IN VIVO PRODUCTION OF SMALL INTERFERING RNAs THAT MEDIATE GENE SILENCING

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

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5 National Institutes of Health. The Government has certain rights in the invention.

TECHNICAL FIELD

This invention relates to ribonucleic acid interference (RNAi), and more particularly to RNAi in vivo.

BACKGROUND

10 RNAi is the sequence-specific, post-transcriptional silencing of a gene's expression by double-stranded RNA. RNAi is mediated by 21-22 nucleotide, double-stranded RNA molecules referred to as small interfering RNAs (siRNAs) that are derived by enzymatic cleavage of long, double-stranded RNA in cells (see, e.g., Fire et al., 1998, "Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans," Nature,
15 391:806-11; Tuschl et al., 1999, "Targeted mRNA degradation by double-stranded RNA in vitro," Genes Dev., 13:3191-7; Zamore et al., 2000, "RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals," Cell, 101:25-33.; Elbashir et al., 2001a, "Duplexes of 21-nucleotide RNAs mediate RNA interference in mammalian cell culture," Nature, 411:494-498; and Elbashir et al., 2001b, "RNA interference
20 is mediated by 21- and 22-nucleotide RNAs," Genes Dev., 15:188-200.

Double-stranded siRNAs mediate gene silencing by targeting for cleavage messenger RNAs (mRNAs) that contain the sequence of one strand of the siRNA. siRNAs introduced into mammalian cells by transfection mediate sequence-specific gene silencing, whereas long, double-stranded RNA induces sequence non-specific responses.

25 Small temporal RNAs, such as *lin-4* and *let-7*in *Caenorhabditis elegans* and *let-7* in *Drosophila melanogaster* and humans, encode no protein, but instead appear to block the productive translation of mRNA by binding sequences in the 3′ untranslated region (3′ UTR) of their target mRNAs.

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Currently, all work in the field of RNAi has been conducted in cell-based systems.

SUMMARY

The invention is based on the discovery that wild-type small temporal RNA (stRNA) precursors can be modified to produce engineered RNA precursors that when expressed in a cell in vivo are processed by the cell to produce double-stranded, targeted siRNAs that selectively silence targeted genes (by targeting specific mRNAs for cleavage) using the cell's RNAi pathway. By introducing nucleic acid molecules that encode these engineered RNA precursors into cells in vivo with appropriate regulatory sequences, expression of the engineered precursors can be selectively controlled both temporally and spatially, i.e., at particular times and/or in particular tissues, organs, or cells.

In general, the invention features an engineered RNA precursor that includes (i) 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; (ii) a second stem portion including a 19 to 22 nucleotide long sequence that is complementary to the specific targeted gene, wherein the first and second stem portions can hybridize with each other to form a duplex stem; and (iii) a loop portion that connects the two stem portions. The RNA precursor can target a portion of the specific targeted gene, e.g., from 100 to 300 nucleotides 3' of the start of translation.

The invention also features nucleic acid molecules (transgenes) that includes a regulatory sequence operably linked to a nucleic acid sequence that encodes the new engineered RNA precursors.

In another aspect, the invention features transgenic, non-human animals (e.g., a rodent such as a mouse, or other mammals, such as goats or cows, or birds), one or more of whose cells include a transgene including a regulatory sequence operably linked to a nucleic acid sequence encoding an engineered ribonucleic acid (RNA) precursor, wherein the transgene is expressed in one or more cells of the transgenic animal resulting in the animal exhibiting ribonucleic acid interference (RNAi) of a specific gene targeted by the engineered RNA precursor.

In this animal, the transgene can be expressed in one or more cardiac cells or lymphocytes, and the regulatory sequence can be constitutive or inducible. The regulatory

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sequence can be, for example, a Pol III or Pol II promoter. The animal can be a rodent, e.g., a mouse.

The invention also features vectors including the new nucleic acid molecules, e.g., plasmid or viral vectors, and cells containing the nucleic acid molecules. The invention also features cells derived from the transgenic animals.

In another aspect, the invention features a method of inducing ribonucleic acid interference (RNAi) of a specific gene in a cell within an animal, by obtaining a transgenic animal including a transgene that includes a nucleic acid sequence encoding an engineered RNA precursor and an inducible promoter; and inducing the cell to express the precursor to form a small interfering ribonucleic acid (siRNA) within the cell, thereby inducing RNAi of the specific gene. Various expression systems as described herein can be used. Any cell, e.g., hematopoietic cells, cardiac cells, vascular cell, or lymphocytes, can express the transgene. The regulatory sequence can be any regulatory sequence, and the regulatory sequence can be constitutive or inducible.

