non Final Office Action mailed on August 26, 2009, for US Application No 10/997,086 filed November 23, 2004	
	CL	Non Final Office Action mailed on February 12, 2007, for US Application No 10/997,086 filed November 23, 2004	

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Electronic Patent Application Fee Transmittal

Application Number:	11894676
Filing Date:	20-Aug-2007
Title of Invention:	Methods and compositions for RNA interference
First Named Inventor/Applicant Name:	Gregory J. Hannon
Filer:	Anne-Marie Yvon/sophie murray
Attorney Docket Number:	287000.130US3

Filed as Small Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
Submission- Information Disclosure Stmt	1806	1	180	180
		Total in USD ($)		**180**

Electronic Acknowledgement Receipt

EFS ID:	9590257
Application Number:	11894676
International Application Number:	
Confirmation Number:	8161
Title of Invention:	Methods and compositions for RNA interference
First Named Inventor/Applicant Name:	Gregory J. Hannon
Customer Number:	84834
Filer:	Anne-Marie Yvon/sophie murray
Filer Authorized By:	Anne-Marie Yvon
Attorney Docket Number:	287000.130US3
Receipt Date:	04-MAR-2011
Filing Date:	20-AUG-2007
Time Stamp:	18:48:26
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Credit Card
Payment was successfully received in RAM	$180
RAM confirmation Number	4972
Deposit Account	080219
Authorized User	LADD,CATHLEEN

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

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Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	287000_130US3_IDS_03042011.pdf	61865 48e44cec7dbfb93b9d15c477cf559a2214aa8eb7	no	1
Warnings:					
Information:					
2	Information Disclosure Statement (IDS) Filed (SB/08)	287000_130US3_SB08_03042011.pdf	117377 8226697deb4e667e1acf6d8dd6745e218cfc4fd4	no	3
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
3	Foreign Reference	EP1462525.pdf	1601571 716b580fe8ebe128ddbd624bea86d36d6e6ec6f2	no	94
Warnings:					
Information:					
4	NPL Documents	287000_127EP1_SR_02172010.pdf	87134 2b86ce221749c2bfeb8a0c6fbfc72ed1e2b26b57	no	2
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5	NPL Documents	287000_127EP1_SR_09222009.pdf	61497 c8154c611607346d4083268addbb568332eff0c2	no	2
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Information:					
6	NPL Documents	Miller.pdf	952413 67a665c0f4bbd837c7edaced25c6b347cc966c49	no	14
Warnings:					
Information:					
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Warnings:					
Information:					

8	NPL Documents	287000_127US1_OA_11082005.pdf	637587 91180eacdce6062c19bf87a1a2b433883a86003d	no	16
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Information:					
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Warnings:					
Information:					
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Information:					
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Information:					
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Information:					
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Information:					
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Warnings:					
Information:					

Total Files Size (in bytes):	7927953

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New Applications Under 35 U.S.C. 111

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National Stage of an International Application under 35 U.S.C. 371

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New International Application Filed with the USPTO as a Receiving Office

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Docket No.: 0287000.00130US3
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Gregory J. Hannon et al.	Confirmation No.:	8161
Application No.:	11/894,676	Art Unit:	1635
Filed:	August 20, 2007	Examiner:	K. Chong
Title:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

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Applicants request that the Examiner initial and return a copy of the enclosed Form PTO SB-08 with the next communication.

Respectfully submitted,

Dated: March 4, 2011

/Anne-Marie C. Yvon/
Anne-Marie C. Yvon
Registration No.: 52,390
Attorney for Applicant(s)

Wilmer Cutler Pickering Hale and Dorr LLP
399 Park Avenue
New York, New York 10022
(212) 230-8800 (telephone)
(212) 230-8888 (facsimile)

PTO/SB/08a (07-09)
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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

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Sheet 1 of 2

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FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

PTO/SB/08b (07-09)
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Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 2 of 2

Complete if Known	
Application Number	11/894,676-Conf. #8161
Filing Date	August 20, 2007
First Named Inventor	Gregory J. HANNON
Art Unit	1635
Examiner Name	K. Chong
Attorney Docket Number	0287000.00130US3

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
	CA	Brummelkamp et al., "Stable suppression of tumorigenicity by virus-mediated RNA interference," Cancer cell, Vol 2, pp. 243-247 (2002)	
	CB	Final Office Action mailed on MArch 18, 2011 for US Application No 12/152837 filed may 16, 2008	
	CC	Mcmanus et al., "Gene silencing using micro-RNA designed hairpins," RNA, Vol 8, pp. 842-850 (2002)	
	CD	Sorensen et al., "Gene Silencing by systemic delivery of Synthetic siRNAs in adult Mice," J. Mol. Biol., Vol 327, pp. 761-766 (2003)	

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. ** CITE NO.: Those document(s) which are marked with an double asterisk (**) next to the Cite No. are not supplied because they were previously cited by or submitted to the Office in a prior application relied upon in this application for an earlier filing date under 35 U.S.C. 120.

[1]Applicant's unique citation designation number (optional). [2]Applicant is to place a check mark here if English language Translation is attached.

Electronic Patent Application Fee Transmittal

Application Number:	11894676
Filing Date:	20-Aug-2007
Title of Invention:	Methods and compositions for RNA interference
First Named Inventor/Applicant Name:	Gregory J. Hannon
Filer:	Julia Anne Grimes/sophie murray
Attorney Docket Number:	287000.130US3

Filed as Small Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
Submission- Information Disclosure Stmt	1806	1	180	180
		Total in USD ($)		**180**

Electronic Acknowledgement Receipt

EFS ID:	9724541
Application Number:	11894676
International Application Number:	
Confirmation Number:	8161
Title of Invention:	Methods and compositions for RNA interference
First Named Inventor/Applicant Name:	Gregory J. Hannon
Customer Number:	84834
Filer:	Julia Anne Grimes/sophie murray
Filer Authorized By:	Julia Anne Grimes
Attorney Docket Number:	287000.130US3
Receipt Date:	23-MAR-2011
Filing Date:	20-AUG-2007
Time Stamp:	17:53:37
Application Type:	Utility under 35 USC 111(a)

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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	287000_130US3_IDS_03232011.pdf	61870 eead3fa1ff449be910978a78331ea2764fd1c143	no	2
Warnings:					
Information:					
2	Information Disclosure Statement (IDS) Filed (SB/08)	287000_130US3_SB08_03232011.pdf	109070 4ea76bdc0321f125dc7d667c3dbfe7dc2959c573	no	2
Warnings:					
Information:					
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3	NPL Documents	Brummelkamp_Stable.pdf	491071 13c06a1f6351f3df8533ea446fe748768e396e0e	no	5
Warnings:					
Information:					
4	NPL Documents	Final_Office_Action_287000_127US2.pdf	732809 15016eeb6d19f93c7131623fd0cb6958c4f89a03	no	20
Warnings:					
Information:					
5	NPL Documents	Mcmanus.pdf	746088 c0806e2817109cbab5a1feb31f1f409761d115af	no	9
Warnings:					
Information:					
6	NPL Documents	Sorensen.pdf	596156 f26510ccc7f81473120688ce3e78ab786e0284f4	no	6
Warnings:					
Information:					
7	Fee Worksheet (PTO-875)	fee-info.pdf	30030 b247b2ade301e60a03fb7e49931f9400e96a4c53	no	2
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		Total Files Size (in bytes):	2767094		

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If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Docket No.: 0287000.00130US3
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Gregory J. Hannon et al.	Confirmation No.:	8161
Application No.:	11/894,676	Art Unit:	1635
Filed:	August 20, 2007	Examiner:	K. Chong
Title:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT (IDS)

Dear Sir:

This Information Disclosure Statement is being filed after the mailing date of the first Office Action on the merits and before the mailing date of a final Office Action or a Notice of Allowance.

The$ 180.00 fee is included herewith.

Applicants request that the Examiner initial and return a copy of the enclosed Form PTO SB-08 with the next communication.

Respectfully submitted,

Dated: March 23, 2011

/Julia A. Grimes/
Julia Grimes
Registration No.: 66,170
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Hannon et al.	Confirmation No.:	8161
Application No:	11/894,676	Art Unit:	1635
Filed:	August 20, 2007	Examiner:	K. CHONG
Title:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INTERVIEW SUMMARY

Pursuant to 37 C.F.R. §1.133(b), applicants are filing an interview summary of the interview held on March 22, 2011 with Examiner Chong, SPE Calamita, SPE Celsa, SPE Woitach (hereinafter "the Examiners"), Professor Hernandez by telephone (a 132 Declarant in this case), Dr. Vladimir Drozdoff of Cold Spring Harbor Laboratory, the assignee of this application, and the undersigned. The interview was held in connection with the two related applications U.S. Serial No. 10/997,086 and U.S. Serial No. 11/894,676. This Interview Summary is being filed concurrently in each case.

The applicants provided a slide set to the Examiner, which is attached hereto as **Exhibit 1**. The following topics were discussed:

A. Claims as Amended Are Described in the Specification as Required Under 35 U.S.C. § 112

Four days before this interview, applicants noticed that Examiner Chong had issued an Office Action in a related continuation-in-part application, U.S. Serial No. 12/152,837[1] which had similar claim amendments as were last presented in this case. Examiner Chong had rejected the claims based on an alleged lack of written description support. In view of this rejection and in order

[1] Applicants note that the '837 is a CIP application and therefore, has a different specification than either the '086 or '676 application. This discussion is not an admission that the same rejections present in the '837 application would

to make the most efficient use of the interview, applicants presented the Examiner's views to Professor Hernandez along with the specification of the parent application in this case, namely U.S. Serial No. 10/055,797[2] (aka U.S. Publication No. 2003/0084471) in order to obtain Prof. Hernandez's opinion on whether the specification provides sufficient written description support for the claim amendments in question. Prof. Hernandez provided her opinion that the '797 application provides explicit and implicit written description support for the phrases which were added to the claims, namely, "wherein the short hairpin RNA molecule is a substrate for Dicer-dependent cleavage and does not trigger a protein kinase RNA-activated (PKR) response in the mammalian cell" and "and is expressed in the cell without use of a PK inhibitor."

First, applicants referred to well-settled law that explicit recitation of the claim element is not required to satisfy the written description requirement. A patent specification need only describe the claimed invention in sufficient detail that one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention. See, e.g., *Moba, B.V. v. Diamond Automation, Inc.*, 325 F.3d 1306, 1319, 66 USPQ2d 1429, 1438 (Fed. Cir. 2003); *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d at 1563, 19 USPQ2d at 1116. Accordingly, Professor Hernandez has looked at the parent application, the '797 application, and the present application and stated during the interview that the application fully shows possession of the invention and sufficient written description support of the amended claims so that one of skill in the art, like herself, would have understood that Dr. Hannon and the other co-inventors invented and possessed what is claimed.

Next, Professor Hernandez pointed out sections of the parent application providing examples of disclosure that she understood as one of ordinary skill to provide sufficient support for the claim amendments. As to "and is expressed in the cell without use of a PK inhibitor," Prof. Hernandez made at least the following points during the interview:

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apply to either the '086 or the '676. Applicants present this information for the Examiner's convenience in assessing the new claim amendments.

[2] In this paper, applicants refer by page and line number to the '797 application as filed, not the published application.

1. Some of the claims of the '797 application as filed (1-3 and 7) are directed to methods of using ds RNA to attenuate expression of a target gene, but do not further require using a PK inhibitor. . However, claim 16 modifies claims 1-3 and 7 such that the method does require a PK inhibitor. The dependent claim therefore indicates (through the doctrine of claim differentiation) that by the broader claims, the inventors also describe a method of attenuating expression of a target gene in cells using a shRNA (which is a kind of dsRNA described) without using a PK inhibitor.

2. Prof. Hernandez explained that the the experiment described in Example 6 would have had no purpose if a PK inhibitor had been included. In particular, Prof. Hernandez pointed to the third sentence in paragraph C on page 53 of the '797 application. It reads: "Additionally, we wanted to demonstrate that unlike long dsRNAs, short dsRNAs do not provoke a non-specific PKR or PKR-like response." In order to demonstrate this, the experiment had to be conducted without using a PK inhibitor.

3. The language "in an amount sufficient to attenuate expression of a target gene" also implies that expression of shRNA is alone sufficient to attenuate target gene expression, with no other required step in the method, , such as using a PK inhibitor. See page 4 of the '797 application as an example.

4. Page 19 of the '797 application was pointed out during the interview. Professor Hernandez pointed out that a primary purpose of the invention was in using RNAi to attenuate target gene expression without using a PK inhibitor. In this regard, the paragraph starting on line 19 states: "As described herein, Applicants have demonstrated that the PKR response can be overcome in favor of the sequence-specific RNAi response. The application also explains that in contrast to this first approach, "However, in certain instances, it may be desirable to treat the cells with agents which inhibit expression of PKR, cause its destruction, and/or inhibit the kinase activity of PKR, and such methods are specifically contemplated for use in the present invention."

Professor Hernandez also stated that the specification of the parent application and the '086 and '676 applications provide written description support for the phrase: "wherein the short hairpin RNA molecule is a substrate for Dicer-dependent cleavage and does not trigger a protein kinase RNA-activated (PKR) response in the mammalian cell."

1. Prof. Hernandez pointed out that the '797 application has extensive disclosure regarding Dicer and the idea of expressing exogenous Dicer in a cell along with expressing shRNAs. See the disclosure starting at Section A on page 23 of the '797 application, for example, "As used herein, the term "Dicer" refers to a protein which (a) mediates an RNAi response...

   ...Accordingly, the method may comprise introducing a dsRNA construct into a cell in which Dicer has been recombinantly expressed or otherwise ectopically activated."

2. Prof. Hernandez stated that a person of skill in the art would understand these examples to teach that an shRNA could be expressed stably in cells and in those cells would be a substrate for Dicer and that the RNAi response can therefore be potentiated by over-expressing Dicer in the cell. Prof. Hernandez explained that these examples and the presently claimed invention would therefore make no sense if read to mean that the shRNA was not processed by Dicer. One of skill would have understood from the Hannon application that unless processed by Dicer, the shRNA would not function to specifically suppress expression of a target gene.

3. In this regard, Prof. Hernandez also pointed to Example 4 of the '797 application as demonstrating that the presence of Dicer in the cell was necessary to achieve gene silencing by RNAi (here as exemplified by expression of long hairpin RNAs), or as the application alternatively refers to the RNAi process, "post-transcriptional gene silencing" or "specifically suppressing gene expression." In this regard, Prof. Hernandez also pointed to Examples 6 and 7 demonstrating the use of shRNA (transfected and stably expressed) for "specifically suppressing gene expression."

4. Prof. Hernandez stated that a person of skill reading the '797 application and the '086 and '676 applications would have understood that Hannon et al. had possession of the invention as presently claimed, and in particular, the use of short hairpin RNA as a substrate for Dicer for the purpose of specifically suppressing expression of a target gene in a mammalian cell.

B. Symonds et al. Does Not Make the Invention Obvious

Prof. Hernandez also stated that Symonds et al. does not make obvious the claimed invention either alone or in combination with the other references relied upon by the Examiner. Prof. Hernandez's Second Declaration specifically addresses this same issue. This was discussed during the interview.

1. Prof. Hernandez reiterated her views as they are expressed in her Second 132 Declaration. That evidence will not be repeated in this paper.

2. Prof. Hernandez also explained that the Symonds et al. application does not in any way teach or suggest the claimed size range for the double-stranded region of the shRNA which is claimed. Prof. Hernandez pointed out that the Symonds et al. application provides for a gigantic range of possible lengths, and that the defined language in that application amplifies the size of the range even further.

3. For example, as Applicants discussed, the definition of "hybridizing conditions" only requires a length of 7 nucleotides to hybridize. (See paragraph 96 of Symonds '393 publication.) The description relied upon by the Examiner, as well as other description through Symonds, therefore defines the double stranded complex to encompass a wide spectrum of lengths. Prof. Hernandez explained that the description in the Symonds et al. application would have provided no guidance to a person of skill in the art to select and use the specific size range of the double-stranded region required by the Hannon et al. claims.

4. Prof. Hernandez, as a person of skill in the art, stated that Symonds et al. does not make obvious the claimed invention.

Further to Prof. Hernandez comments, Applicants remarked that the two US provisional applications to which the Symonds application claims priority, Nos. 60/258731 and 60/258733 are each expressly and specifically directed to two different solutions to the problem of overcoming the PKR response. Both these solutions differ from the presently claimed short hairpin methods. The '731 application is directed to use of HIV Tat protein as a inhibitor of the PKR/ general antiviral response to the presence of dsRNA (see for example, '731 application at 3 and claim 1). Likewise, the '733 application is directed to use of ribozymes, such that expression of dsRNA is restricted to the nucleus with the aim of avoiding the PKR response (see for example, '733 application at 3 and claim 1, and the Symonds '393 published application, Example 5).

Applicants pointed out that the Symonds '393 published application incorporated seven new Examples. However, despite the alleged obviousness of expressing short hairpins as a solution to the PKR problem, none of these Examples either mention or use the short hairpin RNA solution

described by Dr. Hannon and his co-inventors. In contrast, the Symonds Examples are directed instead to use of long dsRNA and use of ribozymes and PKR inhibitors to avoid the PKR response. Notably, despite the widespread use and commercial success of the short hairpin approach presently claimed in the '086 and '676 applications, the Symonds Examples teach away from that solution by relying on an entirely different approach.

C. Review of the State of the Art and Hannon et al.'s Invention

Prof. Hernandez used the slides attached hereto as Exhibit 1 to discuss the state of the art. Prof. Hernandez explained the state of the art and how the claimed invention of Hannon et al. was an advance over what was known, and explained why it was surprising. This was provided in the responses filed in both the '676 and '086 applications. Prof. Hernandez also pointed out that the very different methods of inhibiting gene expression disclosed in Symonds, Elbashir, Fire, and Caplen are not used commercially today. Prof. Hernandez stated that in contrast, the shRNA methods described by Hannon et al. are very successful and used widely in the industry. In this regard, Applicants presented evidence (also provided in the previously filed responses) showing that the first manuscript that Dr. Hannon published describing the presently claimed invention (Paddison et al. 2002 Genes & Development) was one of the most highly cited papers in the field of molecular biology and genetics during 2002-2006.

D. Declaration Under 37 C.F.R. §1.131

Applicants also pointed out that the 131 Declaration filed in the applications shows conception earlier than Symonds, Caplen and Kreutzer and thereby removes those references as prior art. The Examiners stated they were still looking through the Declaration.Applicants hereby submit this record of the substance of the interview pursuant to 37 C.F.R. §1.133(b). No fees are believed to be due for the filing of this paper. However, in the event that any unforeseen fees are due, the Director is hereby authorized to charge any such fee, or credit any overpayment of fees, to Deposit Account No. 08-0219.

Applicants respectfully request that the Examiner take into account the comments from Dr. Hernandez during this interview. If any further information is needed, the Examiner is invited to contact the undersigned at her convenience.

Dated: March 31, 2011

/Jane M. Love, Ph.D./
Jane M. Love, Ph.D.
Registration No.: 42,812
Attorney for Applicant(s)

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Interview at USPTO

USSN 10/997,086
and
USSN 11/894,676

March 22, 2011

WILMERHALE

WILMER CUTLER PICKERING HALE AND DORR LLP ®

Agenda

1. Status of cases (‘676 and ‘086)
2. Claims pending
3. Rejections
4. Professor Hernandez
   - State of the Art
   - Distinguish Invention Over Cited Art
5. Response and Evidence
6. Review process going forward

Status of USSN 11/894,676

- Response filed on January 31, 2011 to Non-Final Office Action including:
  - Amendment to Claims
  - Response to OA
  - Summary of State of the Art
  - 131 Declaration Antedates Caplen, Kreutzer & Symonds
  - 132 Declarations of Prof. Hernandez

Amended Claim – '676

50. A method for attenuating expression of a target gene in a mammalian cell, the method comprising

introducing into [a] mammalian cells a library of RNA expression constructs, each expression construct comprising:

(i) an RNA polymerase promoter, and

(ii) a sequence encoding a short hairpin RNA molecule comprising a double-stranded region wherein the double-stranded region consists of at least 20 nucleotides but not more than 29 nucleotides,

Representative Claim – '676

50. (cont'd)

[such that the short hairpin RNA does not trigger a protein kinase RNA-activated (PKR) response in the mammalian cells],

wherein the short hairpin RNA molecule is a substrate for Dicer-dependent cleavage and does not trigger a protein kinase RNA-activated (PKR) response in the mammalian cell;

wherein the double-stranded region of the short hairpin RNA molecule comprises a sequence that is complementary to a portion of the target gene, and

wherein the short hairpin RNA molecule is stably expressed in the mammalian cell in an amount sufficient to attenuate expression of the target gene in a sequence specific manner, and is expressed in the cell without use of a PK inhibitor, whereby expression of the target gene is inhibited.

