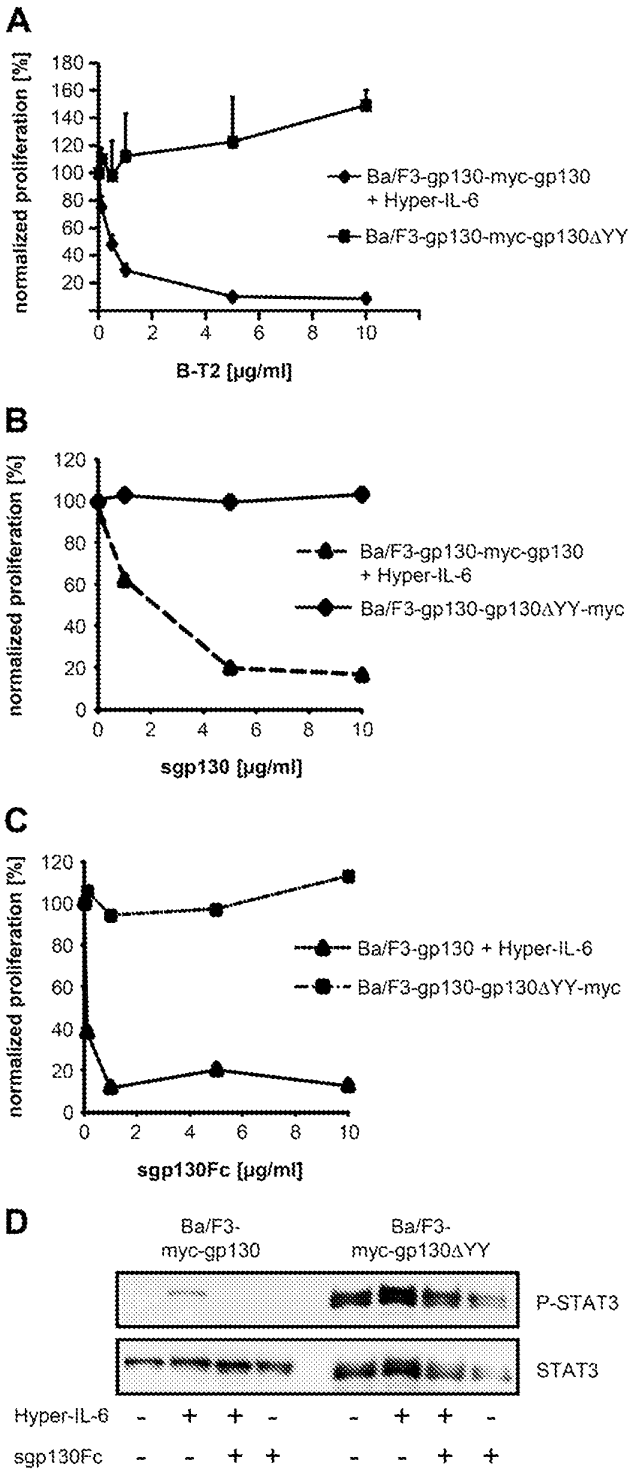

FIGURE 3. **No inhibition of ligand-independent proliferation of Ba/F3-gp130-gp130ΔYY by sgp130 or sgp130Fc or the anti-gp130 mAb B-T2.** *A*, equal numbers of Ba/F3-gp130 cells stably transduced with myc-gp130ΔYY were cultured for 3 days in the absence of Hyper-IL-6 and increasing amounts of B-T2 (0, 0.1, 0.5, 1, 5, and 10 μg/ml). Proliferation was measured as indicated under "Experimental Procedures." As a control, Ba/F3-gp130-myc-gp130 cells were treated with 1 ng/ml Hyper-IL-6 plus B-T2. *B*, equal numbers of Ba/F3-gp130 cells stably transduced with gp130ΔYY-myc were cultured for 3 days in the absence of Hyper-IL-6 and increasing amounts of sgp130 (0, 0.1, 1, 5, and 10 μg/ml). Proliferation was measured as indicated under "Experimental Procedures." As a control, Ba/F3-gp130-myc-gp130 cells were treated with 1 ng/ml Hyper-IL-6 plus sgp130. *C*, equal numbers of Ba/F3-gp130 cells stably transduced with gp130ΔYY-myc were cultured for 3 days in the absence of Hyper-IL-6 and increasing amounts of sgp130Fc (0, 0.1, 1, 5, and 10 μg/ml). Proliferation was measured as indicated under "Experimental Procedures." As a control, Ba/F3-gp130 were treated with 1 ng/ml Hyper-IL-6 and sgp130Fc. *D*, after 6 h of serum starvation, Ba/F3 cells stably transduced with myc-gp130ΔYY or myc-gp130 were stimulated for 5 min with Hyper-IL-6, Hyper-IL-6 + sgp130Fc, or sgp130Fc or left untreated. STAT3 phosphorylation was analyzed by Western blot analysis.

oncostatin M, or ciliary neurtrophic factor (19). As a control, B-P4 did not inhibit the proliferation of Ba/F3-gp130-gp130ΔYY cells stimulated with IL-3, indicating that B-P4 specifically blocked the activity of gp130ΔYY in Ba/F3-gp130-gp130ΔYY-cells (Fig. 4*D*).

DISCUSSION

Constitutive activation of the gp130-dependent transcription factor STAT3 has been implicated in many human neoplastic malignancies, including multiple myeloma (4, 22, 23), prostate cancer, melanoma, ovarian cancer, renal carcinoma (24), as well as gastric cancer (25). Artificially dimerized STAT3 has been shown to exhibit oncogenic