A "transgene" is any nucleic acid molecule, which is inserted by artifice into a cell, and becomes part of the genome of the organism that develops from the cell. Such a transgene may include a gene that is partly or entirely heterologous (i.e., foreign) to the transgenic organism, or may represent a gene homologous to an endogenous gene of the organism. The term "transgene" also means a nucleic acid molecule that includes one or more selected nucleic acid sequences, e.g., DNAs, that encode one or more engineered RNA precursors, to be expressed in a transgenic animal, which is partly or entirely heterologous, i.e., foreign, to the transgenic animal, or homologous to an endogenous gene of the transgenic animal, but which is designed to be inserted into the animal's genome at a location which differs from that of the natural gene. A transgene includes one or more promoters and any other DNA, such as introns, necessary for expression of the selected nucleic acid sequence, all operably linked to the selected sequence, and may include an enhancer sequence.

A "transformed cell" is a cell into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a nucleic acid molecule or transgene encoding an engineered RNA precursor.

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As used herein, the term "operably linked" means that a selected nucleic acid sequence, e.g., encoding an engineered RNA precursor, is in proximity with a promoter, e.g., a tissue-specific promoter, to allow the promoter to regulate expression of the selected nucleic acid sequence. In addition, the promoter is located upstream of the selected nucleic acid sequence in terms of the direction of transcription and translation.

By "promoter" is meant a nucleic acid sequence that is sufficient to direct transcription. A tissue-specific promoter effects expression of the selected nucleic acid sequence in specific cells, e.g., hematopoietic cells, or cells of a specific tissue within an animal, e.g., cardiac muscle or vascular endothelium. The term also covers so-called "leaky" promoters, which regulate expression of a selected nucleic acid sequence primarily in one tissue, but cause expression in other tissues as well. Such promoters also may include additional DNA sequences that are necessary for expression, such as introns and enhancer sequences.

By "transgenic" is meant any cell that includes a nucleic acid, e.g., DNA, sequence that is inserted by artifice into a cell and becomes part of the genome of an organism that develops from that cell. A "transgenic animal" means an animal that includes a transgene that is inserted into an embryonal cell and becomes a part of the genome of the animal which develops from that cell, or an offspring of such an animal. In the transgenic animals described herein, the transgene causes specific tissue cells to express an engineered RNA precursor. Any animal that can be produced by transgenic technology is included in the invention, although mammals are preferred. Preferred mammals include non-human primates, sheep, goats, horses, cattle, pigs, rabbits, and rodents such as guinea pigs, hamsters, rats, gerbils, and, preferably, mice.

A "substantially pure nucleic acid molecule or sequence" is a nucleic acid molecule or sequence that is not immediately contiguous with both of the coding sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally-occurring genome of the organism from which it is derived. The term therefore includes, for example, a recombinant DNA that is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g., a cDNA or a genomic DNA fragment produced by PCR or

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restriction endonuclease treatment) independent of other sequences. It also includes a recombinant DNA that is part of a hybrid gene encoding an additional polypeptide sequence.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

The invention provides several advantages. For example, the invention improves on and overcomes a significant deficiency in the prior art. The previous methods for inducing RNAi in mammalian cells using siRNAs were restricted to cell cultures. The new methods extend RNAi to whole animals, e.g., mammals, and thus allow RNAi to be targeted to specific cell types, organs, or tissues, and/or to specific developmental stages.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic diagram of the dual nature of the stRNA and siRNA pathways.

FIG. 2A is a schematic representation of a wild-type, stRNA precursor.

FIGs. 2B to 2E are schematic representations of synthetic, engineered RNA precursors.

FIG. 3 is a schematic diagram of transgene encoding an engineered RNA precursor and the transcription and processing of the precursor to form a double-stranded siRNA.

DETAILED DESCRIPTION

Small temporal RNAs (stRNAs), such as *lin-4* and *let-7*in *Caenorhabditis elegans* and *let-7* in *Drosophila melanogaster* and humans encode no protein, but instead appear to block the productive translation of mRNA by binding sequences in the 3′ untranslated region

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