Rejections in '676 Office Action

- 103(a) over Symonds, Lieber, Fire, Good and Noonberg
- 112 - Indefiniteness claims 62 and 63
- Obviousness-type double patenting
  - Over '086 application

Rebuttals to 103 Rejection

- Symonds, Caplen and Kreutzer
  - Not prior art (131 Declaration)
  - Secondary refs do not remedy shortcomings of Symonds
- 1st and 2nd 132 Declarations of Professor Hernandez
  - No reasonable expectation of success
  - Prior art teaches away from the invention
  - Symonds teaches away
  - Testimony by one of ordinary skill (state of the art, reasonable expectation) is fact, not opinion

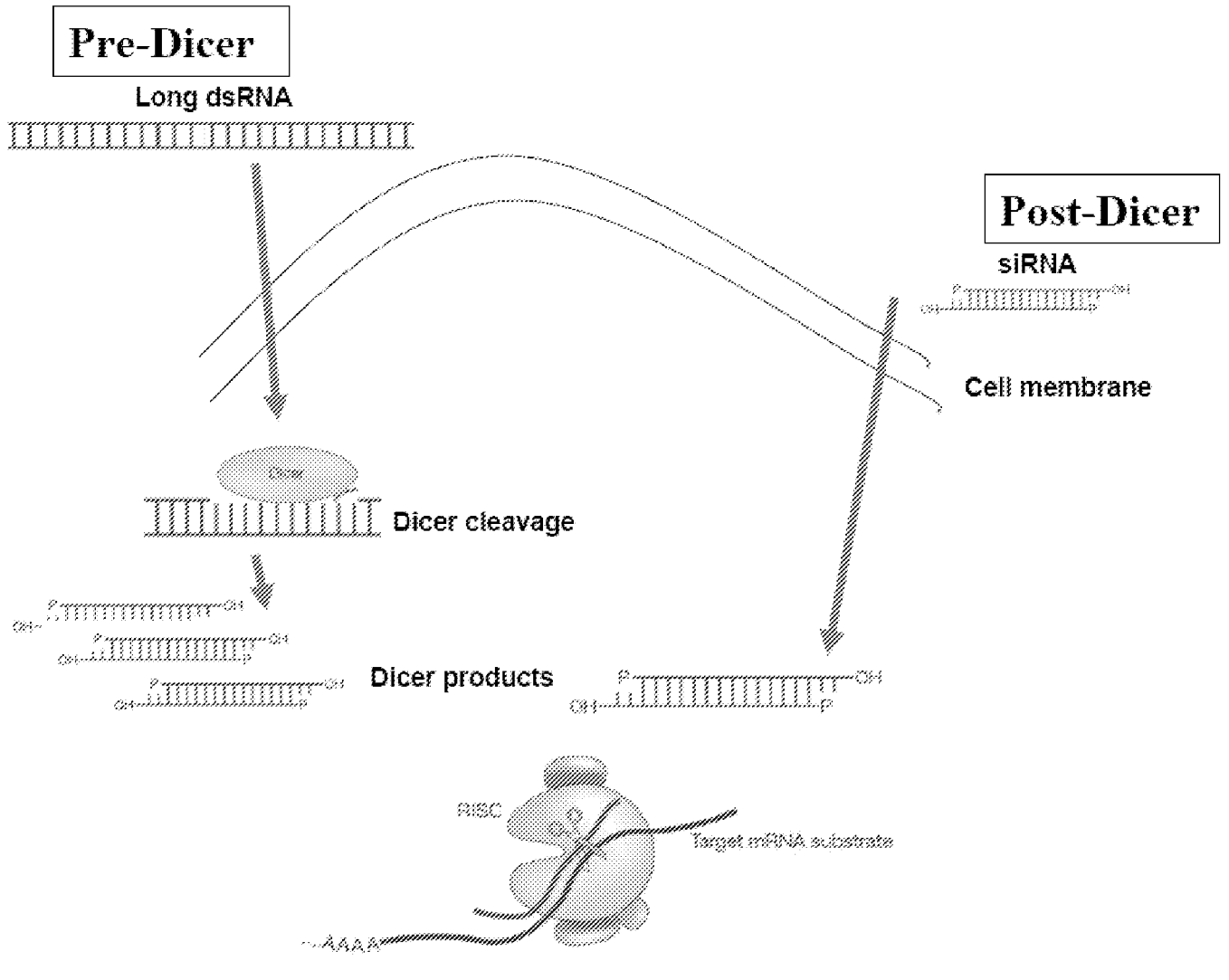
State of the Art – Prof. Hernandez

- Dr. Hannon's goal: to exploit RNAi to study gene function in mammalian cells.
- Dr. Hannon and co-inventors isolated, named and described the critical components of the RNAi pathway

Dicer
Processes dsRNA into **"guide RNAs"**(siRNAs)

RISC
Nuclease complex uses **guide RNAs** to target a specific mRNA for destruction

Dicer Cleaves Long dsRNAs to Make Guide RNAs or siRNAs



RNAi before Dr. Hannon's invention

- Pre-Dicer triggers
  - Long dsRNAs *e.g.*, 300-500 bp (e.g., Fire et al.)
  - When introduced into cell: cleaved by Dicer to act as RNAi triggers

Mammalian cells

Anti-viral/PKR response

Non-specific inhibition

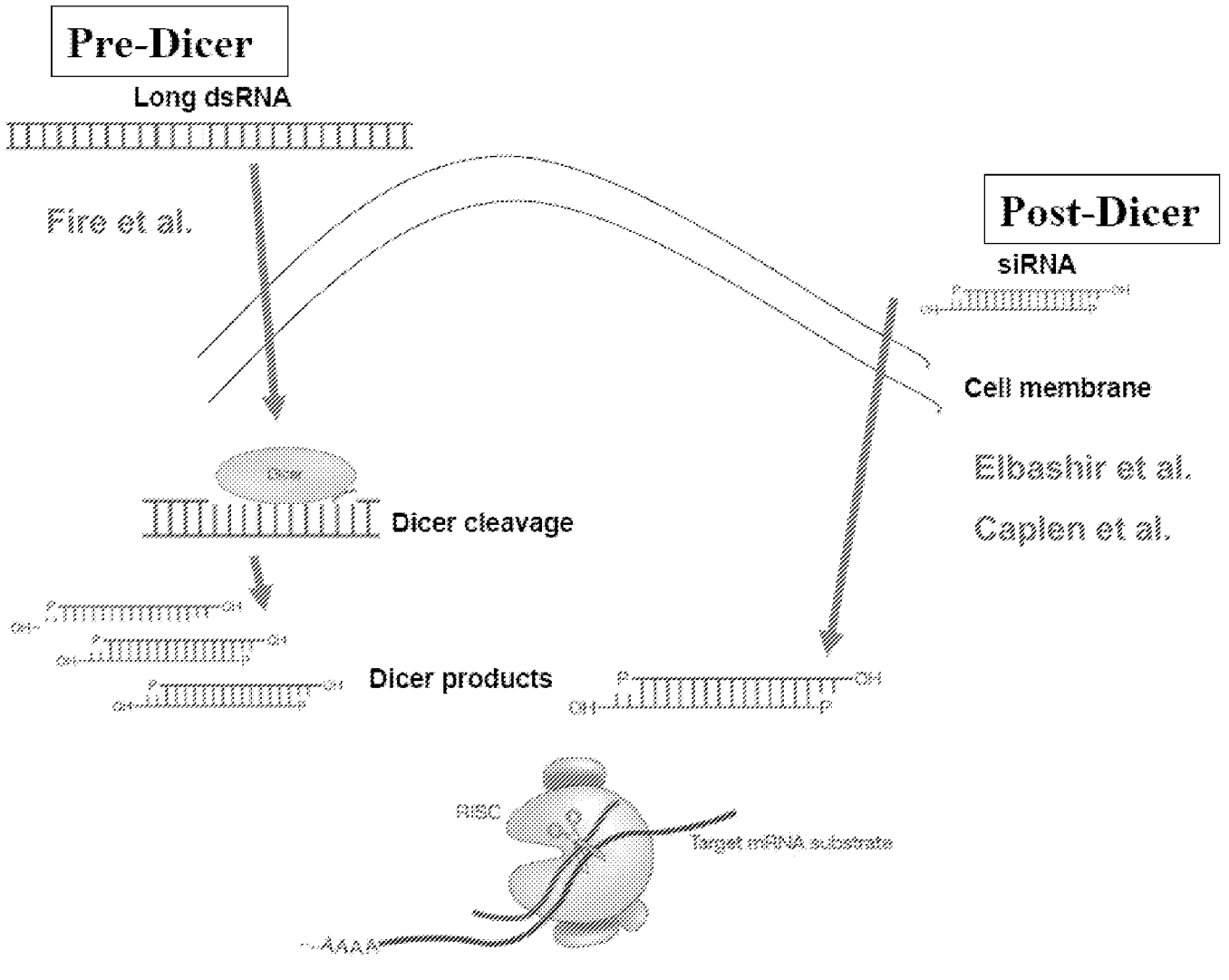
Stable expression kills cells

- Post-Dicer triggers
  - Short siRNAs that mimic Dicer cleavage products (*e.g.*, Elbashir et al., Caplen et al.)
  - When introduced into cell: bypass Dicer to directly act as RNAi triggers

Mammalian cells

Only transient effect

RNAi before Dr. Hannon's invention



Problem

- How could one use RNAi to stably suppress gene expression in mammalian cells without killing the cells or inhibiting all gene expression?
  - Specifically inhibit a single gene in a cell
  - Avoid anti-viral/PKR response
  - Achieve long term inhibition

Dr. Hannon and co-inventors

Described the critical components of the RNAi pathway.

+

Unique insight about Dicer mechanisms

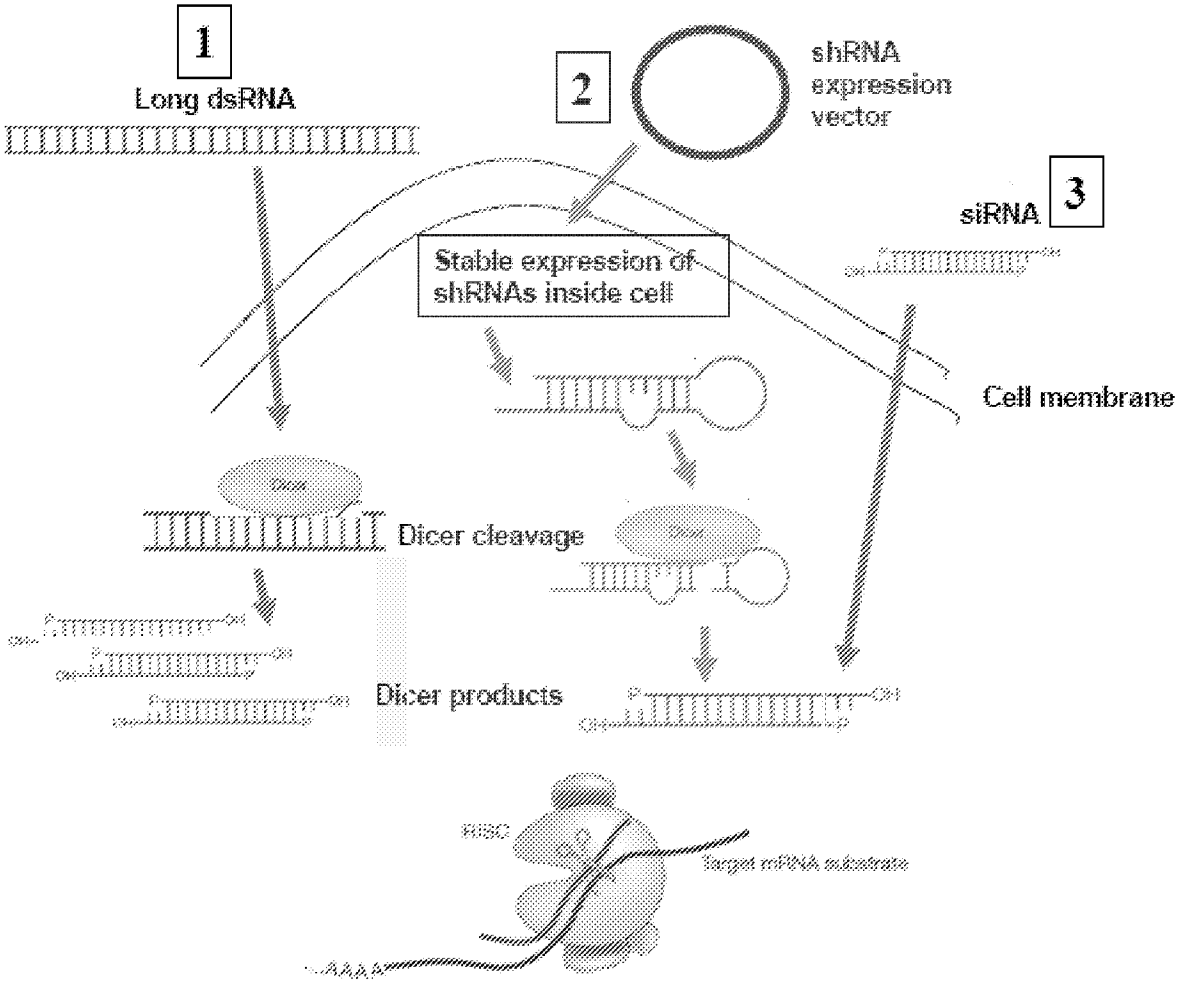
+

Above ordinary skill in the art

Dr. Hannon's Solution

- Short-hairpin RNA in mammalian cells
  - Activate RNAi pathway by expressing shRNA as a substrate for Dicer-dependent cleavage
  - Stably express the shRNA from expression construct
  - Stably expressed shRNA does not activate antiviral/PKR response
  - Stably expressed shRNA acts as RNAi trigger: sequence specific inhibition of single gene

Dr. Hannon's shRNA solution



Industry Acclaim

- Award for Outstanding Achievement in Cancer Research from the AACR (2005)
- Award in Molecular Biology from the NAS and the Paul Marks prize for the contribution of RNAi work to cancer research from MSKCC (2007)
  - "Dr. Hannon is a leader in the relatively new field of RNA interference (RNAi)."

Dr. Hannon discovered several proteins and enzymes that are an essential part of the RNAi mechanism, including Dicer, which cleaves double-stranded RNA into siRNAs; the RISC complex, which helps regulate protein translation and is involved in the body's defense against viral infections; and Argonaute2, which cleaves messenger RNA.

He also has been at the forefront of adapting RNAi techniques to study genes in mammals, and using these techniques to understand the variety of pathways that can lead to the formation of tumors.

Industry Acclaim: Paddison et al. (2002) Genes & Development

- First published paper describing Dr. Hannon's shRNA invention
- In 2002-2006, was one of the most highly cited papers in molecular biology and genetics

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765 records. Paddison, P.J. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells

Publication Year	Record Count
2002	27
2003	122
2004	142
2005	115
2006	106

Authors of High-Impact Papers in Molecular Biology & Genetics, 2002-06
(Ranked by number of high-impact papers)

Rank	Name	Affiliation	Number of high-impact papers	Citations	Citations per high-impact paper
1	Eric S. Lander	MIT Broad Institute	23	9,710	441.4
2	David P. Bartel	HHMI, MIT, Whitehead Institute	19	4,542	239.1
3	C. David Allis	Rockefeller University	13	1,986	152.8
4	W. James Kent	Univ. Calif., Santa Cruz	12	5,158	429.8
5	Gregory J. Hannon	HHMI, Cold Spring Harbor Lab	12	3,542	295.2
6	Thomas Jenuwein	Res. Inst. Molec. Pathology, Vienna	11	1,762	160.2
7	Yi Zhang	HHMI, University of North Carolina	11	1,249	113.6
8	Mark J. Daly	Harvard University	10	5,428	542.8
9	David Haussler	HHMI, Univ. Calif., Santa Cruz	10	4,196	419.6
10	David Altshuler	Harvard University	10	3,868	386.8

Electronic Acknowledgement Receipt

EFS ID:	9779685
Application Number:	11894676
International Application Number:	
Confirmation Number:	8161
Title of Invention:	Methods and compositions for RNA interference
First Named Inventor/Applicant Name:	Gregory J. Hannon
Customer Number:	84834
Filer:	Jane Maureen Love/sophie murray
Filer Authorized By:	Jane Maureen Love
Attorney Docket Number:	287000.130US3
Receipt Date:	31-MAR-2011
Filing Date:	20-AUG-2007
Time Stamp:	14:00:17
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Applicant summary of interview with examiner	287000_130US3_Interview_Summary_03312011.pdf	135966 c259e01ea42f855f3a991f96b8dbf28a9d317cd9	no	7

Warnings:	
Information:	

2	Miscellaneous Incoming Letter	287000_130US3_Exhibit_1_03312011.pdf	365672 cfef89c9569fce463a25ab5ff6273c2c4a13012 6	no	17

Warnings:	
Information:	
Total Files Size (in bytes):	501638

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

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United States Patent and Trademark Office
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/894,676	08/20/2007	Gregory J. Hannon	287000.130US3	8161

84834 7590 04/06/2011

WilmerHale/Cold Spring Harbor Laboratory
399 Park Avenue
New York, NY 10022

EXAMINER
CHONG, KIMBERLY

ART UNIT	PAPER NUMBER
1635	

NOTIFICATION DATE	DELIVERY MODE
04/06/2011	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

Teresa.carvalho@wilmerhale.com
whipusptopairs@wilmerhale.com

Interview Summary	Application No.	Applicant(s)	
	11/894,676	HANNON ET AL.	
	Examiner	Art Unit	
	KIMBERLY CHONG	1635	

All participants (applicant, applicant's representative, PTO personnel):

(1) *KIMBERLY CHONG, HEATHER CALAMITA*. (3) *JANE LOVE, VLADIMIR DROZDOFF*.

(2) *JOSEPH WOITACH, BENNETT CELSA*. (4) *PROFESSOR HERNANDEZ*.

Date of Interview: *22 March 2011*.

Type: a)☐ Telephonic b)☐ Video Conference
c)☒ Personal [copy given to: 1)☐ applicant 2)☐ applicant's representative]

Exhibit shown or demonstration conducted: d)☒ Yes e)☐ No.
If Yes, brief description: *handout attached*.

Claim(s) discussed: _____.

Identification of prior art discussed: _____.

Agreement with respect to the claims f)☐ was reached. g)☐ was not reached. h)☐ N/A.

Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: *Discussed applicant's arguments and points presented in the handout that is attached. The Examiner will consider all declarations and arguments which will be addressed in the next Office action. No agreement was made as to claim amendments or withdrawal of rejections of record*.

(A fuller description, if necessary, and a copy of the amendments which the examiner agreed would render the claims allowable, if available, must be attached. Also, where no copy of the amendments that would render the claims allowable is available, a summary thereof must be attached.)

THE FORMAL WRITTEN REPLY TO THE LAST OFFICE ACTION MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP Section 713.04). If a reply to the last Office action has already been filed, APPLICANT IS GIVEN A NON-EXTENDABLE PERIOD OF THE LONGER OF ONE MONTH OR THIRTY DAYS FROM THIS INTERVIEW DATE, OR THE MAILING DATE OF THIS INTERVIEW SUMMARY FORM, WHICHEVER IS LATER, TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW. See Summary of Record of Interview requirements on reverse side or on attached sheet.

/Kimberly Chong/
Primary Examiner AU1635

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:

1) A brief description of the nature of any exhibit shown or any demonstration conducted,
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
   (The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiner's version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, "Interview Record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.

UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/894,676	08/20/2007	Gregory J. Hannon	287000.130US3	8161

84834 7590 04/15/2011

WilmerHale/Cold Spring Harbor Laboratory
399 Park Avenue
New York, NY 10022

EXAMINER
CHONG, KIMBERLY

ART UNIT	PAPER NUMBER
1635	

NOTIFICATION DATE	DELIVERY MODE
04/15/2011	ELECTRONIC

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Teresa.carvalho@wilmerhale.com
whipusptopairs@wilmerhale.com

Office Action Summary	Application No.	Applicant(s)	
	11/894,676	HANNON ET AL.	
	Examiner	Art Unit	
	KIMBERLY CHONG	1635	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *31 January 2011*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *50,52 and 54-63* is/are pending in the application.

4a) Of the above claim(s) *61* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *50,52,54-60,62 and 63* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date *02/11/2011,03/04/2011,03/23/2011*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Status of Application/Amendment/Claims

Applicant's response filed 01/31/2011 has been considered. The Finality of the previous Office action mailed 08/30/2010 has been withdrawn in view of the new rejections below. Rejections and/or objections not reiterated from the previous office action are hereby withdrawn. The following rejections and/or objections are either newly applied or are reiterated and are the only rejections and/or objections presently applied to the instant application. The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

With entry of the amendment filed on 01/31/2011, claims 50, 52, 54-63 are pending in the application.

Information Disclosure Statement

The submission of the Information Disclosure Statements on 02/11/2011, 03/04/2011 and 03/23/2011 is in compliance with 37 CFR 1.97. The information disclosure statements have been considered by the examiner and signed copies have been placed in the file.

Response to Declaration

The declaration filed on 101/31/2011 under 37 CFR 1.132 by Professor Hernandez is sufficient evidence to overcome the rejection of claims 50, 52, 54-60, 62 and 63 under 35 U.S.C. 103(a) as being unpatentable over Symonds et al. (US 2002/0160393), Lieber et al. (US Patent No. 6,130,092 cited on Applicant's IDS filed

01/10/2008), Fire et al. (US Patent Number 6,506,559 cited on Applicant's IDS filed 01/10/2008), Good et al. (Gene Therapy 1997 cited on Applicant's IDS filed 01/10/2008) and Noonberg et al. (US Patent No. 5,624,803).

The declaration filed on 01/31/2011 under 37 CFR 1.131 by Gregory Hannon, Patrick Paddison, Scott Hammond, Amy Caudy, Emily Bernstein and Douglass Conklin will not be addressed as the declaration above is sufficient to overcome the 103 rejection of record.

Response to Rejections

Double Patenting

The rejection of claims 50, 52, 54-60, 62 and 63 as provisionally rejected under the judicially created doctrine of double patenting over claims 3, 40, 42-47 and 49-51 of copending Application No. 10/997,086 is maintained for the reasons of record as Applicant's have asked that this rejection be held in abeyance.

Claim Rejections - 35 USC § 112

The rejection of claims 62 and 63 under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention is maintained as Applicant's have not addressed this rejection in the previous response.

Claim Rejections - 35 USC § 103

The rejection of claims 50, 52, 54-60, 62 and 63 under 35 U.S.C. 103(a) as being unpatentable over Symonds et al. (US 2002/0160393), Lieber et al. (US Patent No. 6,130,092 cited on Applicant's IDS filed 01/10/2008), Fire et al. (US Patent Number 6,506,559 cited on Applicant's IDS filed 01/10/2008), Good et al. (Gene Therapy 1997 cited on Applicant's IDS filed 01/10/2008) and Noonberg et al. (US Patent No. 5,624,803) is withdrawn in response to Applicant's arguments and the declaration of Professor Hernandez above.

Conclusion

THIS ACTION IS MADE FINAL. Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Kimberly Chong whose telephone number is 571-272-

3111. The examiner can normally be reached Monday thru Thursday between 6 and 3 pm.

If attempts to reach the examiner by telephone are unsuccessful please contact the SPE for 1635 Heather Calamita at 571-272-2876. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Patent applicants with problems or questions regarding electronic images that can be viewed in the Patent Application Information Retrieval system (PAIR) can now contact the USPTO's Patent Electronic Business Center (Patent EBC) for assistance. Representatives are available to answer your questions daily from 6 am to midnight (EST). The toll free number is (866) 217-9197. When calling please have your application serial or patent number, the type of document you are having an image problem with, the number of pages and the specific nature of the problem. The Patent Electronic Business Center will notify applicants of the resolution of the problem within 5-7 business days. Applicants can also check PAIR to confirm that the problem has been corrected. The USPTO's Patent Electronic Business Center is a complete service center supporting all patent business on the Internet. The USPTO's PAIR system provides Internet-based access to patent application status and history information. It also enables applicants to view the scanned images of their own application file folder(s) as well as general patent information available to the public. For more information about the PAIR system, see http://pair-direct.uspto.gov.

For all other customer support, please call the USPTO Call Center (UCC) at 800-786-9199.

/Kimberly Chong/
Primary Examiner
Art Unit 1635

Search Notes	Application/Control No.	Applicant(s)/Patent under Reexamination
	11/894,676	HANNON ET AL.
	Examiner	Art Unit
	KIMBERLY CHONG	1635

SEARCHED			
Class	Subclass	Date	Examiner

INTERFERENCE SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES (INCLUDING SEARCH STRATEGY)		
	DATE	EXMR
updated	4/11/2011	KC

PTO/SB/08a (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 3

Complete if Known	
Application Number	11/894,676-Conf. #8161
Filing Date	August 20, 2007
First Named Inventor	Gregory J. HANNON
Art Unit	1635
Examiner Name	K. Chong
Attorney Docket Number	0287000.00130US3

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	AA*	US-20040001811	01-01-2004	Kreutzer et al.	
	AB*	US-20040086884	05-06-2004	Beach et al.	
	AC*	US-5,814,500	09-29-1998	Dietz	
	AD*	US-6,541,248	04-01-2003	Kingsman et al.	

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]
	BA**	WO-01/68836	09-20-2001	David Beach et al.		
	BB**	WO-03/020931	03-13-2003	Galapagos Genomics NV		

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. * CITE NO.: Those application(s) which are marked with an single asterisk (*) next to the Cite No. are not supplied (under 37 CFR 1.98(a)(2)(iii)) because that application was filed after June 30, 2003 or is available in the IFW. ** CITE NO.: Those document(s) which are marked with an double asterisk (**) next to the Cite No. are not supplied because they were previously cited by or submitted to the Office in a prior application relied upon in this application for an earlier filing date under 35 U.S.C. 120. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /K.C./

PTO/SB/08b (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 2 of 3

Complete if Known	
Application Number	11/894,676-Conf. #8161
Filing Date	August 20, 2007
First Named Inventor	Gregory J. HANNON
Art Unit	1635
Examiner Name	K. Chong
Attorney Docket Number	0287000.00130US3

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
	CA**	Buchholz et al., "Enzymatically prepared RNAi libraries," Nature Methods, Vol 3, No 9, pp. 696-700 (September 2006)	
	CB**	Caplen et al., "Rescue of polyglutamine-mediated cytotoxicity by double-stranded RNA-mediated RNA interference," Human Molecular Genetics, Vol 11, pp. 175-184 (2002)	
	CC**	Chang et al., "Lessons from Nature: microRNA-based ShRNA libraries," Nature Methods, Vol 3, No 9, pp. 707-714 (September 2006)	
	CD**	Cullen, "Enhancing and confirming the specificity of RNAi experiments," Nature Methods, Vol 3, pp. 677-681 (September 2006)	
	CE**	Elbashir et al., "Duplexes of 21-nucleotide RNA's mediate RNA interference in cultured mammalian cells," Nature, Vol 411, pp. 494-498 (May 2001)	
	CF**	Elbashir et al., "RNA interference is mediated by 21- and 22-nucleotide RNA,s," Gene and Development, Vol 15, pp188-200 (2001)	
	CG**	Gil et al., "Induction of apoptosis by the DsRNA-dependent protein Kinase (PKR): mechanism of Action," Apoptosis, Vol 5, pp. 107-114 (2000)	
	CH**	Hutvagner et al., 'A Cellular Function for the RNA-Interference Enzyme Dicer i the maturation of the let-7 Small Temporal RNA," Science, Vol 293, pp. 834-838 (August 2001)	
	CI**	McManus et al., "Gene Silencing in mammals by small interfering RNA's," Nature Reviews, Vol 3, pp. 737-747 (October 2002)	
	CJ**	Pei et al., "On the art of identifying effective and specific siRNAs," Nature Methods, Vol 3, No 9, pp. 670-676 (September 2006)	

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. ** CITE NO.: Those document(s) which are marked with an double asterisk (**) next to the Cite No. are not supplied because they were previously cited by or submitted to the Office in a prior application relied upon in this application for an earlier filing date under 35 U.S.C. 120.