potential, and STAT3 was therefore designated as an oncogene (26). The IL-6/gp130 signaling pathway is a candidate for constitutive STAT3 activation in tumors (27). Increased STAT3 phosphorylation was found in IHCAs (2). Interestingly, gp130 gene mutations were found in 60% of the analyzed IHCAs. It turned out that these mutations resulted in ligand-independent dimerization of gp130 receptor chains and constitutive STAT3 phosphorylation. This was the first report on somatic mutation of gp130 in tumors (2), and in combination with the potential to induce cytokine-independent cellular proliferation shown in this study, gp130 can be defined as an oncogene involved in benign human tumors that contributes to the inflammatory phenotype (2).

All mutations of gp130 found in IHCAs were deletions within the cytokine binding interface of domain 2 (2). Here, we analyzed a frequently occurring gp130 mutation (gp130ΔY186-Y190, gp130ΔYY) found in four of 26 IHCA patients. Six more patients carried mutations from Ser-187-Y190 (gp130ΔSY) that were also covered in Tyr-186 to Tyr-190 (2). We show that gp130ΔYY leads to ligand-independent, long-term proliferation of Ba/F3 cells and constitutive STAT3 phosphorylation.

Interestingly, deletion of domain 1 from gp130ΔYY resulted in a signaling-incompetent receptor chain, indicating that domain 1 contributes to ligand-independent receptor activation. However, dimerization of gp130ΔYY was independent of the presence of the D1 domain. The neutralizing anti-gp130 mAb B-T2 directed against D1 did not inhibit receptor activation of gp130ΔYY, indicating that the gp130 homodimerization induced by IL-6/IL-6R is fundamentally different from the homodimerization of gp130ΔYY. Homodimerization of the wild-type gp130 receptor is facilitated by contacts of gp130 CBM (domain 2 and 3) to the binding site II of IL-6 and of gp130 D1 to the binding site III of IL-6, whereas the IL-6R contacts IL-6 via the binding site I (16). We speculate that homodimerization of gp130ΔYY is facilitated by the interaction of the mutated D2 (CBM) of one receptor with the D2 of the