[1]Applicant's unique citation designation number (optional). [2]Applicant is to place a check mark here if English language Translation is attached.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /K.C./

PTO/SB/08b (07-09)
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 3 of 3

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	CK**	Sen et al., "A brief history of RNAi: the silence of the genes," FASEB J., Vol 20, pp. 1293-1299 (2006)	
	CL**	Snove Jr et al., "Expressing short Hairpin RNAs in vivo," Nature Methods, Vol 3 No 9, pp. 689-695 (September 2006)	
	CM**	Svoboda et al., " RNAI in mouse Oocytes and Preimplantation Embryos: effectiveness of Hairpin dsRNA," Biochem. Biophys. Res. Commum. Vol 287, pp. 1099-1104 (2001)	
	CN**	Vermeulen et al., "the contributions of DsRNA structure to Dicer specificity and efficiency," RNA, Vol 11, pp. 674-682 (2005)	
	CO**	Brummelkamp et al., "A system for stable expression of short interfering RNAs in mammalian cells," Science, Vol 296, pp. 550-553 (April 2002)	

Examiner Signature	/Kimberly Chong/	Date Considered	04/11/2011

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. ** CITE NO.: Those document(s) which are marked with an double asterisk (**) next to the Cite No. are not supplied because they were previously cited by or submitted to the Office in a prior application relied upon in this application for an earlier filing date under 35 U.S.C. 120.

[1]Applicant's unique citation designation number (optional). [2]Applicant is to place a check mark here if English language Translation is attached.

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Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 3

Complete if Known	
Application Number	11/894,676-Conf. #8161
Filing Date	August 20, 2007
First Named Inventor	Gregory J. HANNON
Art Unit	1635
Examiner Name	K. Chong
Attorney Docket Number	0287000.00130US3

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]
	BA	EP-1462525	09-29-2004	Toudai TLO LTD		

Examiner Signature		Date Considered	

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 2 of 3

Complete if Known	
Application Number	11/894,676-Conf. #8161
Filing Date	August 20, 2007
First Named Inventor	Gregory J. HANNON
Art Unit	1635
Examiner Name	K. Chong
Attorney Docket Number	0287000.00130US3

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
	CA	European Search Result mailed on February 17, 2010, for European Application No. EP 03732052 filed January 22, 2003	
	CB	European Search Result mailed on September 22, 2009 for European Application No. EP 03732052 filed January 22, 2003	
	CC	Miller et al., "Improved retroviral vectors for gene transfer and expression," Biotechniques, Vol 7(9), pp. 980-990 (1989)	
	CD	Non final office action mailed on February 9, 2005 for US Application No. 10/055,797 filed January 22, 2002	
	CE	Non final office action mailed on November 8, 2005 for US Application No. 10/055,797 filed January 22, 2002	
	CF	Non final office action mailed on June 23, 2010, for US Application No. 12/152,837 filed January 22, 2002	
	CG	Final office action mailed on April 17, 2007, for US Application No. 10/055,797 filed January 22, 2002	
	CH	Non final office action mailed on July 26, 2006, for US Application No. 10/055,797 filed January 22, 2002	
	CI	Final Office Action mailed on May 12, 2009, for US Application No 10/997,086 filed November 23, 2004	
	CJ	Final Office Action mailed on July 2, 2010, for US Application No 10/997,086 filed November 23, 2004	

Examiner Signature		Date Considered	

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 3 of 3

Complete if Known	
Application Number	11/894,676-Conf. #8161
Filing Date	August 20, 2007
First Named Inventor	Gregory J. HANNON
Art Unit	1635
Examiner Name	K. Chong
Attorney Docket Number	0287000.00130US3

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
	CK	Non Final Office Action mailed on August 26, 2009, for US Application No 10/997,086 filed November 23, 2004	
	CL	Non Final Office Action mailed on February 12, 2007, for US Application No 10/997,086 filed November 23, 2004	

Examiner Signature	/Kimberly Chong/	Date Considered	04/11/2011

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. ** CITE NO.: Those document(s) which are marked with an double asterisk (**) next to the Cite No. are not supplied because they were previously cited by or submitted to the Office in a prior application relied upon in this application for an earlier filing date under 35 U.S.C. 120.

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Sheet 1 of 2

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Application Number	11/894,676-Conf. #8161
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First Named Inventor	Gregory J. HANNON
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Examiner Name	K. Chong
Attorney Docket Number	0287000.00130US3

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FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document: Country Code[3]-Number[4]-Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]

Examiner Signature		Date Considered	

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Sheet 2 of 2

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NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
	CA	Brummelkamp et al., "Stable suppression of tumorigenicity by virus-mediated RNA interference," Cancer cell, Vol 2, pp. 243-247 (2002)	
	CB	Final Office Action mailed on MArch 18, 2011 for US Application No 12/152837 filed may 16, 2008	
	CC	Mcmanus et al., "Gene silencing using micro-RNA designed hairpins," RNA, Vol 8, pp. 842-850 (2002)	
	CD	Sorensen et al., "Gene Silencing by systemic delivery of Synthetic siRNAs in adult Mice," J. Mol. Biol., Vol 327, pp. 761-766 (2003)	

Examiner Signature	/Kimberly Chong/	Date Considered	04/11/2011

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Hannon et al.	Confirmation No.:	8161
Application No:	11/894,676	Art Unit:	1635
Filed:	August 20, 2007	Examiner:	K. CHONG
Title:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT IN RESPONSE TO FINAL OFFICE ACTION UNDER 37 CFR §1.116

This Amendment is filed in response to the April 15, 2011 final Office Action for which a response is due on July 15, 2011. Accordingly, this paper is being timely filed. The Commissioner is authorized to charge any fees due, or to credit any overpayment in fees, to Deposit Account No. 08-0219.

Claim Listing

This listing of the claims will replace all prior versions and listings of claims in the application:

1-49. (Cancelled)

50. (Previously presented) A method for attenuating expression of a target gene in a mammalian cell, the method comprising

introducing into mammalian cells a library of RNA expression constructs, each expression construct comprising:

(i) an RNA polymerase promoter, and

(ii) a sequence encoding a short hairpin RNA molecule comprising a double-stranded region wherein the double-stranded region consists of at least 20 nucleotides but not more than 29 nucleotides,

wherein the short hairpin RNA molecule is a substrate for Dicer-dependent cleavage and does not trigger a protein kinase RNA-activated (PKR) response in the mammalian cell,

wherein the double-stranded region of the short hairpin RNA molecule comprises a sequence that is complementary to a portion of the target gene, and

wherein the short hairpin RNA molecule is stably expressed in the mammalian cell in an amount sufficient to attenuate expression of the target gene in a sequence specific manner, and is expressed in the cell without use of a PK inhibitor, whereby expression of the target gene is inhibited.

51. (Cancelled)

52. (Previously presented) The method of claim 50, wherein the expression construct further comprises LTR sequences located 5' and 3' of the sequence encoding the short hairpin RNA molecule.

53. (Cancelled)

54. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of at least 21 nucleotides.

55. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of at least 22 nucleotides.

56. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of at least 25 nucleotides.

57. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of 29 nucleotides.

58. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule has a total length of about 70 nucleotides.

59. (Previously presented) The method of claim 50, wherein the RNA polymerase promoter comprises a pol II promoter or a pol III promoter.

60. (Previously presented) The method of claim 59, wherein the pol III promoter comprises a U6, an H1, or an SRP promoter.

61. (Withdrawn) The method of claim 59, wherein the pol II promoter comprises a U1 or a CMV promoter.

62-63. (Cancelled)

REMARKS

Claims 50, 52, and 54-63 are pending and under examination. Upon allowability of claims 50, 52, and 54-59, Applicants request rejoinder of claim 61, which would fall within the scope of the allowable generic claims. Claims 62-63 have been cancelled in order to expedite prosecution of the application and without prejudice to pursue the subject matter of these claims in a future application.

I. Double Patenting

Claims 50, 52, 54-60, 62, and 63 were rejected under the judicially created doctrine of obviousness-type double patenting as allegedly being unpatentable over claims 3, 40, 42-47, and 49-51 of co-pending application Serial No. 10/997,086 ("the '086 application"). Applicants assume that the reference to claim 3 of the '086 application, which has been cancelled, was a typographical error and was intended to reference pending claim 38.

In view of the requirements for restriction issued in the '086 application on October 19, 2006, and in the present application on October 9, 2008, Applicants believe this rejection to be improper. Nonetheless, solely to advance prosecution, a Terminal Disclaimer to the '086 application is attached, obviating the double patenting rejection. Applicants request reconsideration and withdrawal.

II. 35 U.S.C. § 112, Second Paragraph

Claims 62 and 63 were rejected under the second paragraph of Section 112 as allegedly being indefinite. Without acquiescing to the substance of the rejection and solely to advance prosecution, claims 62 and 63 are cancelled without prejudice. Applicants reserve the right to pursue the subject matter of claims 62 and 63 in one or more continuing applications. Applicants request reconsideration and withdrawal of the indefiniteness rejection.

CONCLUSION

Consideration of this paper and allowance of this application are requested. If it would advance prosecution, the Examiner is invited to contact the undersigned to discuss the contents of this paper.

Dated: April 18, 2011

Respectfully submitted,

/Jane M. Love, Ph.D./

Jane M. Love, Ph.D.
Registration No. 42,812
Attorney for Applicants

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PTO/SB/96 (07-09)
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STATEMENT UNDER 37 CFR 3.73(b)

Applicant/Patent Owner: Gregory J. Hannon, Patrick Paddison, Emily Bernstein, Amy Caudy, Douglas Conklin, and Scott Hammond

Application No./Patent No.: 11/894,676 Filed/Issue Date: August 20, 2007

Titled: METHODS AND COMPOSITIONS FOR RNA INTERFERENCE

Cold Spring Harbor Laboratory (Name of Assignee) , a Corporation (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.)

states that it is:

1. [X] the assignee of the entire right, title, and interest in;

2. [ ] an assignee of less than the entire right, title, and interest in
(The extent (by percentage) of its ownership interest is _____ %); or

3. [ ] an assignee of an undivided interest in the entirety of (a complete assignment from one of the joint inventors was made) the patent application/patent identified above by virtue of either:

A. [X] An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel 020427, Frame 0756, or for which a copy thereof is attached.

OR

B. [ ] A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as follows:

1. From: _____ To: _____
The document was recorded in the United States Patent and Trademark Office at
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2. From: _____ To: _____
The document was recorded in the United States Patent and Trademark Office at
Reel _____, Frame _____, or for which a copy thereof is attached.

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[ ] Additional documents in the chain of title are listed on a supplemental sheet(s).

[X] As required by 37 CFR 3.73(b)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was, or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.

[NOTE: A separate copy (*i.e.*, a true copy of the original assignment document(s)) must be submitted to Assignment Division in accordance with 37 CFR Part 3, to record the assignment in the records of the USPTO. See MPEP 302.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

Signature	4/18/11 Date
JOHN MARONEY Printed or Typed Name	
Vice President Office of Technology Transfer Legal Counsel	Title

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TERMINAL DISCLAIMER TO OBVIATE A PROVISIONAL DOUBLE PATENTING REJECTION OVER A PENDING "REFERENCE" APPLICATION	Docket Number (Optional) 0287000.00130US3

In re Application of: Gregory J. HANNON et al.

Application No.: 11/894,676-Conf. #8161

Filed: August 20, 2007

For: METHODS AND COMPOSITIONS FOR RNA INTERFERENCE

The owner*, Cold Spring Harbor Laboratory, of 100 percent interest in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of any patent granted on pending **reference** Application Number 10/997,086, filed on November 23, 2004, as such term is defined in 35 U.S.C. 154 and 173, and as the term of any patent granted on said **reference** application may be shortened by any terminal disclaimer filed prior to the grant of any patent on the pending **reference** application. The owner hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and any patent granted on the **reference** application are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.

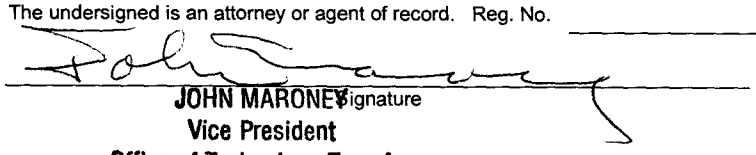
In making the above disclaimer, the owner does not disclaim the terminal part of any patent granted on the instant application that would extend to the expiration date of the full statutory term as defined in 35 U.S.C. 154 and 173 of any patent granted on said **reference** application, "as the term of any patent granted on said **reference** application may be shortened by any terminal disclaimer filed prior to the grant of any patent on the pending **reference** application," in the event that: any such patent: granted on the pending **reference** application: expires for failure to pay a maintenance fee, is held unenforceable, is found invalid by a court of competent jurisdiction, is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321, has all claims canceled by a reexamination certificate, is reissued, or is in any manner terminated prior to the expiration of its full statutory term as shortened by any terminal disclaimer filed prior to its grant.

Check either box 1 or 2 below, if appropriate.

1. [X] For submissions on behalf of a business/organization (e.g., corporation, partnership, university, government agency, etc.), the undersigned is empowered to act on behalf of the business/organization.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

2. [ ] The undersigned is an attorney or agent of record. Reg. No. _____



JOHN MARONEY Signature — 4/19/11 Date

Vice President
Office of Technology Transfer
Legal Counsel
Typed or printed name

(516) 367-8800
Telephone Number

[X] Terminal disclaimer fee under 37 CFR 1.20(d) is included.

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

*Statement under 37 CFR 3.73(b) is required if terminal disclaimer is signed by the assignee (owner). Form PTO/SB/96 may be used for making this statement. See MPEP § 324.

Electronic Patent Application Fee Transmittal

Application Number:	11894676
Filing Date:	20-Aug-2007
Title of Invention:	Methods and compositions for RNA interference
First Named Inventor/Applicant Name:	Gregory J. Hannon
Filer:	Jane Maureen Love/sophie murray
Attorney Docket Number:	287000.130US3

Filed as Small Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
Statutory or terminal disclaimer	2814	1	70	70
		Total in USD ($)		**70**

Electronic Acknowledgement Receipt

EFS ID:	9900400
Application Number:	11894676
International Application Number:	
Confirmation Number:	8161
Title of Invention:	Methods and compositions for RNA interference
First Named Inventor/Applicant Name:	Gregory J. Hannon
Customer Number:	84834
Filer:	Jane Maureen Love/sophie murray
Filer Authorized By:	Jane Maureen Love
Attorney Docket Number:	287000.130US3
Receipt Date:	18-APR-2011
Filing Date:	20-AUG-2007
Time Stamp:	16:45:59
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Credit Card
Payment was successfully received in RAM	$70
RAM confirmation Number	3444
Deposit Account	080219
Authorized User	LADD,CATHLEEN

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Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		287000_130US3_Response_OA_04182011.pdf	92257 85a1bcaf8628c08d1ac1ca11b864349ec8e610ef	yes	5

	Multipart Description/PDF files in .zip description		
	Document Description	Start	End
	Amendment After Final	1	1
	Claims	2	3
	Applicant Arguments/Remarks Made in an Amendment	4	5

Warnings:					
Information:					
2	Assignee showing of ownership per 37 CFR 3.73(b).	287000_130US3_Statement_04182011.pdf	55809 2d4450ba84d28d7f74b8d5cc91988e556b1bf3d1	no	1
Warnings:					
Information:					
3	Terminal Disclaimer Filed	287000_130US3_Terminal_Disclaimer_04182011.pdf	70547 09b32b6f7297e9d5127c63c6e67b2790b76c2e59	no	1
Warnings:					
Information:					
4	Fee Worksheet (PTO-875)	fee-info.pdf	29798 0d2d32bab0c51000a2acedeea7f026a9b2f3f125	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	248411		

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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/06 (07-06)
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PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number	Filing Date	☐ To be Mailed
11/894,676	08/20/2007	

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY ☒ RATE ($)	SMALL ENTITY ☒ FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT	04/18/2011	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 10	Minus	** 20	= 0	X $26 =	0	OR	X $ =	
	Independent (37 CFR 1.16(h))	* 1	Minus	***3	= 0	X $110 =	0	OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE	0	OR	TOTAL ADD'L FEE	

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)	OR	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/KIMBERLY PANNELL/

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Application Number	Application/Control No.	Applicant(s)/Patent under Reexamination	
	11/894,676	HANNON ET AL.	

Document Code - DISQ	Internal Document – DO NOT MAIL

TERMINAL DISCLAIMER	☒ APPROVED	☐ DISAPPROVED
Date Filed : 4/18/11	This patent is subject to a Terminal Disclaimer	

Approved/Disapproved by:
jean proctor

U.S. Patent and Trademark Office

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
11/894,676	08/20/2007	1635	970	287000.130US3	14	2

CONFIRMATION NO. 8161

CORRECTED FILING RECEIPT

84834
WilmerHale/Cold Spring Harbor Laboratory
399 Park Avenue
New York, NY 10022

Date Mailed: 05/20/2011

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Applicant(s)

Gregory J. Hannon, Huntington, NY;
Patrick J. Paddison, Northport, NY;
Emily Bernstein, New York, NY;
Amy Caudy, Lawrenceville, NJ;
Douglas Conklin, Cold Spring Harbor, NY;
Scott Hammond, Cold Spring Harbor, NY;

Power of Attorney: The patent practitioners associated with Customer Number 28089

Domestic Priority data as claimed by applicant

This application is a CON of 10/997,086 11/23/2004
which is a CIP of 10/350,798 01/24/2003 ABN
which is a CIP of 10/055,797 01/22/2002 ABN
which is a CIP of PCT/US01/08435 03/16/2001
which claims benefit of 60/189,739 03/16/2000
and claims benefit of 60/243,097 10/24/2000
and said 10/350,798 01/24/2003
is a CIP of 09/866,557 05/24/2001 ABN
which is a CIP of PCT/US01/08435 03/16/2001
and said 10/350,798 01/24/2003
is a CIP of 09/858,862 05/16/2001 PAT 7,732,417
which is a CIP of PCT/US01/08435 03/16/2001

Foreign Applications (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.)

If Required, Foreign Filing License Granted: 11/02/2007

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 11/894,676**

Projected Publication Date: Not Applicable

Non-Publication Request: No

Early Publication Request: No
**** SMALL ENTITY ****
Title

Methods and compositions for RNA interference

Preliminary Class

435

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4158).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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www.uspto.gov

NOTICE OF ALLOWANCE AND FEE(S) DUE

84834 7590 06/13/2011

WilmerHale/Cold Spring Harbor Laboratory
399 Park Avenue
New York, NY 10022

EXAMINER
CHONG, KIMBERLY

ART UNIT	PAPER NUMBER
1635	

DATE MAILED: 06/13/2011

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/894,676	08/20/2007	Gregory J. Hannon	287000.130US3	8161

TITLE OF INVENTION: METHODS AND COMPOSITIONS FOR RNA INTERFERENCE

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	YES	$755	$300	$0	$1055	09/13/2011

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the SMALL ENTITY status shown above.

If the SMALL ENTITY is shown as YES, verify your current SMALL ENTITY status:

A. If the status is the same, pay the TOTAL FEE(S) DUE shown above.

B. If the status above is to be removed, check box 5b on Part B - Fee(s) Transmittal and pay the PUBLICATION FEE (if required) and twice the amount of the ISSUE FEE shown above, or

If the SMALL ENTITY is shown as NO:

A. Pay TOTAL FEE(S) DUE shown above, or

B. If applicant claimed SMALL ENTITY status before, or is now claiming SMALL ENTITY status, check box 5a on Part B - Fee(s) Transmittal and pay the PUBLICATION FEE (if required) and 1/2 the ISSUE FEE shown above.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail **Mail Stop ISSUE FEE**
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

84834 7590 06/13/2011

WilmerHale/Cold Spring Harbor Laboratory
399 Park Avenue
New York, NY 10022

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/894,676	08/20/2007	Gregory J. Hannon	287000.130US3	8161

TITLE OF INVENTION: METHODS AND COMPOSITIONS FOR RNA INTERFERENCE

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	YES	$755	$300	$0	$1055	09/13/2011

EXAMINER	ART UNIT	CLASS-SUBCLASS
CHONG, KIMBERLY	1635	536-024500

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

❑ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

(1) the names of up to 3 registered patent attorneys or agents OR, alternatively,

(2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 _____

2 _____

3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ❑ Individual ❑ Corporation or other private group entity ❑ Government

4a. The following fee(s) are submitted:

❑ Issue Fee

❑ Publication Fee (No small entity discount permitted)

❑ Advance Order - # of Copies _____

4b. Payment of Fee(s): **(Please first reapply any previously paid issue fee shown above)**

❑ A check is enclosed.

❑ Payment by credit card. Form PTO-2038 is attached.

❑ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment, to Deposit Account Number _____ (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)

❑ a. Applicant claims SMALL ENTITY status. See 37 CFR 1.27.

❑ b. Applicant is no longer claiming SMALL ENTITY status. See 37 CFR 1.27(g)(2).

NOTE: The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____

This collection of information is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.

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United States Patent and Trademark Office
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/894,676	08/20/2007	Gregory J. Hannon	287000.130US3	8161

84834 7590 06/13/2011

WilmerHale/Cold Spring Harbor Laboratory
399 Park Avenue
New York, NY 10022

EXAMINER	
CHONG, KIMBERLY	

ART UNIT	PAPER NUMBER
1635	

DATE MAILED: 06/13/2011

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment to date is 0 day(s). If the issue fee is paid on the date that is three months after the mailing date of this notice and the patent issues on the Tuesday before the date that is 28 weeks (six and a half months) after the mailing date of this notice, the Patent Term Adjustment will be 0 day(s).

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Notice of Allowability	Application No.	Applicant(s)
	11/894,676	HANNON ET AL.
	Examiner	Art Unit
	KIMBERLY CHONG	1635

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to *04/18/2011*.
2. ☒ The allowed claim(s) is/are *50,52,54-61*.
3. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
   a) ☐ All b) ☐ Some* c) ☐ None of the:
      1. ☐ Certified copies of the priority documents have been received.
      2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
      3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

4. ☐ A SUBSTITUTE OATH OR DECLARATION must be submitted. Note the attached EXAMINER'S AMENDMENT or NOTICE OF INFORMAL PATENT APPLICATION (PTO-152) which gives reason(s) why the oath or declaration is deficient.
5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   (a) ☐ including changes required by the Notice of Draftsperson's Patent Drawing Review (PTO-948) attached
      1) ☐ hereto or 2) ☐ to Paper No./Mail Date _____.
   (b) ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).
6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)	5. ☐ Notice of Informal Patent Application
2. ☐ Notice of Draftperson's Patent Drawing Review (PTO-948)	6. ☐ Interview Summary (PTO-413), Paper No./Mail Date _____ .
3. ☐ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____	7. ☐ Examiner's Amendment/Comment
4. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material	8. ☐ Examiner's Statement of Reasons for Allowance
	9. ☐ Other _____.
	/Kimberly Chong/ Primary Examiner AU1635

Search Notes	Application/Control No.	Applicant(s)/Patent under Reexamination
	11/894,676	HANNON ET AL.
	Examiner	Art Unit
	KIMBERLY CHONG	1635

SEARCHED			
Class	Subclass	Date	Examiner

INTERFERENCE SEARCHED			
Class	Subclass	Date	Examiner
536	**24.5**	**05/20/2011**	**KC**

SEARCH NOTES (INCLUDING SEARCH STRATEGY)		
	DATE	EXMR
updated	05/20/2011	KC

Issue Classification	Application/Control No.	Applicant(s)/Patent under Reexamination	
	11/894,676	HANNON ET AL.	
	Examiner	Art Unit	
	KIMBERLY CHONG	1635	

ISSUE CLASSIFICATION

ORIGINAL			CROSS REFERENCE(S)						
CLASS	SUBCLASS	CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)						
536	24.5	536	24.31	24.1					
INTERNATIONAL CLASSIFICATION		435	6	325	375				
C 0 7 H 21/04		514	44						
/									
/									
/									
/									

(Assistant Examiner) (Date)

(Legal Instruments Examiner) (Date)

/Kimberly Chong/
Primary Examiner AU1635
05/22/2011
(Primary Examiner) (Date)

Total Claims Allowed: 10

O.G. Print Claim(s)	O.G. Print Fig.
1	none

☒ Claims renumbered in the same order as presented by applicant ☐ CPA ☒ T.D. ☐ R.1.47

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original
	1		31		61		91		121		151		181
	2		32		62		92		122		152		182
	3		33		63		93		123		153		183
	4		34		64		94		124		154		184
	5		35		65		95		125		155		185
	6		36		66		96		126		156		186
	7		37		67		97		127		157		187
	8		38		68		98		128		158		188
	9		39		69		99		129		159		189
	10		40		70		100		130		160		190
	11		41		71		101		131		161		191
	12		42		72		102		132		162		192
	13		43		73		103		133		163		193
	14		44		74		104		134		164		194
	15		45		75		105		135		165		195
	16		46		76		106		136		166		196
	17		47		77		107		137		167		197
	18		48		78		108		138		168		198
	19		49		79		109		139		169		199
	20		50		80		110		140		170		200
	21		51		81		111		141		171		201
	22		52		82		112		142		172		202
	23		53		83		113		143		173		203
	24		54		84		114		144		174		204
	25		55		85		115		145		175		205
	26		56		86		116		146		176		206
	27		57		87		117		147		177		207
	28		58		88		118		148		178		208
	29		59		89		119		149		179		209
	30		60		90		120		150		180		210

Claim Listing

This listing of the claims will replace all prior versions and listings of claims in the application:

1-49. (Cancelled)

50. (Previously presented) A method for attenuating expression of a target gene in a mammalian cell, the method comprising

introducing into mammalian cells a library of RNA expression constructs, each expression construct comprising:

(i) an RNA polymerase promoter, and

(ii) a sequence encoding a short hairpin RNA molecule comprising a double-stranded region wherein the double-stranded region consists of at least 20 nucleotides but not more than 29 nucleotides,

wherein the short hairpin RNA molecule is a substrate for Dicer-dependent cleavage and does not trigger a protein kinase RNA-activated (PKR) response in the mammalian cell,

wherein the double-stranded region of the short hairpin RNA molecule comprises a sequence that is complementary to a portion of the target gene, and

wherein the short hairpin RNA molecule is stably expressed in the mammalian cell in an amount sufficient to attenuate expression of the target gene in a sequence specific manner, and is expressed in the cell without use of a PK inhibitor, whereby expression of the target gene is inhibited.

51. (Cancelled)

52. (Previously presented) The method of claim 50, wherein the expression construct further comprises LTR sequences located 5' and 3' of the sequence encoding the short hairpin RNA molecule.

53. (Cancelled)

54. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of at least 21 nucleotides.

55. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of at least 22 nucleotides.

56. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of at least 25 nucleotides.

57. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of 29 nucleotides.

58. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule has a total length of about 70 nucleotides.

59. (Previously presented) The method of claim 50, wherein the RNA polymerase promoter comprises a pol II promoter or a pol III promoter.

60. (Previously presented) The method of claim 59, wherein the pol III promoter comprises a U6, an H1, or an SRP promoter.

61. (Withdrawn) The method of claim 59, wherein the pol II promoter comprises a U1 or a CMV promoter.

62-63. (Cancelled)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Hannon et al.	Confirmation No.:	8161
Application No:	11/894,676	Art Unit:	1635
Filed:	August 20, 2007	Examiner:	K. CHONG
Title:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

REQUEST UNDER 37 CFR 1.28(c) TO CORRECT ENTITY STATUS

Dear Sir:

Small entity status was established for this application in good faith, and fees as a small entity have been paid in good faith. It has been discovered that such status as a small entity was established in error. As required by Rule 28(c)(2)(ii), an itemization of the total deficiency payment, which was calculated as required by Rule 28(c)(2)(i), is set forth below.

Type of Fee	Date of Payment	Current Fee for Large Entity	Amount of Fee Paid	Amount Owed
Basic filing fee	01/07/2008	330	155	175
Search fee	01/07/2008	540	255	285
Examination fee	01/07/2008	220	105	115
Application size fee (over 100 pages)	01/07/2008	$270/50 pages x 3 = 810	$130/50 pages x 3 = 390	420
Oath or Declaration surcharge	01/07/2008	130	65	65
5-month extension of time	04/09/2009	2,350	1,175	1,175
3-month extension of time	11/04/2009	1,110	555	555
Petition fee under 37 CFR 1.17(h) (Group III)	04/08/2010	130	130	0
3-month extension of time	07/19/2010	1,110	555	555
2-month extension of time	01/31/2011	490	245	245
Information Disclosure Statement	02/11/2011	180	180	0

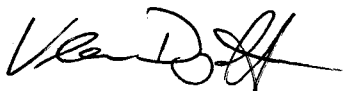
Type of Fee	Date of Payment	Current Fee for Large Entity	Amount of Fee Paid	Amount Owed
Information Disclosure Statement	03/04/2011	180	180	0
Information Disclosure Statement	03/23/2011	180	180	0
Terminal Disclaimer	04/18/2011	140	70	70
			Total Owed	$3,660

The total deficiency payment accompanies this paper. The Commissioner is authorized to charge any additionally required fees, or to credit any overpayment in such fees, to Deposit Account No. 08-0219.

Dated:

7/5/2011

Respectfully submitted,



Vladimir Drozdoff, Ph.D.
Registration No. 51,333

Attorney for Applicant(s)

Office of Technology Transfer
Cold Spring Harbor Laboratory
1 Bungtown Road
Cold Spring Harbor, NY 11724
(516) 367-5010

PTO/SB/17 (10-08)
Approved for use through 09/30/2010. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no person are required to respond to a collection of information unless it displays a valid OMB control number

Effective on 12/08/2004.
Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).

FEE TRANSMITTAL
For FY 2009

☐ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 3,660.00

Complete if Known	
Application Number	11/894,676-Conf. #8161
Filing Date	August 20, 2007
First Named Inventor	Gregory J. HANNON
Examiner Name	K. Chong
Art Unit	1635
Attorney Docket No.	0287000.00130US3

METHOD OF PAYMENT (check all that apply)

☐ Check ☐ Credit Card ☐ Money Order ☐ None ☐ Other (please identify): _____

☒ Deposit Account Deposit Account Number: 08-0219 Deposit Account Name: Wilmer Cutler Pickering Hale and Dorr LLP

For the above-identified deposit account, the Director is hereby authorized to: (check all that apply)

☒ Charge fee(s) indicated below ☐ Charge fee(s) indicated below, **except for the filing fee**

☒ Charge any additional fee(s) or underpayments of fee(s) under 37 CFR 1.16 and 1.17 ☒ Credit any overpayments

FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES

	FILING FEES		SEARCH FEES		EXAMINATION FEES		
Application Type	**Fee ($)**	**Small Entity Fee ($)**	**Fee ($)**	**Small Entity Fee ($)**	**Fee ($)**	**Small Entity Fee ($)**	**Fees Paid ($)**
Utility	330	165	540	270	220	110	575.00
Design	220	110	100	50	140	70	
Plant	220	110	330	165	170	85	
Reissue	330	165	540	270	650	325	
Provisional	220	110	0	0	0	0	

2. EXCESS CLAIM FEES

Fee Description	**Fee ($)**	**Small Entity Fee ($)**
Each claim over 20 (including Reissues)	52	26
Each independent claim over 3 (including Reissues)	220	110
Multiple dependent claims	390	195

Total Claims	**Extra Claims**	**Fee ($)**	**Fee Paid ($)**
_____ - 20 or HP	_____ x	_____ =	_____

HP = highest number of total claims paid for, if greater than 20.

Multiple Dependent Claims	
Fee ($)	**Fee Paid ($)**
_____	_____

Indep. Claims	**Extra Claims**	**Fee ($)**	**Fee Paid ($)**
_____ - 3 or HP =	_____ x	_____ =	_____

HP = highest number of independent claims paid for, if greater than 3.

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), the application size fee due is $270 ($135 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets	**Extra Sheets**	**Number of each additional 50 or fraction thereof**	**Fee ($)**	**Fee Paid ($)**
_____ - 100 =	_____	/50 = _____ (round **up** to a whole number) x	_____ =	420.00

4. OTHER FEE(S) **Fees Paid ($)**

Non-English Specification, $130 fee (no small entity discount) _____

Other (e.g., late filing surcharge): Oath or declaration surcharge, Extension of Time, Terminal Disclaimer 2,665.00

SUBMITTED BY

Signature	/Julia A. Grimes/	Registration No. (Attorney/Agent)	66,170	Telephone	(212) 230-8800
Name (Print/Type)	Julia Grimes, Ph.D.			Date	July 20, 2011

Electronic Acknowledgement Receipt

EFS ID:	10557863
Application Number:	11894676
International Application Number:	
Confirmation Number:	8161
Title of Invention:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE
First Named Inventor/Applicant Name:	Gregory J. Hannon
Customer Number:	84834
Filer:	Julia Anne Grimes/sophie murray
Filer Authorized By:	Julia Anne Grimes
Attorney Docket Number:	287000.130US3
Receipt Date:	20-JUL-2011
Filing Date:	20-AUG-2007
Time Stamp:	14:40:02
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Miscellaneous Incoming Letter	287000_130US3Correction_Entity_Status_07202011.pdf	65790 f9104891a682b9bd7a348cc408bd7fb3de5c4b2d	no	2

Warnings:

Information:

2	Fee Worksheet (SB06)	287000_130US3_Fee_transmittal_07202011.pdf	131767 353f1cb5f87fead662d5df5c03da2b66ee9ffce2	no	1

Warnings:	
Information:	
Total Files Size (in bytes):	197557

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

Bib Data Sheet

CONFIRMATION NO. 8161

SERIAL NUMBER	FILING OR 371(c) DATE / RULE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
11/894,676	08/20/2007	536	1635	287000.130US3

APPLICANTS

Gregory J. Hannon, Huntington, NY;
Patrick J. Paddison, Northport, NY;
Emily Bernstein, New York, NY;
Amy Caudy, Lawrenceville, NJ;
Douglas Conklin, Cold Spring Harbor, NY;
Scott Hammond, Cold Spring Harbor, NY;

**** CONTINUING DATA ***************************

This application is a CON of 10/997,086 11/23/2004
which is a CIP of 10/350,798 01/24/2003 ABN
which is a CIP of 10/055,797 01/22/2002 ABN
which is a CIP of PCT/US01/08435 03/16/2001
which claims benefit of 60/189,739 03/16/2000
and claims benefit of 60/243,097 10/24/2000
and said 10/350,798 01/24/2003
is a CIP of 09/866,557 05/24/2001 ABN
which is a CIP of PCT/US01/08435 03/16/2001
and said 10/350,798 01/24/2003
is a CIP of 09/858,862 05/16/2001 PAT 7,732,417
which is a CIP of PCT/US01/08435 03/16/2001

**** FOREIGN APPLICATIONS *********************

IF REQUIRED, FOREIGN FILING LICENSE GRANTED
** 11/02/2007

		STATE OR COUNTRY	SHEETS DRAWING	TOTAL CLAIMS	INDEPENDENT CLAIMS
Foreign Priority claimed	☐ yes ☐ no	NY	67	14	2
35 USC 119 (a-d) conditions met	☐ yes ☐ no ☐ Met after Allowance				
Verified and Acknowledged	Examiner's Signature Initials				

ADDRESS
84834

TITLE

METHODS AND COMPOSITIONS FOR RNA INTERFERENCE

FILING FEE RECEIVED	FEES: Authority has been given in Paper No. _____ to charge/credit DEPOSIT ACCOUNT No. _____ for following:	☐ All Fees ☐ 1.16 Fees (Filing) ☐ 1.17 Fees (Processing Ext. of time)
970		

		☐ 1.18 Fees (Issue)
		☐ Other _____
		☐ Credit

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UNITED STATES DEPARTMENT OF COMMERCE
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/894,676	08/20/2007	Gregory J. Hannon	287000.130US3	8161

84834 7590 08/22/2011

WilmerHale/Cold Spring Harbor Laboratory
399 Park Avenue
New York, NY 10022

EXAMINER	
CHONG, KIMBERLY	
ART UNIT	PAPER NUMBER
1635	

NOTIFICATION DATE	DELIVERY MODE
08/22/2011	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

Teresa.carvalho@wilmerhale.com
whipusptopairs@wilmerhale.com

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450
www.uspto.gov

Application No. : 11894676
Applicant : Hannon
Filing Date : 08/20/2007
Date Mailed : 08/22/2011

NOTICE TO FILE CORRECTED APPLICATION PAPERS

Notice of Allowance Mailed

This application has been accorded an Allowance Date and is being prepared for issuance. The application, however, is incomplete for the reasons below.

Applicant is given 2 month(s) from the mail date of this Notice, or the time remaining from the Notice of Allowance and Fee(s) Due, whichever is longer, within which to respond.

The informalities requiring correction are indicated in the attachment(s). If the informality pertains to the abstract, specification (including claims) or drawings, the informality must be corrected with an amendment in compliance with 37 CFR 1.121 (or, if the application is a reissue application, 37 CFR 1.173). Such an amendment may be filed after payment of the issue fee if limited to correction of informalities noted herein. See Waiver of 37 CFR 1.312 for Documents Required by the Office of Patent Publication, 1280 Off. Gaz. Patent Office 918 (March 23, 2004). In addition, if the informality is not corrected until after payment of the issue fee, for purposes of 35 U.S.C. 154(b)(1)(iv), "all outstanding requirements" will be considered to have been satisfied when the informality has been corrected. A failure to respond within the above-identified time period will result in the application being ABANDONED. **This period for reply is NOT extendable under 37 CFR 1.136(a).**

See attachment(s).

A copy of this notice MUST be returned with the reply. Please address response to "Mail Stop Issue Fee,Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450".

/Tamika Tolbert/
Publication Branch
Office of Data Management
(571) 272-4200

Application No. 11894676

IDENTIFICATION OF SPECIFICATION/DRAWING INCONSISTENCIES

☐ On Page of the specification there is a brief description of FIG. , but the drawings filed do not include a drawing with that designation. Applicant must respond either by supplying the omitted drawing or by amending the specification to remove all references to that drawing.

☒ The drawings filed 08/20/2007 include FIG. 2D, but the specification's brief description of the drawings does not describe a drawing with that designation. Applicant must respond either by amending the specification to add a brief description of that drawing or by correcting the drawings to remove the drawing in question.

☐ Drawings are present in the application and are referred to in the detailed description of the invention, but the specification does not contain a brief description of the drawings as required by 37 CFR 1.74 and 37 CFR 1.77(b)(8).

☐ Page of the specification refers to FIG. , but no drawing with that designation is described in the brief description of the drawings and no drawing with that designation is present in the application. Applicant must respond either by amending the specification to remove all references to that drawing, or by supplying that drawing and amending the specification to add a brief description of it.

☐ OTHER:

☐ COMMENTS:

UNITED STATES PATENT AND TRADEMARK OFFICE

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Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/894,676	08/20/2007	Gregory J. Hannon	287000.130US3	8161

84834 7590 09/12/2011

WilmerHale/Cold Spring Harbor Laboratory
399 Park Avenue
New York, NY 10022

EXAMINER	
CHONG, KIMBERLY	

ART UNIT	PAPER NUMBER
1635	

NOTIFICATION DATE	DELIVERY MODE
09/12/2011	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

Teresa.carvalho@wilmerhale.com
whipusptopairs@wilmerhale.com

Applicant-Initiated Interview Summary	Application No.	Applicant(s)	
	11/894,676	HANNON ET AL.	
	Examiner	Art Unit	
	KIMBERLY CHONG	1635	

All participants (applicant, applicant's representative, PTO personnel):

(1) *KIMBERLY CHONG*. (3)_____.

(2) *JANE LOVE*. (4)_____.

Date of Interview: *19 August 2011*.

Type: ☒ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☐ No.
If Yes, brief description: _____.

Issues Discussed ☐101 ☐112 ☐102 ☐103 ☒Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: _____.

Identification of prior art discussed: _____.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

Jane Love contacted SPE Heather Calamita and the Examiner about patent 7,691,995. This patent was brought to the attention of the representatives and Jane Love asked about getting this patent considered. The Examiner stated there are several options one being submit and IDS with the proper certification before issue fees are paid. The other option would be to file an RCE. The Examiner stated that this patent had not been considered and in briefly reviewing the patent stated this could be considered prior art. The Examiner stated this patent would need to be made of record.

Applicant recordation instructions: The formal written reply to the last Office action must include the substance of the interview. (See MPEP section 713.04). If a reply to the last Office action has already been filed, applicant is given a non-extendable period of the longer of one month or thirty days from this interview date, or the mailing date of this interview summary form, whichever is later, to file a statement of the substance of the interview

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☐ Attachment

	/Kimberly Chong/ Primary Examiner AU1635

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:
1) A brief description of the nature of any exhibit shown or any demonstration conducted,
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
   (The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiner's version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, "Interview Record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.

PTO/SB/30EFS (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

REQUEST FOR CONTINUED EXAMINATION(RCE)TRANSMITTAL
(Submitted Only via EFS-Web)

Application Number	11894676	Filing Date	2007-08-20	Docket Number (if applicable)	0287000.00130US3	Art Unit	1635
First Named Inventor	Gregory J. HANNON			Examiner Name	K. Chong		

This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application.
Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, or to any design application. The Instruction Sheet for this form is located at WWW.USPTO.GOV

SUBMISSION REQUIRED UNDER 37 CFR 1.114

Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).

☐ Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.

- ☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____
- ☐ Other _____

☒ Enclosed

- ☐ Amendment/Reply
- ☒ Information Disclosure Statement (IDS)
- ☐ Affidavit(s)/ Declaration(s)
- ☒ Other Amendment and Response to Notice to File Corrected Application Papers

MISCELLANEOUS

☐ Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a period of months _____
(Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)

☐ Other _____

FEES

☒ **The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.**
The Director is hereby authorized to charge any underpayment of fees, or credit any overpayments, to Deposit Account No 080219

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

☒ Patent Practitioner Signature

☐ Applicant Signature

Signature of Registered U.S. Patent Practitioner			
Signature	/Anne-Marie C. Yvon/	Date (YYYY-MM-DD)	2011-09-13
Name	Anne-Marie C. Yvon	Registration Number	52390

This collection of information is required by 37 CFR 1.114. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Hannon et al.	Confirmation No.:	8161
Application No:	11/894,676	Art Unit:	1635
Filed:	August 20, 2007	Examiner:	K. CHONG
Title:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT AND RESPONSE TO NOTICE TO FILE CORRECTED APPLICATION PAPERS, ACCOMPANIED BY REQUEST FOR CONTINUED EXAMINATION AND INFORMATION DISCLOSURE STATEMENT

This is a response to the Notice to File Corrected Application Papers mailed on August 22, 2011, setting a two-month period for reply. A response is due October 22, 2011. Therefore, this paper is timely filed.

A Notice of Allowance was mailed on June 13, 2011, and the issue fee is due September 13, 2011. Instead of paying the issue fee, Applicants submit an RCE and IDS for consideration by the Examiner, prior to payment of the issue fee.

The Commissioner is authorized to charge any fees due, or to credit any overpayment in fees, to Deposit Account No. 08-0219.

AMENDMENT

In the Drawings

Please replace Figures 2A-2D with the accompanying figures 2A-2C, which are marked "Replacement Sheet" in accordance with 37 C.F.R. § 1.121(d).

REMARKS

I. Status of Claims and Formal Matters

Claims 50, 52, and 54-61 are pending and were allowed in the June 13, 2011 Notice of Allowance. A Request for Continued Application accompanies this paper in order to cite U.S. Patent No. 7,691,995 and its provisional application Serial No. 60/305,185, such that they are considered and entered into the record.

The Notice to File Corrected Application Papers, dated August 22, 2011, stated that the specification does not include a description of Figure 2D and required either an amendment to the specification to add a brief description, or a correction of the drawing. Upon review of Figure 2, it has come to Applicant's attention that the transcripts depicted in Figure 2D were erroneously separated from Figures 2B and 2C. See, for example, the description of Figure 2B at page 10, lines 18-23, which refers to the "cross hatched box, below." The attached Replacement Sheet for Figure 2 includes the transcripts as originally intended, as part of Figures 2B and 2C. No new matter is added.

Applicants submit that the requirements of the Notice to File Corrected Application Papers have been met and that the application is complete.

II. Telephone Conference with Examiner Chong

On August 24, 2011, Dr. Jane Love of WilmerHale and Dr. Vladimir Drozdoff of Cold Spring Harbor Laboratory, the assignee, spoke with Examiner Chong to discuss U.S. Patent No. 7,691,995, which came to the Applicant's attention. Applicant indicated that the '995 patent has the same classification as the present application, *i.e.*, class 536, sub-class 24.5, and that the '995 patent, and its underlying '185 provisional application, do not qualify as prior art under §102(e). The Examiner confirmed that the '955 patent and '185 provisional were not yet of record. Because the present application was already allowed, the Examiner and Applicant agreed that Applicant would file an RCE in order to make the '955 patent and '185 provisional of record.

III. Information Disclosure Statement – U.S. Patent No. 7,691,995 and Provisional Application 60/305,185

This IDS is filed before payment of the issue fee and is filed concurrently with an RCE. As discussed below, neither the '995 patent, nor its corresponding published application US

2006/0009402 A1, nor its underlying '185 provisional application, is prior art to the pending claims. Applicants respectfully request the Examiner to make the '995 patent and '185 provisional of record and allow the claims to proceed to issue.

A. The 35 U.S.C. §102(e) Date Of A Published U.S. Application Is Not Entitled To A Provisional Filing Date Under 35 U.S.C. §119(e) If The Provisional Does Not Meet 35 U.S.C. § 112, First Paragraph For The Subject Matter Relied Upon To Make A Rejection

Section 2136.03 of the MPEP sets forth when a patent can be accorded a critical reference date, and qualify as prior art, based on the filing date of an underlying provisional application. Importantly, it can do so only, "if the provisional applications(s) properly supports the subject matter relied upon to make the rejection in compliance with 35 U.S.C. § 112, first paragraph." Accordingly, if a provisional application is not in compliance with the enablement and written description standards set out in 35 U.S.C. § 112, first paragraph for the subject matter of the rejection (either anticipation or obviousness), it cannot be prior art under 35 U.S.C. § 102(e). See MPEP 2136.03, III. Priority from Provisional Application Under 35 U.S.C. § 119(e). *See also, Ex parte* Yamaguchi, 88 U.S.P.Q.2d 1606 (B.P.A.I. 2008).

B. The '995 Patent is Not Prior Art Under 35 U.S.C. § 102

U.S. Serial No. 10/195,034, which issued as the '995 patent, was filed on July 12, 2002 which is *after* the filing date of the present application, January 22, 2002. The '034 application claims priority to a provisional application, U.S. Serial No. 60/305,185, filed on July 12, 2001. The '034 application published as US 2006/0009402 A1 on July 12, 2006. As discussed more fully below, the underlying '185 provisional does not describe or enable the presently claimed subject matter. Nor does the '185 provisional provide any evidence to counter the evidence of record establishing the non-obviousness of the presently claimed subject matter. It does not meet the requirements under 35 U.S.C. § 112, first paragraph for the presently claimed subject matter and therefore is not prior art under 35 U.S.C. § 102(e).