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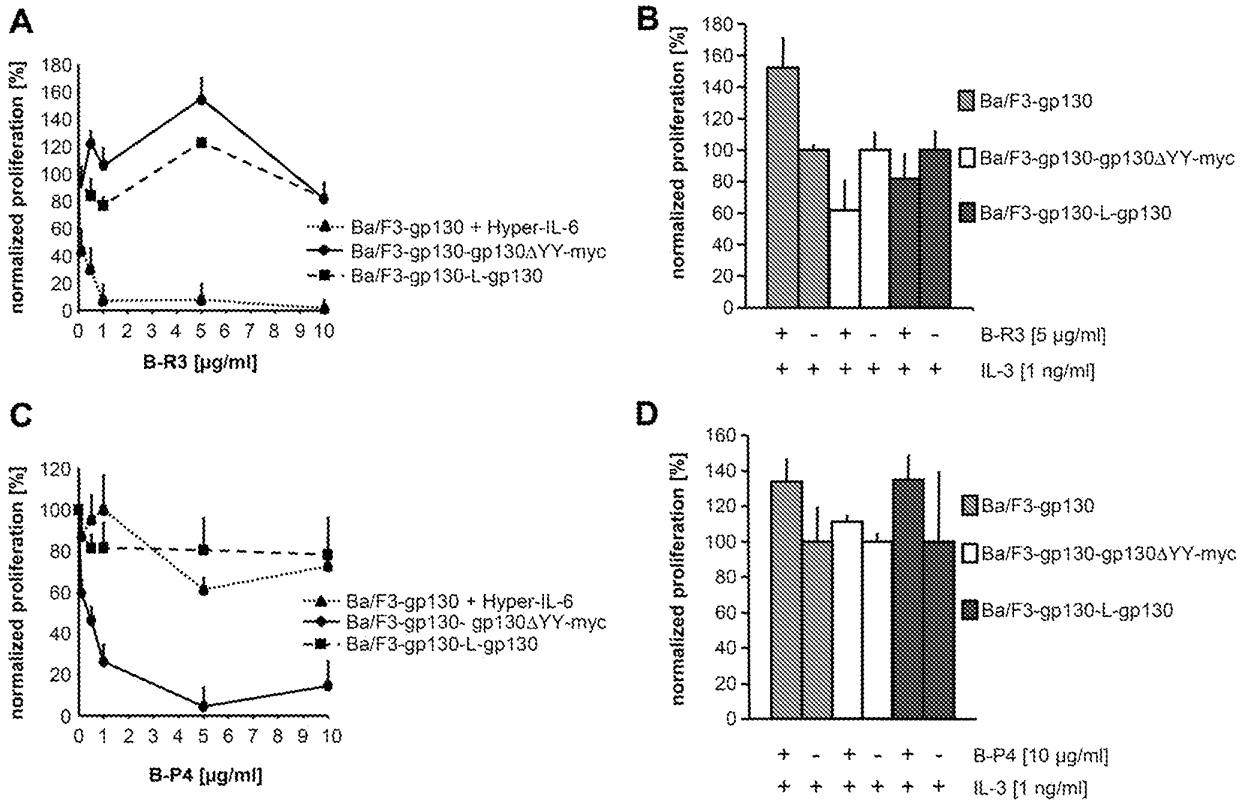