1. Critical Aspects of the Presently Claimed Methods Are Not Disclosed in the '185 Provisional

The short hairpin method invented by Dr. Hannon and his co-inventors, and claimed here, provides a way to use RNAi to stably attenuate expression of a target gene in mammalian cells. The breakthrough of Dr. Hannon's invention was in providing a way to stably suppress

expression of a gene in a sequence specific manner, that is, without activating a harmful non-specific PK/anti-viral response in the cells, and without using a PK inhibitor to prevent such a response. To do this in mammalian cells, as a critical aspect of the method, the Hannon application teaches one should express a "short hairpin RNA" consisting of a double-stranded region less than 30 nucleotides in length. Accordingly, the presently claimed method requires: (1) expressing a short hairpin RNA, defined as having a double stranded region consisting of at least 20 nucleotides but not more than 29 nucleotides in length, and (2) expressing such a hairpin without using a PK inhibitor.

Neither of these critical aspects are described or taught in the '185 provisional. When drafting the '185 provisional, the '185 applicants had neither conceived of nor described using a short hairpin RNA to suppress genes in a mammalian cell without using a PK inhibitor. The '185 provisional instead discloses generally the engineering (i.e., replacing or modifying portions of the nucleotide sequence) of wild-type small temporal RNA precursors (pre-stRNAs) to make engineered RNA precursors (pre-siRNA) that could potentially be expressed in cells to suppress genes.

Notably, the '185 applicants did not know what feature(s) could be manipulated in these engineered precursors to avoid a non-sequence specific (PK) response. The '185 provisional indicates only hypothetically that the engineered RNA precursors "as a defining feature" would not induce, or would induce a lower sequence non-specific response "as a consequence of their length, sequence and/or structure." (See page 8, lines 11-15 of the '185 provisional.) In other words, the '185 disclosure provides no guidance as to which of these variables one would need to manipulate to avoid a non-sequence specific (PK) response. The '185 provisional therefore does not disclose when and how the engineered precursors could be expressed in a mammalian cell to suppress gene expression in a sequence-specific manner without using a PK inhibitor. Not surprisingly, the engineered precursors disclosed in the '185 provisional are never described in terms of a double-stranded region, including a double-stranded region consisting of less than 30 nucleotides in length. Thus, the '185 applicants did not conceive of and describe using short hairpin RNA.

In March 2002, eight months after the '185 provisional was filed, the paper by Paddison et al., "Short Hairpin RNAs (shRNAs) Induce Sequence-Specific Silencing in Mammalian

Cells." *Genes and Development*, 2002, 16:948-958, was published. It was in this paper that Dr. Hannon reported much of the work underlying the presently claimed invention, including the unexpected and surprising finding, in view of the prior art, that short hairpins with a double-stranded region under 30 nucleotides in length could mediate suppression of genes in mammalian cells and avoid inducing a harmful non-specific/ anti-viral (PK) response. (See, for example, pages 7-10 of Amendment filed January 31, 2011, Second Declaration of Professor Nouria Hernandez Under 35 U.S.C. §1.132 ¶¶ 8-15).

Four months after the Paddison paper, and seven months after the prior date of the present application, the '185 applicants filed the '995 patent and described for the first time that it was necessary to limit the length of the double-stranded region when expressing the engineered precursors in mammalian cells: "When used in mammalian cells, the length of the stem portions should be less than about 30 nucleotides to avoid provoking non-specific responses like the interferon pathway." See ''995 patent at col. 6:45-49. The '185 provisional lacks any such disclosure. Instead of limiting the length of the stem, the '185 provisional teaches "introducing additional base-paired nucleotides to one or both of the stem portions of the natural pre-stRNA." (See page 7, line 12 of the '185 provisional).

In sum, the two crucial limitations of Hannon's method as presently claimed-- use of a short hairpin RNA, and expressing that short hairpin RNA in a mammalian cell without use of a PK inhibitor -- are not described in the '185 provisional. Accordingly, the '185 provisional does not reasonably convey to one skilled in the relevant art that the Applicants had possession of the invention as presently claimed (*i.e,* described or enabled in compliance with 35 U.S.C. 112, first paragraph). As such, neither the '995 patent, nor its corresponding published application, nor the underlying '185 provisional can qualify as prior art under § 102(e).

2. The '185 Provisional Is Entirely Prophetic And Does Not Counter The Evidence Of Record Regarding The State Of The Art And The Non-Obviousness Of The Claimed Invention

The disclosure of the '185 provisional application is entirely prophetic. It provides no actual data. In contrast, the present record provides data, expert testimony, and published data showing unexpected results. For example, the record includes two substantive Declarations submitted by Professor Hernandez, as well as several interviews with Professor Hernandez, in which Dr. Hernandez, as a person of ordinary skill in the art at the time of the invention,

provided testimony regarding the understanding of one of skill in the art as to the state of the art, including published and cited references representative of the state of the art. Because its disclosure includes no actual data, the '185 provisional does not add to this evidentiary record.

The Examiner has also considered a plethora of evidence submitted by applicants in the course of the prosecution of this application. Neither this evidentiary record nor the finding of non-obviousness is changed by the '185 provisional application.

IV. CONCLUSION

In conclusion, neither the '995 patent nor the '185 provisional application are prior art as to the pending application. The filing date of the '995 patent is after the filing date of the present application. The '185 provisional application fails to disclose the subject matter of the presently pending claims to meet the requirements of 35 U.S.C. § 112, first paragraph and therefore is not prior art under 35 U.S.C. § 102. Applicants request that the Examiner make the '995 patent of record and permit the pending claims to issue as a patent.

Consideration of this paper and allowance of this application are requested. If it would advance prosecution, the Examiner is invited to contact the undersigned to discuss the contents of this paper.

Dated: September 13, 2011

Respectfully submitted,

/Anne-Marie C. Yvon/

Anne-Marie C. Yvon, Ph.D.
Registration No. 52,390

Jane M. Love, Ph.D.
Registration No. 42,812

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Docket No.: 0287000.00130US3
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Gregory J. Hannon et al.	Confirmation No.:	8161
Application No.:	11/894,676	Art Unit:	1635
Filed:	August 20, 2007	Examiner:	K. Chong
Title:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT (IDS)

Dear Sir:

This Information Disclosure Statement is being filed concurrently with a Request for Continued Examination and before the mailing of the First Office Action. No fee is required.

Applicants request that the Examiner initial and return a copy of the enclosed Form PTO SB-08 with the next communication. Applicant believes no fee is due with this response. However, if a fee is due, please charge our Deposit Account No. 08-0219, under Order No. 0287000.00130US3 from which the undersigned is authorized to draw.

Dated: September 13, 2011

Respectfully submitted,

/Anne-Marie C. Yvon/

Anne-Marie C. Yvon, Ph.D.
Registration No. 52,390

Jane M. Love, Ph.D.
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PTO/SB/08a (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 2

Complete if Known	
Application Number	11/894,676-Conf. #8161
Filing Date	August 20, 2007
First Named Inventor	Gregory J. HANNON
Art Unit	1635
Examiner Name	K. Chong
Attorney Docket Number	0287000.00130US3

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	AA*	US-7,691,995	04-06-2010	Zamore et al.	

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. * CITE NO.: Those application(s) which are marked with an single asterisk (*) next to the Cite No. are not supplied (under 37 CFR 1.98(a)(2)(iii)) because that application was filed after June 30, 2003 or is available in the IFW. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

PTO/SB/08b (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 2 of 2

Complete if Known	
Application Number	11/894,676-Conf. #8161
Filing Date	August 20, 2007
First Named Inventor	Gregory J. HANNON
Art Unit	1635
Examiner Name	K. Chong
Attorney Docket Number	0287000.00130US3

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
	CA	Provisional application Serial No. 60/305,185 filed July 12, 2001	

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1]Applicant's unique citation designation number (optional). [2]Applicant is to place a check mark here if English language Translation is attached.

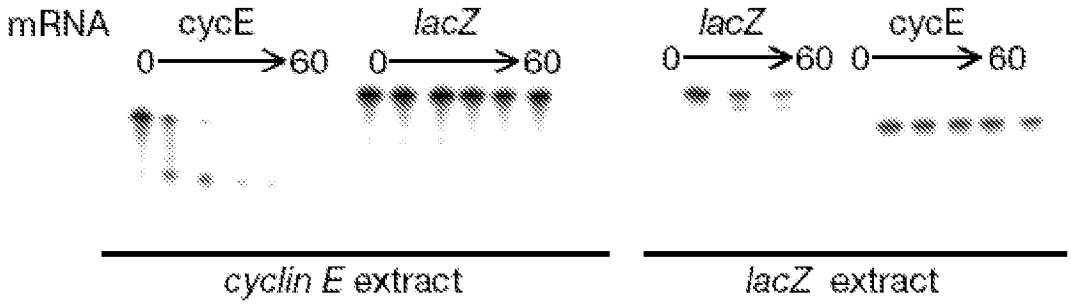
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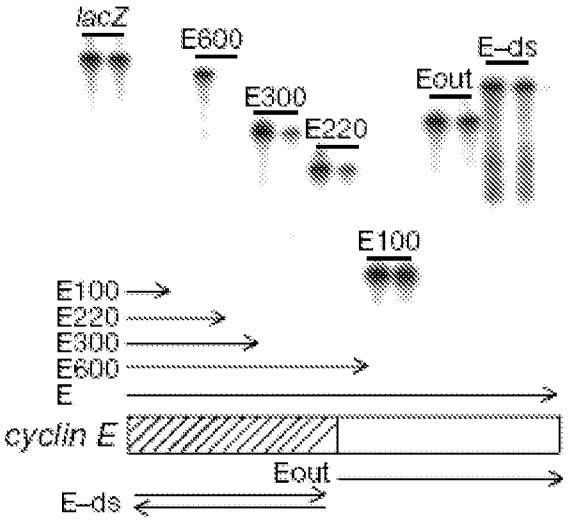
Fig. 2A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E–ds
E100
E100
E220
E300
E600
E
cyclin E
Eout
E–ds

Fig. 2B

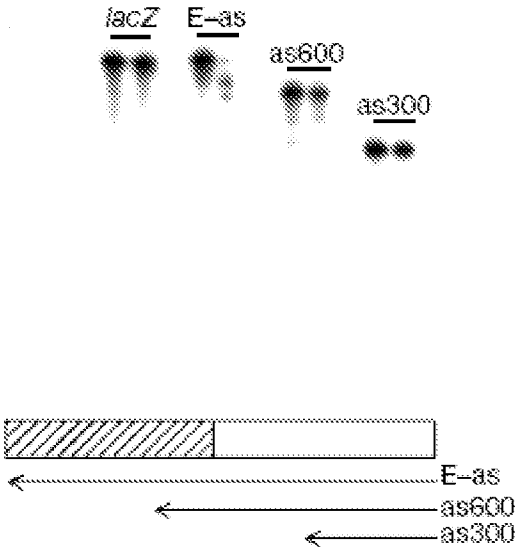

Fig. 2C

Electronic Patent Application Fee Transmittal

Application Number:	11894676
Filing Date:	20-Aug-2007
Title of Invention:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE
First Named Inventor/Applicant Name:	Gregory J. Hannon
Filer:	Anne-Marie Yvon/sophie murray
Attorney Docket Number:	287000.130US3

Filed as Large Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
Request for continued examination	1801	1	810	810
		Total in USD ($)		**810**

Electronic Acknowledgement Receipt

EFS ID:	10937802
Application Number:	11894676
International Application Number:	
Confirmation Number:	8161
Title of Invention:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE
First Named Inventor/Applicant Name:	Gregory J. Hannon
Customer Number:	84834
Filer:	Anne-Marie Yvon/sophie murray
Filer Authorized By:	Anne-Marie Yvon
Attorney Docket Number:	287000.130US3
Receipt Date:	13-SEP-2011
Filing Date:	20-AUG-2007
Time Stamp:	17:41:20
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Credit Card
Payment was successfully received in RAM	$810
RAM confirmation Number	4838
Deposit Account	080219
Authorized User	LADD,CATHLEEN

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

Charge any Additional Fees required under 37 C.F.R. Section 1.16 (National application filing, search, and examination fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.17 (Patent application and reexamination processing fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.19 (Document supply fees)

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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Request for Continued Examination (RCE)	287000_130US3_RCE_09132011.pdf	36456 4473139a0318c23c8ee7b922d8d19f50d3c355d9	no	3
Warnings:					
This is not a USPTO supplied RCE SB30 form.					
Information:					
2		287000_130US3_Amendment_09132011.pdf	114140 555e030a2ebe3a13e0d835376520966l9d394462	yes	7

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Amendment Submitted/Entered with Filing of CPA/RCE	1	1
	Applicant Arguments/Remarks Made in an Amendment	2	7

Warnings:					
Information:					
3	Transmittal Letter	287000_130US3_IDS.pdf	60608 049c2158b1e9e6c7b1c8946ca22ac9579910a401	no	1
Warnings:					
Information:					
4	Information Disclosure Statement (IDS) Form (SB08)	287000_130US3_SB08_09132011.pdf	107980 1dd221016328f12be1dcd3ef69abbf135a411683	no	2
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
5	Non Patent Literature	60305185.pdf	1564645 1c347d71219dcfc08299d560c5825290ae466ff6	no	30
Warnings:					
Information:					
6	Drawings-only black and white line drawings	287000_130US3_Replacement_drawings.pdf	69055 21496509ec4e829c29f86b94d1d2975c69d1091f	no	2
Warnings:					

Information:					
7	Fee Worksheet (SB06)	fee-info.pdf	30519 e2dfa53e87c262de775ff07d7874443ee4896bb0	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	1983403	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

SCORE Placeholder Sheet for IFW Content

Application Number: 11894676 Document Date: 9/13/2011

The presence of this form in the IFW record indicates that the following document type was received in electronic format on the date identified above. This content is stored in the SCORE database.

- Drawings – Other than Black and White Line Drawings

Since this was an electronic submission, there is no physical artifact folder, no artifact folder is recorded in PALM, and no paper documents or physical media exist. The TIFF images in the IFW record were created from the original documents that are stored in SCORE.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/894,676	08/20/2007	Gregory J. Hannon	287000.130US3	8161

84834 7590 10/31/2011

WilmerHale/Cold Spring Harbor Laboratory
399 Park Avenue
New York, NY 10022

EXAMINER
CHONG, KIMBERLY

ART UNIT	PAPER NUMBER
1635	

NOTIFICATION DATE	DELIVERY MODE
10/31/2011	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

Teresa.carvalho@wilmerhale.com
whipusptopairs@wilmerhale.com

Office Action Summary	Application No.	Applicant(s)	
	11/894,676	HANNON ET AL.	
	Examiner	Art Unit	
	KIMBERLY CHONG	1635	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *13 September 2011*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

5)☒ Claim(s) *50,52 and 54-63* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *50,52 and 54-63* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

12)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

13)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date *09/13/2011*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

The indicated allowability of the instant claims has been withdrawn in response to identification of potential prior art as explained in the Examiner Interview Summary filed 09/12/2011.

Status of the Application

Claims 50, 52 and 54-63 are pending and currently under examination.

Information Disclosure Statement

The submission of the Information Disclosure Statement on 09/13/2011 is in compliance with 37 CFR 1.97. The information disclosure statement has been considered by the examiner and signed copies have been placed in the file.

New Rejections

Claim Rejections - 35 USC § 112

The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 58, 62 and 63 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

The claims recite the "expression of the target gene is attenuated by at least 60%" or "about 60% to about 90%". These claims are indefinite because it is unclear what the decrease in expression is being measured against or compared with and

without assumption the skilled artisan would not reasonably be apprised of the scope of the invention. For purposes of examination, the claims are interpreted to mean the attenuation is being measure against a normal control cell consisting of an expression construct encoding a shRNA that does not target the target gene.

Claim 58 is drawn to a shRNA having a total length of about 70 nucleotides in length. The shRNA of “about 70 nucleotides” is indefinite because it is not particularly pointed out how long the shRNA would be. The use of the term "about" is indefinite because it could encompass any number of nucleotides such as 100 nucleotides. 100 nucleotides could be about 70 nucleotides. This recitation does not distinctly point out a size of the shRNA and thus the claim is indefinite.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

Claims 50, 52, 54-60, 62 and 63 are rejected under 35 U.S.C. 103(a) as being unpatentable over Zamore et al. (US Patent No. 7,691,995 cited on IDS filed 09/13/2011), Lieber et al. (US Patent No. 6,130,092 cited on Applicant’s IDS filed 01/10/2008), Symond et al. (US 2002/0160393 of record), Elbashir et al. (Nature 2001

of record), Good et al. (Gene Therapy 1997 cited on Applicant's IDS filed 01/10/2008) and Noonberg et al. (US Patent No. 5,624,803).

The claims are drawn to a method of attenuating expression of a target gene in a mammalian cells comprising introducing into mammalian cells a library of RNA expression constructs wherein each construct comprises a promoter and a shRNA wherein the shRNA is a substrate for Dicer-dependent cleavage and does not trigger a PKR response, wherein the shRNA is stably expressed in a mammalian cell and is expressed in a cell without the use of a PK inhibitor, wherein the construct comprises LTR sequences and wherein the shRNA comprises at least 20 but less than 29 nucleotide double stranded region and wherein the promoter is a pol III, U6 promoter.

Zamore et al. teach the use of shRNA for attenuating expression of a target gene wherein the shRNA consists of stem portions that are about 18 to about 40 or more nucleotides in length. Zamore et al. teach methods of using said shRNA for targeting genes in mammalian cells and teach said shRNA can be expressed from DNA constructs comprising pol II or pol III promoters and regulatory sequences (see priority application '185 at pages 2-7).

Zamore et al. teach in paragraph [0048] that [a]nother defining feature of these engineered RNA precursors is that as a consequence of their length, sequence, and/or structure, they do not induce sequence non-specific responses, such as induction of the interferon response or apoptosis, or that they induce a lower level of such sequence non-specific responses than long, double-stranded RNA (>150 bp) currently used to

induce RNAi. For example, the interferon response is triggered by dsRNA longer than 30 base pairs." (see priority application '185 at page 8).

Zamore et al. further teach the shRNA vector can be stably expressed from cells (see priority application '185 at pages 8-9) and teach methods of mediating RNAi in cells using the shRNA constructs wherein the constructs are introduced into cells and, processed by Dicer to yield siRNA that reduce target gene expression (see priority application '185 at pages 19-20).

Zamore et al. do not specifically teach the use of shRNA library expression constructs and do not specifically state the shRNA is expressed in cells without the use of a PK inhibitor.

Methods of attenuating expression of a target gene and searching for the function gene comprising making randomized inhibitory nucleic acid libraries were known in the art at the time of filing of the instant invention. Lieber et al. teach the use of ribozyme libraries and introducing the ribozyme libraries into mammalian cells, selecting cells into which the library expression systems were introduced and analyzing the phenotypes of the cells (see Figure 2 and columns 3 and 8 and claims 1-8). Lieber et al. teach the ribozymes are chemically synthesized by transcription using expression cassettes comprising pol II (CMV) or pol III promoters (see column 3). Further, Symonds et al. teach the use of attenuation of expression of a target gene using expression vectors expression shRNA wherein the vectors can be retroviral expression constructs comprising LTR sequences flanking the hairpin RNA (see paragraph 0136, 0158 and Figure 9).

Thus the use of expression systems as instantly claimed was well known in the art as well as the use of libraries for expression multiple RNA constructs as well as the use of various pol II or pol III promoters in constructs for expression of inhibitory nucleic acid molecules as shown by Good et al. who teach an expression construct comprising a U6 promoter and a coding sequence for a hairpin RNA wherein the expression construct is capable of efficiently expressing small hairpin RNA and LTR sequences flanking the RNA sequences (see entire document and at least Figure 1) and Noonberg et al. who teach an expression construct for generation of short-sequence specific oligonucleotides for the purpose of gene regulation wherein the construct comprises a U6 promoter (see columns 7-8). Noonberg et al. teach such constructs facilitate delivery of oligonucleotides to any target cell.

It would have been obvious to one of ordinary skill in the art to use a library of RNA expression constructs capable of expression the shRNA given Lieber et al. teach identifying a gene responsible for a particular phenotype is crucial to important any biological mechanism and our understanding of disease and teach the use of a library expression system that can identify genes that are specifically involved in producing a particular phenotype by knocking down intracellular expression, one would have clearly been motivated to incorporate a shRNA in the library expression system to attenuate the expression of a target gene and identify the function of said gene and would have expected to be capable as taught by Symonds et al.

Moreover it was well known in the art that pol III promoters such as U6 promoters could be used to efficiently generate inhibitory oligonucleotides as taught by Noonberg

et al. and given Good et al. teach a construct comprising U6 promoters were capable of expressing shRNA, one of ordinary skill in the art would have used a U6 promoter to generate the shRNA as claimed..

Because it was well known in the art at the time of filing of the instant application that dsRNAs greater than 30 base pairs activated an unwanted PKR response in cells (Elbashir et al. Nature 2001 of record), it would have been obvious for one of skill in the art to use the methods of Zamore et al. in cells without the use of a PK inhibitor and would have expected to be capable of attenuating the expression of a target gene in a mammalian cell using the shRNA in the methods of Zamore et al. without the use of a PK inhibitor particularly given Zamore et al. teach the "…defining feature of these engineered RNA precursors is that as a consequence of their length, sequence, and/or structure, they do not induce sequence non-specific responses, such as induction of the interferon response…" as compared to longer dsRNA that were known to cause this effect.

Thus in the absence of evidence to the contrary, the invention as a whole would have been prima facie

Response to Arguments

Applicant argues in the response filed 09/13/2011 that the '185 application discloses generally the use of precursor molecules as claimed but neither conceived nor described using a short hairpin RNA to suppress genes in a mammalian cell without using a PK inhibitor. Applicant further argues the '185 applicants did not know what

features could be manipulated in these shRNA molecules to avoid a non-sequence (PK) response and provided no guidance as to what variables one would need to manipulate to avoid a PK response. Applicant's state the '185 never described the precursors in terms of a double-stranded region of less than 30 nucleotides.

In response the last point of the '185 never describing the precursor in terms of a double-stranded region, page 2 of the '185 clearly describes the precursor as comprising a first and second stem portion of 19 to 22 nucleotides in length that hybridize together and comprises a loop. This is a clear description of a shRNA and therefore Applicant's arguments are not convincing.

Regarding the argument that the '185 application neither conceived nor described using a short hairpin RNA to suppress genes in a mammalian cell without using a PK inhibitor, while Zamore et al. do not specifically state the methods do not use a PK inhibitor is not a persuasive argument that one of skill in the art would not practice the methods taught by Zamore et al. without the use of a PK inhibitor. Applicant's inherent support the exclusion of a PK inhibitor is in Example 7 (as previously pointed out by Applicant), wherein the shRNA is expressed in a cell and siRNA was effective in suppressing gene expression because as further pointed out by Applicant, claim 16 of the priority application 10/055,797 specifically recite the use of a PK inhibitor.

Thus while Applicants argue that the '185 Zamore application did not know what features could be manipulated in these shRNA to avoid a PK response, neither did the instant application. There is no evidence provided in the instant application or priority applications that discuss which features could be manipulated to avoid a PK response

and further there is no guidance provided that shows what variables one would need to manipulate to avoid a PK response. At the time of filing of the instant application, the prior art reference of Zamore et al. taught what was later described by the instant application, smaller precursor molecules could be used to attenuate expression of a target gene without the PK response typically seen by longer dsRNA.

What is clearly taught by Zamore et al. is that the principle design of their shRNA was the size their size, length and structure did not induce a PK response as compared to longer dsRNA shown in the prior art that did induce a PK response. So with respect to features that Zamore et al. new could be manipulated to avoid a PK response, one of skill in the art would have clearly realized Zamore et al. recognized and discussed the use of smaller shRNA precursors that were capable of generating a siRNA in cells without a PK response.

Given this feature as discussed above, one would have used the methods in cells without the use of a PK inhibitor and would have expected to be capable of attenuating the expression of a target gene in a mammalian cell using the shRNA in the methods of Zamore et al. without the use of a PK inhibitor particularly given Zamore et al. teach the "…defining feature of these engineered RNA precursors is that as a consequence of their length, sequence, and/or structure, they do not induce sequence non-specific responses, such as induction of the interferon response…" as compared to longer dsRNA that were known to cause this effect.

Thus the claimed invention was obvious to one of skill in the art at the time the invention was made.