FIGURE 4. **Biological activity of gp130ΔYY can be suppressed by the neutralizing anti-gp130 mAb B-P4 but not by B-R3.** *A*, equal numbers of Ba/F3-gp130-gp130ΔYY-myc cells were cultured for 3 days in the absence of Hyper-IL-6 and increasing amounts of B-R3 (0, 0.1, 0.5, 1, 5, and 10 μg/ml). Proliferation was measured as indicated under "Experimental Procedures." As a control, Ba/F3-gp130 cells were treated with 10 ng/ml Hyper-IL-6 and B-R3. *B*, equal numbers of Ba/F3-gp130-gp130ΔYY-myc cells were cultured for 3 days in the presence of IL-3 (1 ng/ml) and B-R3 (5 μg/ml). Proliferation was measured as indicated under "Experimental Procedures." As a control, Ba/F3-gp130 cells were treated with 1 ng/ml IL-3 and B-R3 (5 μg/ml). *C*, equal numbers of Ba/F3-gp130-gp130ΔYY-myc cells were cultured for 3 days in the absence of Hyper-IL-6 and increasing amounts of B-P4 (0, 0.1, 0.5, 1, 5, and 10 μg/ml). Proliferation was measured as indicated under "Experimental Procedures." As a control, Ba/F3-gp130 cells were treated with 10 ng/ml Hyper-IL-6 and B-P4 (10 μg/ml). *D*, equal numbers of Ba/F3-gp130-gp130ΔYY-myc cells were cultured for 3 days in the presence of IL-3 (1 ng/ml) and B-P4 (10 μg/ml). Proliferation was measured as indicated under "Experimental Procedures."

other receptor. However, future studies are needed to fully explore the mechanism of ligand-independent gp130ΔYY receptor activation.

Moreover, the wild-type gp130 receptor formed stable heterodimers with gp130ΔYY, and overexpression of the wild-type gp130 receptor blocked constitutive activation of gp130ΔSY (2). A likely mechanism for this inhibition is that interaction of the extracellular parts of wild-type and mutated gp130 receptors resulted in inactive gp130/gp130ΔSY heterodimers. Surprisingly, soluble gp130 variants (sgp130 and sgp130Fc) did not inhibit gp130ΔYY-induced cellular proliferation. This paradoxical situation might be explained by a limited access of sgp130 in the sterical correct orientation to the cell surface bound gp130 protein, which might also explain why sgp130 cannot inhibit gp130ΔYY-induced cellular proliferation.

However, constitutive ligand-independent activation of gp130ΔYY was blocked by the neutralizing anti-gp130 mAb B-P4. The epitope of B-P4 is located within the fibronectin type III domain 4 of gp130 (gp130-D4). Truncation of the fibronectin-like type III domains results in gp130 molecules devoid of signaling capacity (28), and it has been speculated that the functional role of the fibronectin type III domains is the assembly of the transmembrane domains in close proximity to allow activation of gp130-associated intracellular JAKs (29). Interestingly, B-P4 has been shown to block only gp130 signaling induced by IL-11 but not by IL-6 or the other members of the IL-6 family, leukemia inhibitory factor, oncostatin M, and ciliary neurotrophic factor (19). Cardiotrophin 1, cardiotrophin-like cytokine, and IL-27 were, however, not investigated so far (19). This might indicate that signaling of gp130ΔYY mimics IL-11 signaling. IL-11 was shown to promote gastric cancer via gp130 and STAT3 phosphorylation (30). This view is supported by the finding that IL-11 but not IL-6 was overexpressed in IHCAs (2). IL-11 was, however, only overexpressed in IHCAs that did not harbor gp130 mutations (2), suggesting that IHCAs are, to some extent, driven by IL-11 via wild-type gp130. After somatic mutation of gp130 into an IL-11-like constitutively active gp130 variant, the necessity of IL-11-driven gp130 signal transduction might be abrogated, resulting in down-regulation of IL-11 expression levels.

No malignant transformation was found in IHCAs with gp130 mutations, but two of 111 analyzed cases of malignant transformation of IHCAs into hepatocellular carcinoma carried mutations in gp130 and in the β-catenin pathway, suggest-

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ing a rare interplay of these pathways in malignant transformation (2). In conclusion, blockade of constitutive activation of mutant gp130 by B-P4 might open a possibility to therapeutically block gp130-induced STAT3 phosphorylation in hepatic adenomas and in a subclass of hepatocellular carcinomas.