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Kimberly Chong whose telephone number is 571-272-3111. The examiner can normally be reached Monday thru Friday between 7-4 pm.

If attempts to reach the examiner by telephone are unsuccessful please contact the SPE for 1635 Heather Calamita at 571-272-2876. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Patent applicants with problems or questions regarding electronic images that can be viewed in the Patent Application Information Retrieval system (PAIR) can now contact the USPTO's Patent Electronic Business Center (Patent EBC) for assistance. Representatives are available to answer your questions daily from 6 am to midnight (EST). The toll free number is (866) 217-9197. When calling please have your application serial or patent number, the type of document you are having an image problem with, the number of pages and the specific nature of the problem. The Patent Electronic Business Center will notify applicants of the resolution of the problem within 5-7 business days. Applicants can also check PAIR to confirm that the problem has been corrected. The USPTO's Patent Electronic Business Center is a complete service center supporting all patent business on the Internet. The USPTO's PAIR system provides Internet-based access to patent application status and history information. It also enables applicants to view the scanned images of their own application file folder(s) as well as general patent information available to the public. For more information about the PAIR system, see http://pair-direct.uspto.gov.

For all other customer support, please call the USPTO Call Center (UCC) at 800-786-9199.

/Kimberly Chong/
Primary Examiner
Art Unit 1635

Search Notes	Application/Control No.	Applicant(s)/Patent under Reexamination
	11/894,676	HANNON ET AL.
	Examiner	Art Unit
	KIMBERLY CHONG	1635

SEARCHED			
Class	Subclass	Date	Examiner

INTERFERENCE SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES (INCLUDING SEARCH STRATEGY)		
	DATE	EXMR
updated	10/20/2011	KC

PTO/SB/08a (07-09)
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Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 2

Complete if Known	
Application Number	11/894,676-Conf. #8161
Filing Date	August 20, 2007
First Named Inventor	Gregory J. HANNON
Art Unit	1635
Examiner Name	K. Chong
Attorney Docket Number	0287000.00130US3

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	AA*	US-7,691,995	04-06-2010	Zamore et al.	

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. * CITE NO.: Those application(s) which are marked with an single asterisk (*) next to the Cite No. are not supplied (under 37 CFR 1.98(a)(2)(iii)) because that application was filed after June 30, 2003 or is available in the IFW. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /K.C./

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet	2	of	2

Complete if Known	
Application Number	11/894,676-Conf. #8161
Filing Date	August 20, 2007
First Named Inventor	Gregory J. HANNON
Art Unit	1635
Examiner Name	K. Chong
Attorney Docket Number	0287000.00130US3

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
	CA	Provisional application Serial No. 60/305,185 filed July 12, 2001	

Examiner Signature	/Kimberly Chong/	Date Considered	10/24/2011

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1]Applicant's unique citation designation number (optional). [2]Applicant is to place a check mark here if English language Translation is attached.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/894,676	08/20/2007	Gregory J. Hannon	287000.130US3	8161

84834 7590 01/09/2012

WilmerHale/Cold Spring Harbor Laboratory
399 Park Avenue
New York, NY 10022

EXAMINER
CHONG, KIMBERLY

ART UNIT	PAPER NUMBER
1635	

NOTIFICATION DATE	DELIVERY MODE
01/09/2012	ELECTRONIC

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The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

Teresa.carvalho@wilmerhale.com
whipusptopairs@wilmerhale.com

Applicant-Initiated Interview Summary	Application No.	Applicant(s)	
	11/894,676	HANNON ET AL.	
	Examiner	Art Unit	
	KIMBERLY CHONG	1635	

All participants (applicant, applicant's representative, PTO personnel):

(1) *KIMBERLY CHONG*.

(2) *HEATHER CALAMITA*.

(3) *BENNETT CELSA*.

(4) *JANE LOVE, VLADIMIR DROZDOFF*.

Date of Interview: *12/20/2011*.

Type: ☐ Telephonic ☐ Video Conference
☒ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☒ Yes ☐ No.
If Yes, brief description: *powerpoint slides attached*.

Issues Discussed ☐101 ☐112 ☐102 ☒103 ☐Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: *pending*.

Identification of prior art discussed: _____.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

See Continuation Sheet.

Applicant recordation instructions: The formal written reply to the last Office action must include the substance of the interview. (See MPEP section 713.04). If a reply to the last Office action has already been filed, applicant is given a non-extendable period of the longer of one month or thirty days from this interview date, or the mailing date of this interview summary form, whichever is later, to file a statement of the substance of the interview

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☒ Attachment

	/Kimberly Chong/ Primary Examiner AU1635

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:
1) A brief description of the nature of any exhibit shown or any demonstration conducted,
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
   (The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiner's version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, "Interview Record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.

Continuation of Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments: Discussed 103 rejection of record, particularly prior art reference Zamore '034. Applicant's representatives requested the non-final action mailed on 10/31/2011 withdrawn as there was no reasonable expectation of success at using a short hairpin RNA of less than 30 nucleotides in length based on the prior art and the previous declaration of Dr. Hernandez. Applicant's representatives argue the Zamore '034 application does not have support in the provisional '185 application for a shRNA of less than 30 nucleotides. It was pointed out that the Zamore '185 provisional application teach a shRNA precursor with stem portions of 21 nucleotides in length which meet the instant claim limitations. Applicant's representatives contend the RNA precursor referred to in the '185 provisional application is a longer shRNA which contains a "stem portion" of 21 nucleotides in length and therefore because Zamore does not teach a shRNA with a duplex region of less than 30 nucleotides and there was no expectation of success at using a short siRNA in the prior art, the 103 rejection should be withdrawn. It was suggested that a proper response to the non-final rejection be submitted as there is a new 103 rejection and the argument of Zamore not teaching a shRNA was not raised in the inerview on 09/13/2011 wherein the Zamore reference was brought to the attention of the Examiner nor was it raised in the remarks filed with the RCE on 09/13/2011.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Hannon et al.	Confirmation No.:	8161
Application No:	11/894,676	Art Unit:	1635
Filed:	August 20, 2007	Examiner:	K. CHONG
Title:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT IN RESPONSE TO OCTOBER 31, 2011 NON-FINAL OFFICE ACTION

This Amendment is filed in response to the October 31, 2011 Non-Final Office Action for which a response is due January 31, 2012.

Applicants request the Examiner to consider the remarks provided in the Amendment filed on September 13, 2011 in connection with this paper responding to the Office Action. This Amendment is also filed in view of the in-person interview held at the PTO on December 20, 2011 with the Examiner, SPE Calamita and Examiner Celsa regarding this Office Action. No fees are believed to be due. However, the Commissioner is authorized to charge any unforeseen fees that may be due, or to credit any overpayment in fees, to Deposit Account No. 08-0219.

Claim Listing begin on page 2.
Remarks begin on page 4.

Claim Listing

This listing of the claims will replace all prior versions and listings of claims in the application:

1-49. (Cancelled)

50. (Previously presented) A method for attenuating expression of a target gene in a mammalian cell, the method comprising

introducing into mammalian cells a library of RNA expression constructs, each expression construct comprising:

(i) an RNA polymerase promoter, and

(ii) a sequence encoding a short hairpin RNA molecule comprising a double-stranded region wherein the double-stranded region consists of at least 20 nucleotides but not more than 29 nucleotides,

wherein the short hairpin RNA molecule is a substrate for Dicer-dependent cleavage and does not trigger a protein kinase RNA-activated (PKR) response in the mammalian cell,

wherein the double-stranded region of the short hairpin RNA molecule comprises a sequence that is complementary to a portion of the target gene, and

wherein the short hairpin RNA molecule is stably expressed in the mammalian cell in an amount sufficient to attenuate expression of the target gene in a sequence specific manner, and is expressed in the cell without use of a PK inhibitor, whereby expression of the target gene is inhibited.

51. (Cancelled)

52. (Previously presented) The method of claim 50, wherein the expression construct further comprises LTR sequences located 5' and 3' of the sequence encoding the short hairpin RNA molecule.

53. (Cancelled)

54. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of at least 21 nucleotides.

55. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of at least 22 nucleotides.

56. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of at least 25 nucleotides.

57. (Previously presented) The method of claim 50, wherein the short hairpin RNA molecule comprises a double-stranded region consisting of 29 nucleotides.

58. (Currently Amended) The method of claim 50, wherein the short hairpin RNA molecule has a total length of [about]70 nucleotides.

59. (Previously presented) The method of claim 50, wherein the RNA polymerase promoter comprises a pol II promoter or a pol III promoter.

60. (Previously presented) The method of claim 59, wherein the pol III promoter comprises a U6, an H1, or an SRP promoter.

61. (Withdrawn) The method of claim 59, wherein the pol II promoter comprises a U1 or a CMV promoter.

62-63. (Cancelled).

REMARKS

Claims 50, 52, and 54-60 are pending and under examination, with claim 61 withdrawn from consideration. Claim 58 has been amended without prejudice to pursue the previous subject matter of the claim in another application, and in order to expedite prosecution of this application. An Interview Summary regarding the in-person interview held on December 20, 2011 to discuss the outstanding Office Action was mailed by the Patent Office today – January 9, 2012. The present paper further addresses the issues discussed at the in-person interview of December 20, 2011.

I. Rejection of Claims Under 35 U.S.C. §103

The Examiner rejected claims 50, 52, 54-60, 62 and 63 as allegedly obvious over Zamore ('995 patent), Lieber et al. ('092 patent), Symonds et al. (2002), Elbashir (2001), Good et al. (1997) and Noonberg et al. ('803 patent). This rejection was discussed in detail at the interview at the USPTO on December 20, 2011. The rebuttal points raised at the interview are summarized here. In particular, the Examiner relies on Zamore (USPN 7,691,995, issued from USSN 10/195,034 filed July 12, 2002) as a primary prior art reference under 35 U.S.C. 103(a).

A. Applicants Response

In response, Applicants respectfully traverse. The Zamore '995 patent itself, which has a filing date after the January 22, 2002 filing date of the present application, is not prior art under 35 U.S.C. §102(e) and therefore under 35 U.S.C. §103. The only disclosure of Zamore potentially available as prior art is limited to subject matter that is disclosed in the underlying Zamore '185 provisional application in compliance with 35 U.S.C. § 112, first paragraph. See MPEP 2136.03, III. Priority from Provisional Application Under 35 U.S.C. § 119(e)(emphasis

added). See also, Ex parte Yamaguchi, 88 U.S.P.Q.2d 1606 (B.P.A.I. 2008). The Office Action fails to set forth factual findings identifying any such subject matter that would support a conclusion that the claimed invention as a whole would have been obvious, nor does it articulate a reasoned rationale for such a conclusion. See MPEP 2141, 2141.02.

As discussed below, the presently claimed method requires a number of critical elements: "a short hairpin RNA molecule comprising a double-stranded region wherein the double-stranded region consists of at least 20 nucleotides but not more than 29 nucleotides," which is "expressed in the cell without use of a PK inhibitor," and attenuation of target gene expression "in a sequence specific manner." None of these critical aspects is described or taught in the '185 provisional, either alone or in combination with any of the secondary references the Office Action relies upon.

In particular, the Office Action relies upon five secondary references for the rejection under 35 U.S.C. § 103. All these references were already considered in detail, along with rebuttal evidence, in one or both of the Declarations Under 37 C.F.R. §1.132 submitted in this case by Prof. Nouria Hernandez. This evidence was also discussed by Prof. Hernandez with the three participants from the USPTO (Examiner Chong, SPE Calamita and Ex. Celsa) in two prior interviews in connection with this application. Dr. Hernandez provided her written and oral statements as a person of skill in the art as of the effective filing date who at that time was familiar with the state of the art. Among other things, this evidence demonstrated that the skilled practitioner would have had no reasonable expectation of success in using the presently claimed methods to achieve sequence specific inhibition of a target gene without use of a PK inhibitor. See Declarations of Dr. Hernandez dated October 29, 2009 and January 4, 2011.

As discussed below, nothing in the '185 provisional provides any teaching or suggestion missing from the secondary references (either by itself or in combination) that would have rendered the presently claimed methods obvious, including any teaching or suggestion that would have provided the skilled practitioner with a reasonable expectation of success of the claimed methods. Thus, the Examiner cannot now continue to rely upon those references, and conclude the opposite, that they instead would have provided a reasonable expectation of success.

1. The Invention Claimed in the Present Application

Applicants previously provided a detailed discussion of the presently claimed methods as contrasted with the state of the art, and for more detail we respectfully refer the Examiner to previously filed papers, including the Amendment and Response to the August 30, 2010 Non-Final Office Action, dated January 31, 2011, in particular, pages 4-11. The presently pending claims are directed to methods for achieving stable, long-term silencing of genes in mammalian cells by expressing a pre-Dicer RNAi trigger (precursor) without the use of a PK inhibitor. In particular, the pre-Dicer RNAi trigger is engineered in such a way (i.e., in the form of a short hairpin RNA where the double stranded region consists of no more than 29 base pairs) that its expression in the cell does not invoke non-specific anti-viral responses, and therefore it is able to silence gene expression in a sequence-specific manner.

Before the invention of the presently claimed methods, the use of RNA interference to suppress expression of specific genes in mammalian cells having a PKR response was limited. Use of post-Dicer triggers (siRNA as described in Elbashir (2001)) achieved only transient suppression. See Amendment and Response to August 30, 2010 Non-Final Office Action dated January 31, 2011, pages 7-8. On the other hand, expression of long hairpins required continued

use of a PKR inhibitor to inhibit general antiviral responses against double-stranded RNA. See id, page 8, U.S. Publication 2003/0084471 ¶¶ 0106, 0254, Example 8. The deleterious effects of these potent antiviral responses, including ultimately cell death via apoptosis, placed a significant limitation on the utility of this approach in mammalian cells. See, for example, U.S. Publication 2003/0084471 ¶¶ 0254, Paddison et al. at 948-9.

In contrast, by expressing a short hairpin RNA as presently claimed, that is, having a double-stranded region consisting of not more than 29 nucleotides, one could achieve sequence-specific suppression without use of PKR inhibitors and at the same time avoid these deleterious effects. See, for example, U.S. Publication 2003/0084471 Example 6, "Generation of Short Hairpin dsRNA and Suppression of Gene Expression Using Such Short Hairpins," Example 7, "Encoded Short Hairpins Function in vivo," (e.g., "The specific suppression observed in HeLa cells in the presence of short dsRNAs is contrary to the non-specific effects observed when HeLa cells were treated with long dsRNAs and demonstrate that short dsRNAs do not provoke a non-specific PKR or PKR-like response.").

Evidencing the substantial advance that the presently claimed methods represented over the prior art, the Paddison et al. paper, in which the inventors published these methods, was after its publication among the most cited "high impact" papers in molecular biology and genetics. See Declaration Under 37 C.F.R. §1.131 of Gregory J. Hannon dated January 31, 2011 ¶¶ 33-36, Exhibits M-N. Commercial recognition of the value of Dr. Hannon's shRNA invention is further demonstrated by numerous prestigious awards Dr. Hannon received for his development of short hairpin RNA as a genetic tool. See id, ¶¶ 37-38, Exhibits O-Q.

2. The Zamore '185 Provisional Application Does Not Disclose or Suggest Critical Aspects of the Invention

As referred to above, the '185 provisional application fails to describe or teach a number of critical elements required by the presently claimed method: "a short hairpin RNA molecule comprising a double-stranded region wherein the double-stranded region consists of at least 20 nucleotides but not more than 29 nucleotides," which is "expressed in the cell without use of a PK inhibitor" and attenuation of target gene expression "in a sequence specific manner."

The '185 provisional is instead directed to use of certain engineered RNA precursors (pre-siRNAs) to silence target genes in mammalian and other cells. These engineered precursors are expressly defined as molecules that are altered or modified from naturally occurring wild-type stRNA precursors (pre-stRNAs) by modifying or replacing portions of the nucleotide sequence of the wild-type stRNA. '185 provisional, page 7, lines 5-7. ("Engineered RNA precursors (pre-siRNAs) are similar to naturally occurring pre-stRNAs, but are altered from the wild-type precursor sequences to promote their processing into duplex siRNAs rather than single-stranded stRNAs in vitro and in vivo.")

The same page explains specifically what such altering entails. Namely, one selects a desired 21 nucleotide sequence corresponding to a sequence that will hopefully be processed into a desired siRNA. To make the engineered precursor, this 21 nucleotide sequence is then used in place of (i.e., it replaces) a 21 or 22 nucleotide portion of a duplex stem of the naturally occurring stRNA. See '185 provisional, page 7, line 23- page 8, line 1. The resulting stem of the engineered precursor will therefore be longer and consist of two portions, a 21 or 22 nucleotide stem portion that has been replaced with a selected siRNA sequence and a stem portion in the stRNA that has not been replaced. As discussed during the interview, the reference on page 7 at line 9 to a "stem portion" of 21 nucleotides in length therefore does not mean the total length of the duplex stem in the engineered precursor, which, as taught on page 7, must be longer.

Nowhere does the '185 provisional teach or suggest that one should shorten the length of the resulting duplex stem (or double-stranded region). In fact, the '185 provisional only teaches the opposite, i.e., "introducing additional base-paired nucleotides to one or both of the stem portions of the natural pre-stRNA." '185 provisional, page 7, lines 11-15. (emphasis added). Indeed, the '185 provisional never describes the critical element of the presently claimed method that is essential for avoiding a non-sequence specific (PK) response in mammalian cells, i.e., limiting the double-stranded region to no more than 29 base pairs.

The '185 provisional provides no guidance or suggestion as to how the engineered precursor should be designed to avoid such a response. The '185 provisional merely hypothesizes (it includes no data) that the engineered RNA precursors "as a defining feature" would not induce, or would induce a lower level of such sequence non-specific response "as a consequence of their length, sequence and/or structure." (See page 8, lines 11-15 of the '185 provisional.) There is no description as to what such a defining feature is. The '185 disclosure provides no guidance as to which one (or more) of these variables (length, sequence and/or structure) would need to be manipulated, or how, to avoid a non-sequence specific (PK) response.

In this regard, the "19 to 22 nucleotide sequence" referred to on page 2 again does not mean the total length of the stem. It refers to the portion of the wild type stRNA stem that has been replaced with a selected siRNA sequence and is included in the entire stem of the engineered RNA precursor '185 provisional, page 2, lines 11-18. ("an engineered RNA precursor includes "a first stem portion including a 19 to 22 nucleotide long sequence (although the portion can be longer) that is identical to a specific targeted gene…)(emphasis added). The reference to "19" logically follows from the statement on page 7 that the "last two nucleotides of the 21

nucleotide sequence [of the selected siRNA sequence] will typically be UU." '185 provisional, page 7, line 30. These last two nucleotides would therefore not necessarily be identical to the target gene, in which case the selected siRNA sequence identical to the target gene would be 19 nucleotides long (21 minus 2). In short, the description of the engineered precursor on page 2 does not describe or suggest any limit on the length of the stem or double stranded region.

Moreover, the '185 provisional does not disclose a method of attenuating gene expression in a sequence-specific manner without the use of a PK inhibitor. It instead teaches away from such a method. Since the '185 provisional teaches that the engineered precursors would in some cases induce a sequence non-specific response (and therefore would not achieve sequence-specific attenuation), to achieve sequence specific attenuation by following the '185 disclosure, the only evident approach would have been to use a PK inhibitor.

In sum, the '185 provisional fails to describe two crucial limitations of Hannon's method as presently claimed-- use of a short hairpin RNA having a double-stranded region consisting of no more than 29 base pairs, and expressing that short hairpin RNA in a mammalian cell without use of a PK inhibitor. Accordingly, the '185 provisional does not reasonably convey to one skilled in the relevant art that the Applicants had possession of the invention as presently claimed (*i.e,* described and enabled in compliance with 35 U.S.C. 112, first paragraph). As such, neither the '995 patent, nor its corresponding published application, nor the underlying '185 provisional can qualify as prior art under § 102(e), or under § 103 as allegedly describing or suggesting these critical elements or rendering the presently claimed invention obvious.

3. The '185 Provisional Does Not Teach Use of "shRNA"

The Office Action contends that "Zamore et al. teach the use of shRNA for attenuating expression of a target gene wherein the shRNA consists of stem portions that are about 18 to

about 40 or more nucleotides in length." (Office Action, page 4). There is no such language or range disclosed in the cited pages of the '185 provisional. Moreover, as discussed above, the "engineered RNA precursors (pre-siRNAs)" disclosed in the '185 provisional are not shRNAs as claimed in the present application. Indeed, the '185 provisional never describes the critical element of the presently claimed method, i.e., a double-stranded region of no more than 29 base pairs. The Office Action improperly imports the language of the presently pending claims – shRNA – into the '185 provisional. The Office Action repeats the erroneous reference to "shRNA" on page 5, namely "Zamore et al. further teach the shRNA vector…and teach methods of mediating RNAi in cells using the shRNA constructs…" The '185 provisional has no such disclosure.

4. The Critical Aspects of the Presently Claimed Method Were Added to the Zamore Application Only After the Publication of Paddison et al.

The Paddison et al. *Genes & Development* paper published in April 2002. In that paper, Dr. Hannon et al. reported much of the work underlying the presently claimed invention, including the unexpected and surprising finding, in view of the prior art, that short hairpins with a double-stranded region under 30 base pairs in length could mediate suppression of genes in mammalian cells and avoid inducing a harmful non-specific / anti-viral (PK) response. (See, for example, pages 7-10 of Amendment filed January 31, 2011, Second Declaration of Professor Nouria Hernandez Under 35 U.S.C. §1.132 ¶¶ 8-15).

Three months after the Paddison paper was published, and seven months after the effective filing date of the present application, Zamore et al. filed the '034 application. Only then did the Zamore applicants revise the definition of an engineered precursor and include key aspects of the presently claimed method that can be found in Paddison et al. For example, the '034 application added that "[w]hen used in mammalian cells, the length of the stem portions

should be less than about 30 nucleotides to avoid provoking non-specific responses like the interferon pathway." See ''995 patent at col. 6:45-49. The '185 provisional lacks any such disclosure.

In this regard, the conclusion that the Zamore application would have rendered the presently claimed methods obvious lacks a factual underpinning. While the '185 provisional expressly identifies reducing or eliminating the PK response as advantageous, it does not teach how to do so. It only hypothesizes (and states prophetically, without any data) that some feature of the engineered precursors would do so, but without any guidance as to whether that would involve its length, sequence or structure or instead some combination of these features.

Nonetheless, the Office Action contends that what that key feature was, how to modify that key feature to avoid the PK response, and how to achieve sequence-specific attenuation without using a PK inhibitor, would have all been obvious. Yet, despite the fact that the Zamore applicants at the time were among the most experienced scientists in the RNAi field, they failed to identify or disclose these key aspects in the '185 provisional. Rather, they disclosed them only in the '034 application and after Dr. Hannon, in Paddison et al., demonstrated that short hairpin RNAs with a double-stranded region under 30 base pairs in length could in fact successfully mediate sequence-specific suppression of genes in mammalian cells, and without inducing a PK response.

5. Factual Findings that Improperly Rely on Incomplete Quotations from the '185 Provisional

In several places, the Office Action makes factual findings as to subject matter purportedly disclosed in the '185 provisional regarding the PK response, where the findings rely on partial quotes from the '185 provisional. However, omitting the full sentences substantively changes the meaning of the quoted sentences and renders the findings incorrect.

Among these, a partial quote on page 7 (which also appears on page 9) refers to the "…defining feature of these engineered RNA precursors is that as a consequence of their length, sequence, and/or structure, they do not induce sequence non-specific responses, such as induction of the interferon response…" The incomplete quote erroneously implies that the '185 provisional provided guidance as to how the engineered precursor should be designed to avoid such a response without use of a PK inhibitor. Instead, the omitted language "…or that they induce a lower level of such sequence specific responses than long, double-stranded RNA (> 150 bp) currently used to induce RNAi," illustrates that the Zamore applicants did not know how to modify the engineered precursor to avoid such a response, and thus allow sequence-specific attenuation without use of a PK inhibitor. The teaching, namely, that "[w]hen used in mammalian cells, the length of the stem portions should be less than about 30 nucleotides to avoid provoking non-specific responses like the interferon pathway" was only added after the publication of Paddison et al., demonstrating use of shRNA to achieve sequence specific attenuation in mammalian cells. See ''995 patent at col. 6:45-49.

6. The Evidence of Record Demonstrates That There was No Reasonable Expectation of Success and the Art Taught Away from the Claimed Invention as a Whole

A conclusion that a claimed invention as a whole would have been obvious cannot be sustained if the evidence of record demonstrates that there was no reasonable expectation of success. See MPEP 2143.02. "The question under 35 U.S.C. 103 is not whether the differences themselves would have been obvious, but whether the claimed invention as a whole would have been obvious. MPEP 2141.02" (emphasis in original). If the record demonstrates there was no reasonable expectation of success in carrying out that invention, there can be no reasoned basis to modify the prior art to do so. See MPEP 2143.02.

The presently claimed method, as a whole, is directed to expressing an RNA precursor having a double-stranded region of 29 base pairs or less to specifically silence a target gene in a mammalian cell, without using a PK inhibitor. Here, the record includes substantial evidence addressing whether one of ordinary skill, before Hannon's invention, would have had a reasonable expectation that this method would work. It demonstrates there was no such expectation. Among this evidence, Prof. Hernandez, as a person of ordinary skill and familiar with the state of the art at the time of the invention, provided testimony, two declarations and other direct evidence regarding the state of the art, including published data in the field of the invention.

Taking into account all the secondary references now cited in the Office Action, i.e., Lieber, Symonds, Elbashir, Good and Noonberg, Prof. Hernandez stated that a person of ordinary skill in the art would not have had a reasonable expectation of success. (See *e.g.*, entire First Declaration and Second Declaration ¶¶ 8-15 and 26-27.) Prof. Hernandez explained in detail how the art at that time would have taught away and discouraged one of skill from using the presently claimed invention.

Among this literature, Prof. Hernandez referred to detailed experimental data in Elbashir et al. that "provide a factual basis for my conclusion as a person of ordinary skill in the art as of January 22, 2002. Notably, "Elbashir et al. disclosed negative results that would have caused one to expect that a short hairpin RNA with a double-stranded region consisting of 20-29bp in length (a) would not be processed to the 21 and 22-nt siRNA structures necessary to mediate RNAi and (b) would consequently be ineffective in mediating RNA" Second Declaration ¶ 10.

"In particular, among these results, the data in Elbashir et al. demonstrate a distinct negative linear correlation between the length of a dsRNA (from 500bp to 29bp) and its ability to

act as an RNAi trigger (see Elbashir et al. Fig. 1)…Decreasing the length of the dsRNA to below 100bp resulted in a marked, approximately linear decrease in the effectiveness of the dsRNA as an RNAi trigger. In particular, as referred to in my first Declaration, shortening the length of the dsRNA to 30 or 29 bp completely eliminated the ability of the dsRNA to serve as an RNAi trigger. (Elbashir et al., Fig. 1). Notably, this lack of any RNAi activity for 29 and 30bp dsRNA was observed even under optimized conditions, using a 100:1 molar ration of dsRNA to target. (Elbashir et al., Fig. 1 and page 189, first column). In this regard, dsRNA of 39-bp in length or longer all appeared to be efficiently processed into the 21 and 22-nt (guide) siRNAs ultimately responsible for mediating cleavage of the target RNA (see Elbashir et al., Figs. 2 and 7). In contrast, 29 bp RNA was only slowly processed to such guide fragments, strongly suggesting that without efficient processing to yield sufficient siRNA product, the dsRNA would fail to act as an RNAi trigger." Second Declaration ¶11

Regarding Elbashir, Prof. Hernandez emphasized, "it would have been backwards and contrary to the Elbashir paper's text for a person of ordinary skill in the art to interpret the negative results of Elbashir as providing any reasonable expectation that one could have achieved gene silencing by stably expressing a short hairpin RNA in mammalian cells." Second Declaration ¶15. Rather, "one of skill at the time would have understood these data to indicate that there was a critical minimal length requirement for dsRNA to be able to serve as RNAi triggers. The dsRNA would have to be long enough, i.e, over 30 bp in length to provide for enough production of guide RNAs to result in degradation of the target mRNA." Second Declaration ¶12.

Regarding Symonds, Prof. Hernandez explained how Symonds, including its two priority applications (the '731 and '733), not only fails to describe or suggest the presently claimed

methods, or (itself or in combination) make such methods obvious, it is directed to approaches (using Tat type or ribozyme-type structures) that are entirely different from and would have taught away from the presently claimed methods. See Second Declaration ¶¶28-43, in particular ¶¶30-31.

The '185 provisional application provides no data or experimental results. The examples are entirely prophetic. As an entirely prophetic document, the '185 provisional would not have altered the reasonable expectation of the skilled scientist, where that expectation was based on actual experimental data that taught away from the claimed invention and indicated that expressing a double-stranded RNA with a double-stranded region of 30 base pairs or less would fail to serve as an RNAi trigger. With nothing to change this expectation, one of skill would not have had any motivation to modify the methods taught in the '185 provisional to express an shRNA as presently claimed to attenuate target gene expression in a mammalian cell.

In this regard, the '185 provisional includes only a single, entirely prophetic example relating to expressing an engineered precursor in a mammalian cell. Nothing in this example teaches or suggests modifying the engineered precursor to avoid a PK response. In fact, the specific precursor the method teaches (that of Example 1 and Figure 2B) depicts a double-stranded region consisting of 31 base pairs, outside the range required by the instant Hannon claims and teaching away from the presently claimed methods.

B. In Asserting a Prima Facie Case of Obviousness, the Office Action Has Failed to Consider the Evidence of Record

1. The PTO Is Required To Consider All of the Evidence of Record

The MPEP instructs that "rejections on obviousness cannot be sustained with mere conclusory statements; instead, there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness." *In re Kahn*, 441 F.3d 977, 988, 78

USPQ2d 1329, 1336 (Fed. Cir. 2006). See also KSR, 550 U.S. at ___ , 82 USPQ2d at 1396 (quoting Federal Circuit statement with approval). Regarding rejections under 35 U.S.C. 103, the examiner bears the initial burden of factually supporting any *prima facie* conclusion of obviousness. To establish a *prima facie* case of obviousness, the PTO:

> (1) must consider any evidence supporting the patentability of the claimed invention, such as any evidence in the specification or any other evidence submitted by the applicant,
>
> (2) must provide sufficient evidence, based on the record as a whole, including evidence submitted by the applicant, to establish a *prima facie* case of obviousness by a preponderance of evidence,
>
> (3) must clearly articulate of the reason(s) why the claimed invention would have been obvious, making explicit the analysis supporting the rejection.
>
> See, MPEP 2142.

The Examiner should consider all rebuttal arguments and evidence of record presented by applicants, including declarations and all evidence relating to secondary considerations of non-obviousness. See, MPEP 2145, 716.01(a).

2. The PTO Provides No Countervailing Evidence and Zamore Provides No Actual Data

In alleging a *prima facie* case of obviousness, the Office Action improperly does not consider or discuss any of the above evidence (including declarations and evidence relating to secondary considerations of non-obviousness), which in the previous allowance was found persuasive in demonstrating the non-obviousness of the presently claimed methods. See, MPEP 2145, 716.01(a). Moreover, the present Office Action has not provided any countervailing evidence or basis to discount the evidence provided by Prof. Hernandez of the lack of any reasonable expectation of success. The '185 provisional application provides no data or experimental results and does not add to this evidentiary record.

In sum, applicants assert that the claims are not rendered obvious by the combination of Zamore et al., Lieber, Symonds, Elbashir, Good and Noonberg. The evidence provided in the First and Second Declarations from Prof. Hernandez supports a finding of non-obviousness. Applicants respectfully request the Examiner reconsider and withdraw this ground of rejection.

C. Rebuttal to Specific Factual Findings and Statements in the Office Action

In traverse of the pending rejection, applicants respectfully include the following additional comments regarding specific factual findings made in the Office Action. See MPEP 2141.

1. The '185 Priority Application Does Not Disclose Using an Engineered Precursor with Less Than 30 Base Pairs to Avoid an Antiviral/PK Response

The Office Action includes a purported quote from the '185 provisional allegedly indicating that it teaches use of an engineered precursor with a double stranded region of less than 30 base pairs to avoid a PK response. However, the critical language quoted here, "[f]or example, the interferon response is triggered by dsRNA longer than 30 base pairs," does not appear in the '185 provisional. It was only added after the Paddison et al. *Genes & Development* paper was published, to the '034 non-provisional Zamore application. As discussed above (for example, see above pages 9-12), there is no guidance in the '185 provisional for how to modify or alter the described engineered RNA precursor to avoid a non-sequence specific (PK) response in mammalian cells.

Regarding the stem of the engineered RNA precursor, the '185 provisional teaches only to lengthen the stem by "introducing additional base-paired nucleotides to one or both of the stem portions of the natural pre-stRNA." '185 provisional, page 7, lines 11-15. (emphasis added). In this regard, the Office Action at page 9 mistakenly lists "size" as being part of "the

principle design" of Zamore's "shRNA." The '185 provisional never refers to "size." The concept was added to the '034 application only after the Paddison et al. *Genes & Development* paper was published.

2. The Office Action in Alleging a Prima Facie Case of Obviousness Did Not Consider Rebuttal Evidence Already of Record

On page 7, the Office Action appears to allege that "because it was well known in the art at the time of filing of the instant application that dsRNAs greater than 30 base pairs activated an unwanted PKR response in cells…" one of skill would have found it obvious to use an shRNA in a mammalian cell without use of a PK inhibitor, and therefore "in the absence of evidence to the contrary, the invention as a whole would have been prima facie [obvious]."

As discussed above, applicants already submitted extensive evidence on this very issue into the record, including two §132 Declarations of Dr. Hernandez and published data reflecting the state of the art (for example, see above pages 14-16). As discussed above, such evidence demonstrated the opposite. For example, "[a]s of January 22, 2002, a person of ordinary skill in the art would have had no reasonable expectation of success in carrying out sequence specific gene silencing by using an expression vector encoding a short hairpin RNA molecule having a double-stranded region consisting of 20-29 base pairs (bp). As discussed below, the references cited by the Examiner (along with the leading literature in the field) would have taught away from using an expressed short hairpin molecule, which to have gene silencing activity must first be processed in the cell." Second Declaration ¶¶8, 11-13. Nowhere, however, does the Office Action take this evidence into account.

3. Comparing the Instant Specification to the Prior Art Is an Improper Inquiry under 35 U.S.C. §103

The Office Action at page 8 compares the disclosure of the instant specification to the prior art. This is not a proper inquiry in the context of determining obviousness under 35 U.S.C.

§103. Here, the issue is whether the references cited render the claimed invention obvious. The proper comparison is between that claimed invention and the state of the art combined with the references cited. The elements of the presently claimed invention, including the claimed structural features, describe a method one can use to achieve sequence specific attenuation of the target gene in a mammalian cell without use of a PK inhibitor. There is no disclosure of the claimed invention, or suggestion or motivation to practice the claimed invention, in the cited prior art references, either singly or in any combination.

II. Rejections Under 35 U.S.C. §112

The Examiner rejected claims 58, 62 and 63 as indefinite.

As to claims 62 and 63, Applicants canceled these claims in the paper filed on April 18, 2011. Accordingly, these claims are not pending and the rejection of these claims should be withdrawn. As to claim 58, this claim does not include the phrase "expression of the target gene…" as stated by the Examiner on page 2 of the Office Action. In addition, Applicants have amended the claim to remove the word "about" in order to expedite prosecution of this application and without prejudice to pursue the subject matter in another application. Accordingly, Applicants respectfully request the Examiner to withdraw this ground of rejection.

CONCLUSION

Consideration of this paper and allowance of this application are requested. If it would advance prosecution, the Examiner is invited to contact the undersigned to discuss the contents of this paper.

Dated: January 9, 2012

Respectfully submitted,

/Jane M. Love, Ph.D./
Jane M. Love, Ph.D.
Registration No. 42,812

Attorney for Applicants

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Electronic Acknowledgement Receipt

EFS ID:	11792863
Application Number:	11894676
International Application Number:	
Confirmation Number:	8161
Title of Invention:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE
First Named Inventor/Applicant Name:	Gregory J. Hannon
Customer Number:	84834
Filer:	Jane Maureen Love/Carolyn DeCasseres
Filer Authorized By:	Jane Maureen Love
Attorney Docket Number:	287000.130US3
Receipt Date:	09-JAN-2012
Filing Date:	20-AUG-2007
Time Stamp:	17:41:33
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		0287000_00130US3_RESPONSE_010911.pdf	157192 8fb860bd0a4e0c792d748d6d26848f1e5e4d81b1	yes	21

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Amendment/Req. Reconsideration-After Non-Final Reject	1	1
	Claims	2	3
	Applicant Arguments/Remarks Made in an Amendment	4	21
Warnings:			
Information:			
	Total Files Size (in bytes):	157192	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/06 (07-06)
Approved for use through 1/31/2007. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number	Filing Date	☐ To be Mailed
11/894,676	08/20/2007	

APPLICATION AS FILED – PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY ☐ RATE ($)	SMALL ENTITY ☐ FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT 01/09/2012	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	SMALL ENTITY ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 10	Minus	** 20	= 0	X $ =		OR	X $60=	0
Independent (37 CFR 1.16(h))	* 1	Minus	***3	= 0	X $ =		OR	X $250=	0
☐ Application Size Fee (37 CFR 1.16(s))									
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	0

AMENDMENT	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)		RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))									
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/MARTHA NEWMAN/

PTO/SB/08a (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 2

Complete if Known	
Application Number	11/894,676-Conf. #8161
Filing Date	August 20, 2007
First Named Inventor	Gregory J. HANNON
Art Unit	1635
Examiner Name	K. Chong
Attorney Docket Number	0287000.00130US3

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]
	BA	CA-2470903	07-10-2003	Cancer Rec. Tech. Ltd.		

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

PTO/SB/08b (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet	2	of	2

Complete if Known	
Application Number	11/894,676-Conf. #8161
Filing Date	August 20, 2007
First Named Inventor	Gregory J. HANNON
Art Unit	1635
Examiner Name	K. Chong
Attorney Docket Number	0287000.00130US3

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1]Applicant's unique citation designation number (optional). [2]Applicant is to place a check mark here if English language Translation is attached.

Electronic Acknowledgement Receipt

EFS ID:	11810145
Application Number:	11894676
International Application Number:	
Confirmation Number:	8161
Title of Invention:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE
First Named Inventor/Applicant Name:	Gregory J. Hannon
Customer Number:	84834
Filer:	Anne-Marie Yvon/sophie murray
Filer Authorized By:	Anne-Marie Yvon
Attorney Docket Number:	287000.130US3
Receipt Date:	11-JAN-2012
Filing Date:	20-AUG-2007
Time Stamp:	16:56:45
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	287000_130US3_IDS_01112012.pdf	60655 14a069c85cca2a4cc6d5f2b259ea8a4d37c32887	no	1

Warnings:	
Information:	

2	Information Disclosure Statement (IDS) Form (SB08)	287000_130US3_SB08_01112012.pdf	107337 c86a3e7900dfc739b15b3c77420f2f20d3604264	no	2
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
3	Foreign Reference	CA2470903.pdf	3698564 22f612f3b9db113373fb23e398ac5e0d3be53c4c	no	85
Warnings:					
Information:					
			Total Files Size (in bytes):	3866556	

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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Docket No.: 0287000.00130US3
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Gregory J. Hannon et al.	Confirmation No.:	8161
Application No.:	11/894,676	Art Unit:	1635
Filed:	August 20, 2007	Examiner:	K. Chong
Title:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT (IDS)

Dear Madam:

Applicants state that the item contained in the Information Disclosure Statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing date of this Information Disclosure Statement. No fee is required.

Applicants request that the Examiner initial and return a copy of the enclosed Form PTO SB-08 with the next communication. Applicants believe that no fee is due with this response. However, if a fee is due, please charge our Deposit Account No. 08-0219, under Order No. 0287000.00130US3 from which the undersigned is authorized to draw.

Respectfully submitted,

Dated: January 11, 2012

/Anne-Marie C. Yvon/
Anne-Marie C. Yvon
Registration No.: 52,390
Attorney for Applicant(s)

Wilmer Cutler Pickering Hale and Dorr LLP
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(212) 230-8800 (telephone)
(212) 230-8888 (facsimile)

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
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Alexandria, Virginia 22313-1450
www.uspto.gov

NOTICE OF ALLOWANCE AND FEE(S) DUE

84834 7590 03/06/2012

WilmerHale/Cold Spring Harbor Laboratory
399 Park Avenue
New York, NY 10022

EXAMINER
CHONG, KIMBERLY

ART UNIT	PAPER NUMBER
1635	

DATE MAILED: 03/06/2012

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/894,676	08/20/2007	Gregory J. Hannon	287000.130US3	8161

TITLE OF INVENTION: METHODS AND COMPOSITIONS FOR RNA INTERFERENCE

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	NO	$1740	$300	$0	$2040	06/06/2012

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the SMALL ENTITY status shown above.

If the SMALL ENTITY is shown as YES, verify your current SMALL ENTITY status:

A. If the status is the same, pay the TOTAL FEE(S) DUE shown above.

B. If the status above is to be removed, check box 5b on Part B - Fee(s) Transmittal and pay the PUBLICATION FEE (if required) and twice the amount of the ISSUE FEE shown above, or

If the SMALL ENTITY is shown as NO:

A. Pay TOTAL FEE(S) DUE shown above, or

B. If applicant claimed SMALL ENTITY status before, or is now claiming SMALL ENTITY status, check box 5a on Part B - Fee(s) Transmittal and pay the PUBLICATION FEE (if required) and 1/2 the ISSUE FEE shown above.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail **Mail Stop ISSUE FEE**
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

84834 7590 03/06/2012

WilmerHale/Cold Spring Harbor Laboratory
399 Park Avenue
New York, NY 10022

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/894,676	08/20/2007	Gregory J. Hannon	287000.130US3	8161

TITLE OF INVENTION: METHODS AND COMPOSITIONS FOR RNA INTERFERENCE

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	NO	$1740	$300	$0	$2040	06/06/2012

EXAMINER	ART UNIT	CLASS-SUBCLASS
CHONG, KIMBERLY	1635	536-024500

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

❑ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

(1) the names of up to 3 registered patent attorneys or agents OR, alternatively,

(2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 _____

2 _____

3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ❑ Individual ❑ Corporation or other private group entity ❑ Government

4a. The following fee(s) are submitted:

❑ Issue Fee

❑ Publication Fee (No small entity discount permitted)

❑ Advance Order - # of Copies _____

4b. Payment of Fee(s): **(Please first reapply any previously paid issue fee shown above)**

❑ A check is enclosed.

❑ Payment by credit card. Form PTO-2038 is attached.

❑ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment, to Deposit Account Number _____ (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)

❑ a. Applicant claims SMALL ENTITY status. See 37 CFR 1.27.

❑ b. Applicant is no longer claiming SMALL ENTITY status. See 37 CFR 1.27(g)(2).

NOTE: The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____

This collection of information is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.

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www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/894,676	08/20/2007	Gregory J. Hannon	287000.130US3	8161

84834 7590 03/06/2012

WilmerHale/Cold Spring Harbor Laboratory
399 Park Avenue
New York, NY 10022

EXAMINER	
CHONG, KIMBERLY	

ART UNIT	PAPER NUMBER
1635	

DATE MAILED: 03/06/2012

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment to date is 0 day(s). If the issue fee is paid on the date that is three months after the mailing date of this notice and the patent issues on the Tuesday before the date that is 28 weeks (six and a half months) after the mailing date of this notice, the Patent Term Adjustment will be 0 day(s).

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Notice of Allowability	Application No.	Applicant(s)	
	11/894,676	HANNON ET AL.	
	Examiner	Art Unit	
	KIMBERLY CHONG	1635	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to *01/11/2012*.

2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

3. ☒ The allowed claim(s) is/are *50,52 and 54-61*.

4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
   a) ☐ All b) ☐ Some* c) ☐ None of the:
      1. ☐ Certified copies of the priority documents have been received.
      2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
      3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ A SUBSTITUTE OATH OR DECLARATION must be submitted. Note the attached EXAMINER'S AMENDMENT or NOTICE OF INFORMAL PATENT APPLICATION (PTO-152) which gives reason(s) why the oath or declaration is deficient.

6. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   (a) ☐ including changes required by the Notice of Draftsperson's Patent Drawing Review (PTO-948) attached
      1) ☐ hereto or 2) ☐ to Paper No./Mail Date _____.
   (b) ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

7. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☐ Notice of Draftperson's Patent Drawing Review (PTO-948)
3. ☒ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date 01/11/2012
4. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material
5. ☐ Notice of Informal Patent Application
6. ☐ Interview Summary (PTO-413), Paper No./Mail Date _____ .
7. ☐ Examiner's Amendment/Comment
8. ☐ Examiner's Statement of Reasons for Allowance
9. ☐ Other _____.

/Kimberly Chong/
Primary Examiner AU1635

PTO/SB/08a (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 2

Complete if Known	
Application Number	11/894,676-Conf. #8161
Filing Date	August 20, 2007
First Named Inventor	Gregory J. HANNON
Art Unit	1635
Examiner Name	K. Chong
Attorney Docket Number	0287000.00130US3

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]
	BA	CA-2470903	07-10-2003	Cancer Rec. Tech. Ltd.		

Examiner Signature	/Kimberly Chong/	Date Considered	03/01/2012

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /K.C./

PTO/SB/08b (07-09)
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Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet	2	of	2

Complete if Known	
Application Number	11/894,676-Conf. #8161
Filing Date	August 20, 2007
First Named Inventor	Gregory J. HANNON
Art Unit	1635
Examiner Name	K. Chong
Attorney Docket Number	0287000.00130US3

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]

Examiner Signature	/Kimberly Chong/	Date Considered	03/01/2012

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1]Applicant's unique citation designation number (optional). [2]Applicant is to place a check mark here if English language Translation is attached.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /K.C./

Issue Classification	Application/Control No.	Applicant(s)/Patent under Reexamination	
	11/894,676	HANNON ET AL.	
	Examiner	Art Unit	
	KIMBERLY CHONG	1635	

ISSUE CLASSIFICATION

ORIGINAL			CROSS REFERENCE(S)						
CLASS	SUBCLASS	CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)						
536	24.5	536	24.31	24.1					
INTERNATIONAL CLASSIFICATION		435	6	325	375				
C 0 7 H	21/04	514	44						
	/								
	/								
	/								
	/								

(Assistant Examiner) (Date)	/Kimberly Chong/ Primary Examiner AU1635 03/01/2012 (Primary Examiner) (Date)	Total Claims Allowed: 10	
(Legal Instruments Examiner) (Date)		O.G. Print Claim(s): 1	O.G. Print Fig.: none

☒ Claims renumbered in the same order as presented by applicant ☐ CPA ☒ T.D. ☐ R.1.47

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original
	1		31		61		91		121		151		181
	2		32		62		92		122		152		182
	3		33		63		93		123		153		183
	4		34		64		94		124		154		184
	5		35		65		95		125		155		185
	6		36		66		96		126		156		186
	7		37		67		97		127		157		187
	8		38		68		98		128		158		188
	9		39		69		99		129		159		189
	10		40		70		100		130		160		190
	11		41		71		101		131		161		191
	12		42		72		102		132		162		192
	13		43		73		103		133		163		193
	14		44		74		104		134		164		194
	15		45		75		105		135		165		195
	16		46		76		106		136		166		196
	17		47		77		107		137		167		197
	18		48		78		108		138		168		198
	19		49		79		109		139		169		199
	20		50		80		110		140		170		200
	21		51		81		111		141		171		201
	22		52		82		112		142		172		202
	23		53		83		113		143		173		203
	24		54		84		114		144		174		204
	25		55		85		115		145		175		205
	26		56		86		116		146		176		206
	27		57		87		117		147		177		207
	28		58		88		118		148		178		208
	29		59		89		119		149		179		209
	30		60		90		120		150		180		210

Search Notes	Application/Control No.	Applicant(s)/Patent under Reexamination
	11/894,676	HANNON ET AL.
	Examiner	Art Unit
	KIMBERLY CHONG	1635

SEARCHED			
Class	Subclass	Date	Examiner

INTERFERENCE SEARCHED			
Class	Subclass	Date	Examiner
536	**24.5**	**03/01/2012**	**KC**

SEARCH NOTES (INCLUDING SEARCH STRATEGY)		
	DATE	EXMR
updated	03/01/2012	KC

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail **Mail Stop ISSUE FEE**
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INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

84834 7590 03/06/2012

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Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/894,676	08/20/2007	Gregory J. Hannon	287000.130US3	8161

TITLE OF INVENTION: METHODS AND COMPOSITIONS FOR RNA INTERFERENCE

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	NO	$1740	$300	$0	$2040	06/06/2012

EXAMINER	ART UNIT	CLASS-SUBCLASS
CHONG, KIMBERLY	1635	536-024500

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

(1) the names of up to 3 registered patent attorneys or agents OR, alternatively,

(2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 Wilmer Cutler Pickering Hale and Dorr LLP
2
3

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE: Cold Spring Harbor Laboratory

(B) RESIDENCE: (CITY and STATE OR COUNTRY): Cold Spring Harbor, NY

Please check the appropriate assignee category or categories (will not be printed on the patent) : ☐ Individual ☒ Corporation or other private group entity ☐ Government

4a. The following fee(s) are submitted:

☒ Issue Fee

☒ Publication Fee (No small entity discount permitted)

☐ Advance Order - # of Copies _____

4b. Payment of Fee(s): **(Please first reapply any previously paid issue fee shown above)**

☐ A check is enclosed.

☒ Payment by credit card. ~~Form PTO-2038 is attached.~~

☒ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment, to Deposit Account Number 080219 (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)

☐ a. Applicant claims SMALL ENTITY status. See 37 CFR 1.27.

☐ b. Applicant is no longer claiming SMALL ENTITY status. See 37 CFR 1.27(g)(2).

NOTE: The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

Authorized Signature /Jane M. Love, Ph.D./ Date 03/07/2012

Typed or printed name Jane M. Love, Ph.D. Registration No. 42,812

This collection of information is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Hannon et al.	Confirmation No.:	8161
Application No:	11/894,676	Art Unit:	1635
Filed:	August 20, 2007	Examiner:	K. CHONG
Title:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT AFTER ALLOWANCE UNDER 37 C.F.R. §1.312

This paper seeks to have the Bibliographic Data Sheet updated to reflect the correct priority claim to U.S. Ser. No. 10/055,797, filed on January 22, 2002. This paper updates the reference in the specification to the applications to which the present application claims priority. A Supplemental Application Data Sheet accompanies this paper. The Commissioner is authorized to charge any fees due, or to credit any overpayment in fees, to Deposit Account No. 08-0219.

AMENDMENT

In the Specification

On page 1, please amend the paragraph immediately after the heading "Related Applications" as follows:

-- This application is a continuation application of ~~U.S. Serial No. 11/791,554, filed on May23, 2007, which is a national stage filing under 35 U.S.C. § 371 of International Application No. PCT/US2005/042488, filed on November 23,2005, which is a continuation application of~~ U.S. Serial No. 10/997,086, filed on November 23, 2004, ~~which is a continuation-in-part of U.S. Ser. No. 10/350,798, filed on January 24, 2003,~~ which is a continuation-in-part of U.S. Ser. No. 10/055,797, filed on January 22, 2002, which is ~~a continuation-in-part of International Application No. PCT/US01/08435, filed on March 16,2001, which claims the benefit of the filing date from U.S. Provisional Application Nos. 60/189,739, filed on March 16,2000, and 60/243,097, filed on October 24,2000. U.S. Ser. No. 10/350,798 is also a continuation-in-part of U.S. Ser. No. 09/866.557, filed on May 24, 2001, which is also a continuation-in-part of International Application No. PCT/US0l/08435, filed on March 16,2001. U.S. Ser. No. 10/350,798 is also a continuation-in-part of U.S. Ser. No. 09/858,862, filed on May 16,2001, which is also a continuation-in-part of International Application No. PCT/US0l/08435, filed on March 16,2001. The specifications of such applications are~~ incorporated by reference herein. ~~International Application PCT/US0l/08435 and International Application PCT/US2005/042488 were both published under PCT Article 21(2) in English.~~ --

REMARKS

The specification was amended to refer to the applications to which the present application claims priority. In particular, priority was claimed to U.S. application Serial Nos. 10/997,086 and 10/055,797.

This amendment was previously filed on April 8, 2010 and appears on PAIR, along with the EFS Acknowledgement Receipt. A Supplemental Application Data Sheet reflecting the amended priority claim was filed on April 9, 2010, and appears on PAIR, along with its EFS Acknowledgement Receipt. A copy of the Supplemental ADS that was filed on April 9, 2010 accompanies this paper.

Update to Bibliographic Data Sheet Requested

The most recent Filing Receipt, dated May 20, 2011, and Bibliographic Data Sheet, dated July 25, 2011, both list the original priority claim before correction by the April 2010 submissions. PAIR, on the other hand, lists the priority claim in the Continuity Data section as U.S. application Serial No. 10/997,086 only, omitting the claim to Serial No. 10/055,797.

Applicants request entry of this paper and appropriate correction of the priority claim.

Dated: March 7, 2012

Respectfully submitted,

/Jane M. Love, Ph.D./

Jane M. Love, Ph.D.
Registration No. 42,812

Attorney for Applicant(s)

Wilmer Cutler Pickering Hale and Dorr LLP
399 Park Avenue
New York, New York 10022
(212) 230-8800 (telephone)
(212) 230-8888 (facsimile)

Supplemental Application Data Sheet

Application Information

Application number::	11/894,676
Filing Date::	08/20/07
Application Type::	Regular
Subject Matter::	Utility
Suggested classification::	
Suggested Group Art Unit::	1635
CD-ROM or CD-R?::	None
Number of CD disks::	
Number of copies of CDs::	
Sequence submission?::	None
Computer Readable Form (CRF)?::	No
Number of copies of CRF::	
Title::	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE
Attorney Docket Number::	0287000.00130US3
Request for Early Publication?::	No
Request for Non-Publication?::	No
Suggested Drawing Figure::	
Total Drawing Sheets::	67
Small Entity?::	Yes
Petition included?::	No

Petition Type::	
Licensed US Govt. Agency::	
Contract or Grant Numbers::	
Secrecy Order in Parent Appl.?::	No

Applicant Information

Applicant Authority Type::	Inventor
Primary Citizenship Country::	US
Status::	Full Capacity
Given Name::	Gregory
Middle Name::	J.
Family Name::	HANNON
Name Suffix::	
City of Residence::	Huntington
State or Province of Residence::	NY
Country of Residence::	US
Street of mailing address::	34 Griffith Lane
City of mailing address::	Huntington
State or Province of mailing address::	NY
Country of mailing address::	
Postal or Zip Code of mailing address::	11743

Applicant Authority Type::	Inventor
Primary Citizenship Country::	
Status::	Full Capacity
Given Name::	Patrick
Middle Name::	
Family Name::	PADDISON
Name Suffix::	
City of Residence::	~~Oyster Bay~~ Seattle
State or Province of Residence::	~~NY~~ WA
Country of Residence::	US
Street of mailing address::	~~9 Moffett Street~~ 7051 18th Ave. NE
City of mailing address::	~~Oyster Bay~~ Seattle
State or Province of mailing address::	~~NY~~ WA
Country of mailing address::	
Postal or Zip Code of mailing address::	~~11771~~ 98115
Applicant Authority Type::	Inventor
Primary Citizenship Country::	US
Status::	Full Capacity
Given Name::	Emily
Middle Name::	

Family Name::	BERNSTEIN
Name Suffix::	
City of Residence::	New York
State or Province of Residence::	NY
Country of Residence::	US
Street of mailing address::	1161 York Avenue, Apt 11
City of mailing address::	New York
State or Province of mailing address::	NY
Country of mailing address::	
Postal or Zip Code of mailing address::	10021
Applicant Authority Type::	Inventor
Primary Citizenship Country::	US
Status::	Full Capacity
Given Name::	Amy
Middle Name::	
Family Name::	CAUDY
Name Suffix::	
City of Residence::	Lawrenceville
State or Province of Residence::	NJ
Country of Residence::	US
Street of mailing address::	4221 Town Court N
City of mailing address::	Lawrenceville
State or Province of mailing address::	NJ

Country of mailing address::	
Postal or Zip Code of mailing address::	08648
Applicant Authority Type::	Inventor
Primary Citizenship Country::	US
Status::	Full Capacity
Given Name::	Douglas
Middle Name::	
Family Name::	CONKLIN
Name Suffix::	
City of Residence::	Cold Spring Harbor
State or Province of Residence::	NY
Country of Residence::	US
Street of mailing address::	One Bungtown Road
City of mailing address::	Cold Spring Harbor
State or Province of mailing address::	NY
Country of mailing address::	
Postal or Zip Code of mailing address::	11724
Applicant Authority Type::	Inventor
Primary Citizenship Country::	US
Status::	Full Capacity

Given Name::	Scott
Middle Name::	
Family Name::	HAMMOND
Name Suffix::	
City of Residence::	Cold Spring Habor
State or Province of Residence::	NY
Country of Residence::	US
Street of mailing address::	One Bungtown Road, Nichols Bldg.
City of mailing address::	Cold Spring Harbor
State or Province of mailing address::	NY
Country of mailing address::	
Postal or Zip Code of mailing address::	11724

Correspondence Information

Correspondence Customer Number::	84834

Representative Information

Representative Customer Number::	84834

Domestic Priority Information

Application::	Continuity Type::	Parent Application::	Parent Filing Date::
This Application	Continuation of	10/997086	11/23/04
~~10/997086~~	~~Continuation-in-part of~~	~~10/350798~~	~~01/24/03~~
10/997086	Continuation-in-part of	10/055797	01/22/02

Foreign Priority Information

Assignee Information

Assignee name::	Cold Spring Harbor Laboratory
Street of mailing address::	One Bungtown Road
City of mailing address::	Cold Spring Harbor
State or Province of mailing address::	NY
State or Province of mailing address::	
Country of mailing address::	US
Postal or Zip Code of mailing address::	11724

Signature:

A signature of the applicant or representative is required in accordance with 37 CFR 1.33 and 10.18. Please see 37 CFR 1.4(d) for the form of the signature.			
Signature	/Anne-Marie C. Yvon/	Date	April 9, 2010
Name (Print/Type)	Anne-Marie C. Yvon	Registration No. (Attorney/Agent)	52,390

Electronic Patent Application Fee Transmittal

Application Number:	11894676
Filing Date:	20-Aug-2007
Title of Invention:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE
First Named Inventor/Applicant Name:	Gregory J. Hannon
Filer:	Jane Maureen Love/sophie murray
Attorney Docket Number:	287000.130US3

Filed as Large Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Utility Appl issue fee	1501	1	1740	1740
Publ. Fee- early, voluntary, or normal	1504	1	300	300

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	**2040**

Electronic Acknowledgement Receipt

EFS ID:	12244317
Application Number:	11894676
International Application Number:	
Confirmation Number:	8161
Title of Invention:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE
First Named Inventor/Applicant Name:	Gregory J. Hannon
Customer Number:	84834
Filer:	Jane Maureen Love/sophie murray
Filer Authorized By:	Jane Maureen Love
Attorney Docket Number:	287000.130US3
Receipt Date:	07-MAR-2012
Filing Date:	20-AUG-2007
Time Stamp:	18:24:54
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Credit Card
Payment was successfully received in RAM	$2040
RAM confirmation Number	7517
Deposit Account	080219
Authorized User	LADD,CATHLEEN

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

Charge any Additional Fees required under 37 C.F.R. Section 1.16 (National application filing, search, and examination fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.17 (Patent application and reexamination processing fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.19 (Document supply fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.20 (Post Issuance fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Issue Fee Payment (PTO-85B)	287000_130US3_Issue_fee.pdf	90917 595b7c2315e97185cdf8f66fdacff498bdf2788d	no	1
Warnings:					
Information:					
2		287000_130US3_Amendment_312.pdf	80432 3f02ff516f5d6de42c2a378988f8d049075635eb	yes	3

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Amendment after Notice of Allowance (Rule 312)	1	1
	Specification	2	2
	Applicant Arguments/Remarks Made in an Amendment	3	3

Warnings:					
Information:					
3	Application Data Sheet	287000_130US3_suppl_ADS_filed_04092010.pdf	134843 bb99012ed6e9934ee8c8c23b3eb4195849384fc3	no	8
Warnings:					
Information:					
This is not an USPTO supplied ADS fillable form					
4	Fee Worksheet (SB06)	fee-info.pdf	31896 4e0388e5aa4bb76adaf219c229bb86cf95da3e50	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	338088		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Hannon et al.	Confirmation No.:	8161
Application No:	11/894,676	Art Unit:	1635
Filed:	August 20, 2007	Examiner:	K. CHONG
Title:	METHODS AND COMPOSITIONS FOR RNA INTERFERENCE		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT AFTER ALLOWANCE UNDER 37 C.F.R. §1.312

This paper seeks to have the Bibliographic Data Sheet updated to reflect the correct priority claim to U.S. Ser. No. 10/055,797, filed on January 22, 2002. This paper updates the reference in the specification to the applications to which the present application claims priority. A Supplemental Application Data Sheet accompanies this paper. The Commissioner is authorized to charge any fees due, or to credit any overpayment in fees, to Deposit Account No. 08-0219.

AMENDMENT

In the Specification

On page 1, please amend the paragraph immediately after the heading "Related Applications" as follows:

-- This application is a continuation application of ~~U.S. Serial No. 11/791,554, filed on May23, 2007, which is a national stage filing under 35 U.S.C. § 371 of International Application No. PCT/US2005/042488, filed on November 23,2005, which is a continuation application of~~ U.S. Serial No. 10/997,086, filed on November 23, 2004, ~~which is a continuation-in-part of U.S. Ser. No. 10/350,798, filed on January 24, 2003,~~ which is a continuation-in-part of U.S. Ser. No. 10/055,797, filed on January 22, 2002, which is ~~a continuation-in-part of International Application No. PCT/US01/08435, filed on March 16,2001, which claims the benefit of the filing date from U.S. Provisional Application Nos. 60/189,739, filed on March 16,2000, and 60/243,097, filed on October 24,2000. U.S. Ser. No. 10/350,798 is also a continuation-in-part of U.S. Ser. No. 09/866.557, filed on May 24, 2001, which is also a continuation-in-part of International Application No. PCT/US0l/08435, filed on March 16,2001. U.S. Ser. No. 10/350,798 is also a continuation-in-part of U.S. Ser. No. 09/858,862, filed on May 16,2001, which is also a continuation-in-part of International Application No. PCT/US0l/08435, filed on March 16,2001. The specifications of such applications are~~ incorporated by reference herein. ~~International Application PCT/US0l/08435 and International Application PCT/US2005/042488 were both published under PCT Article 21(2) in English.~~ --

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/894,676	08/20/2007	Gregory J. Hannon	287000.130US3	8161

84834 7590 03/14/2012

WilmerHale/Cold Spring Harbor Laboratory
399 Park Avenue
New York, NY 10022

EXAMINER	
CHONG, KIMBERLY	
ART UNIT	PAPER NUMBER
1635	

NOTIFICATION DATE	DELIVERY MODE
03/14/2012	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

Teresa.carvalho@wilmerhale.com
whipusptopairs@wilmerhale.com

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Address : COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450

APPLICATION NO./ CONTROL NO.	FILING DATE	FIRST NAMED INVENTOR / PATENT IN REEXAMINATION	ATTORNEY DOCKET NO.
11/894,676	20 August, 2007	HANNON ET AL.	287000.130US3

WilmerHale/Cold Spring Harbor Laboratory
399 Park Avenue
New York, NY 10022

EXAMINER	
KIMBERLY CHONG	
ART UNIT	PAPER
1635	20120309

DATE MAILED:

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner for Patents

The amendment filed on 03/07/2012 under 37 CFR 1.312 has been entered.

/Kimberly Chong/
Primary Examiner AU1635

PTO-90C (Rev.04-03)

Used in Lieu of PTO/SB/08A/B
(Based on PTO 04-07 version)

Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 7

Complete if Known	
Application Number	11/894,676
Filing Date	August 20, 2007
First Named Inventor	Gregory J. Hannon
Art Unit	1635
Examiner Name	Not Yet Assigned
Attorney Docket Number	CSHL-P08-010

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number: Number-Kind Code[2] (*if known*)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	AA	US-20020086356-A1	07-04-2002	Tuschl et al.	
	AB	US-20020114784-A1	08-22-2002	Li et al.	
	AC	US-20030051263-A1	03-13-2003	Fire et al.	
	AD	US-20030055020-A1	03-20-2003	Fire et al.	
	AE	US-20030056235-A1	03-20-2003	Fire et al.	
	AF	US-20030084471-A1	05-01-2003	Beach et al.	
	AG	US-20040018999-A1	01-29-2004	Beach et al.	
	AH	US-20040086884-A1	05-06-2004	Beach et al.	
	AI	US-20040229266-A1	11-18-2004	Tuschl et al.	
	AJ	US-20050164210-A1	07-28-2005	Mittal et al.	
	AK	US-20050197315-A1	09-08-2005	Taira et al.	
	AL	US-5,246,921	09-21-1993	Reddy et al.	
	AM	US-5,998,148	12-07-1999	Bennett et al.	
	AN	US-6,107,027	August 22, 2000	Kay et al.	
	AO	US-6,130,092	10-10-2000	Lieber et al.	
	AP	US-6,326,193	12-04-2001	Liu et al.	
	AQ	US-6,506,559	01-14-2003	Fire et al.	
	AR	US-6,573,099-A1	06-03-2003	Graham et al.	
	AS	US-6,605,429	08-12-2003	Barber et al.	

Change(s) applied to document, /D.H.P./ 7/22/2011

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document: Country Code[3]-Number[4]-Kind Code[5] (*if known*)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]
	BA	WO-00/01846	01-13-2000	Devgen Nv et al.		
	BB	WO-00/44895	08-03-2000	Kreutzer Roland et al.		
	BC	WO-00/63364	10-26-2000	American Home Prod et al.		
	BD	WO-01/49844	07-12-2001	Univ Rutgers et al.		
	BE	WO-02/44321	06-06-2002	Max Planck Gesellschaft et al.		
	BF	WO-04/029219	04-08-2004	Cold Spring Harbor Laboratory		
	BG	WO-94/01550	01-20-1994	Hybridon Inc et al.		
	BH	WO-99/49029	09-30-1999	Gene Australia Limited Ag et al.		
	BI	WO-00/44914	08-03-2000	Medical College Of Georgia Res et al.		
	BJ	WO-01/29058	04-26-2001	Univ Massachusetts et al.		
	BK	WO-01/36646	05-25-2001	Cancer Res Campaign Tech et al.		
	BL	WO-01/48183	07-05-2001	Devgen Nv et al.		

Examiner Signature		Date Considered	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /K.C./

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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P.O. Box 1450
Alexandria, Virginia 22313-1450
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Bib Data Sheet

CONFIRMATION NO. 8161

SERIAL NUMBER	FILING OR 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
11/894,676	08/20/2007 RULE	536	1635	287000.130US3

APPLICANTS

Gregory J. Hannon, Huntington, NY;
Patrick J. Paddison, Northport, NY;
Emily Bernstein, New York, NY;
Amy Caudy, Lawrenceville, NJ;
Douglas Conklin, Cold Spring Harbor, NY;
Scott Hammond, Cold Spring Harbor, NY;

**** CONTINUING DATA ***************************

This application is a CON of 10/997,086 11/23/2004

**** FOREIGN APPLICATIONS *********************

IF REQUIRED, FOREIGN FILING LICENSE GRANTED
** 11/02/2007

Foreign Priority claimed	STATE OR COUNTRY	SHEETS DRAWING	TOTAL CLAIMS	INDEPENDENT CLAIMS
☐ yes ☐ no	NY	67	14	2

35 USC 119 (a-d) conditions met ☐ yes ☐ no ☐ Met after Allowance

Verified and Acknowledged _____ Examiner's Signature Initials

ADDRESS
84834

TITLE

METHODS AND COMPOSITIONS FOR RNA INTERFERENCE

FILING FEE RECEIVED	FEES: Authority has been given in Paper No. _____ to charge/credit DEPOSIT ACCOUNT No. _____ for following:
970	☐ All Fees ☐ 1.16 Fees (Filing) ☐ 1.17 Fees (Processing Ext. of time) ☐ 1.18 Fees (Issue) ☐ Other _____ ☐ Credit

UNITED STATES PATENT AND TRADEMARK OFFICE

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Alexandria, Virginia 22313-1450
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APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/894,676	04/10/2012	8153776	287000.130US3	8161

84834 7590 03/21/2012

WilmerHale/Cold Spring Harbor Laboratory
399 Park Avenue
New York, NY 10022

ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment is 0 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Data Management (ODM) at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site http://pair.uspto.gov for additional applicants):

Gregory J. Hannon, Huntington, NY;
Patrick J. Paddison, Northport, NY;
Emily Bernstein, New York, NY;
Amy Caudy, Lawrenceville, NJ;
Douglas Conklin, Cold Spring Harbor, NY;
Scott Hammond, Cold Spring Harbor, NY;

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/894,676	08/20/2007	Gregory J. Hannon	287000.130US3	8161

84834 7590 03/30/2012

WilmerHale/Cold Spring Harbor Laboratory
399 Park Avenue
New York, NY 10022

EXAMINER
CHONG, KIMBERLY

ART UNIT	PAPER NUMBER
1635	

NOTIFICATION DATE	DELIVERY MODE
03/30/2012	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

Teresa.carvalho@wilmerhale.com
whipusptopairs@wilmerhale.com

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APPLICATION NO./ CONTROL NO.	FILING DATE	FIRST NAMED INVENTOR / PATENT IN REEXAMINATION	ATTORNEY DOCKET NO.
11/894,676	20 August, 2007	HANNON ET AL.	287000.130US3

		EXAMINER	
WilmerHale/Cold Spring Harbor Laboratory 399 Park Avenue New York, NY 10022		KIMBERLY CHONG	
		ART UNIT	PAPER
		1635	20120326

DATE MAILED:

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner for Patents

A corrected Bib Data Sheet is attached correctly listing the continuing data.	
	/Kimberly Chong/ Primary Examiner AU1635

PTO-90C (Rev.04-03)

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
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Alexandria, Virginia 22313-1450
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BIB DATA SHEET

CONFIRMATION NO. 8161

SERIAL NUMBER	FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
11/894,676	08/20/2007 RULE	536	1635	287000.130US3

APPLICANTS

Gregory J. Hannon, Huntington, NY;
Patrick J. Paddison, Northport, NY;
Emily Bernstein, New York, NY;
Amy Caudy, Lawrenceville, NJ;
Douglas Conklin, Cold Spring Harbor, NY;
Scott Hammond, Cold Spring Harbor, NY;

**** CONTINUING DATA ***************************

This application is a CON of 10/997,086 11/23/2004
which is a CIP of 10/055,797 01/22/2002 ABN

**** FOREIGN APPLICATIONS ***************************

**** IF REQUIRED, FOREIGN FILING LICENSE GRANTED ****

11/02/2007

Foreign Priority claimed ☐ Yes ☑ No 35 USC 119(a-d) conditions met ☐ Yes ☑ No Verified and Acknowledged /KIMBERLY CHONG/ Examiner's Signature	☐ Met after Allowance Initials	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
		NY	67	14	2

ADDRESS

WilmerHale/Cold Spring Harbor Laboratory
399 Park Avenue
New York, NY 10022
UNITED STATES

TITLE

METHODS AND COMPOSITIONS FOR RNA INTERFERENCE

FILING FEE RECEIVED		
1270	FEES: Authority has been given in Paper No._____ to charge/credit DEPOSIT ACCOUNT No._____ for following:	☐ All Fees ☐ 1.16 Fees (Filing) ☐ 1.17 Fees (Processing Ext. of time) ☐ 1.18 Fees (Issue) ☐ Other _____ ☐ Credit