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Constitutively Active Mutant gp130 Receptor Protein from Inflammatory Hepatocellular Adenoma Is Inhibited by an Anti-gp130 Antibody That Specifically Neutralizes Interleukin 11 Signaling

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Application Number:	15988463
International Application Number:	
Confirmation Number:	7597
Title of Invention:	TREATMENT OF FIBROSIS
First Named Inventor/Applicant Name:	Stuart Alexander Cook
Customer Number:	23628
Filer:	Amy Jeanette McMahon/Ashley Cerrone
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Application Type:	Utility under 35 USC 111(a)

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TRANSMITTAL FORM *(to be used for all correspondence after initial filing)*			
		Application Number	15/988,463-Conf. #7597
		Filing Date	May 24, 2018
		First Named Inventor	Stuart Alexander Cook
		Art Unit	1646
		Examiner Name	Prema Maria Mertz
Total Number of Pages in This Submission		Attorney Docket Number	M0546.70012US01

ENCLOSURES (***Check all that apply***)

☐ Fee Transmittal Form	☐ Drawing(s)	☐ After Allowance Communication to TC
☐ Fee Attached	☐ Licensing-related Papers	☐ Appeal Communication to Board of Appeals and Interferences
☐ Amendment/Reply	☐ Petition	☐ Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
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☐ Reply to Missing Parts under 37 CFR 1.52 or 1.53		

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	WOLF, GREENFIELD & SACKS, P.C.		
Signature	/Amy J. McMahon/		
Printed name	Amy J. McMahon, PhD		
Date	July 13, 2018	Reg. No.	73,073

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Stuart Alexander Cook
Application No.:	15/988,463
Confirmation No.:	7597
Filed:	May 24, 2018
For:	TREATMENT OF FIBROSIS
Examiner:	Prema Maria Mertz
Art Unit:	1646

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STATEMENT FILED PURSUANT TO THE DUTY OF DISCLOSURE UNDER 37 C.F.R. §§ 1.56, 1.97 AND 1.98

Sir:

Pursuant to the duty of disclosure under 37 C.F.R. §§ 1.56, 1.97 and 1.98, the undersigned requests consideration of this Information Disclosure Statement.

PART I: Compliance with 37 C.F.R. § 1.97

This Information Disclosure Statement has been filed within three months of the filing date of a national application other than a continued prosecution application under 37 C.F.R. § 1.53(d).

No fee or certification is required.

PART II: Information Cited

The undersigned hereby makes of record in the above-identified application the information listed on the attached form PTO-1449 (modified PTO/SB/08). The order of presentation of the references should not be construed as an indication of the importance of the references.

PART III: Remarks

Documents cited anywhere in the Information Disclosure Statement are enclosed unless otherwise indicated. It is respectfully requested that:

1. The Examiner consider completely the cited information, along with any other information, in reaching a determination concerning the patentability of the present claims;

2. The enclosed form PTO-1449 (modified PTO/SB/08) be signed by the Examiner to evidence that the cited information has been fully considered by the United States Patent and Trademark Office during the examination of this application;

3. The citations for the information be printed on any patent which issues from this application.

By submitting this Information Disclosure Statement, the undersigned makes no representation that a search has been performed, of the extent of any search performed, or that more relevant information does not exist.

By submitting this Information Disclosure Statement, the undersigned makes no representation that the information cited in the Statement is, or is considered to be, material to patentability as defined in 37 C.F.R. § 1.56(b).

By submitting this Information Disclosure Statement, the undersigned makes no representation that the information cited in the Statement is, or is considered to be, in fact, prior art as defined by 35 U.S.C. § 102.

Notwithstanding any statements by the undersigned, the Examiner is urged to form his or her own conclusion regarding the relevance of the cited information.

An early and favorable action is hereby requested.

The Director is hereby authorized to charge any deficiency or credit any overpayment in the fees occasioned by the filing of this Information Disclosure Statement to our Deposit Account No. 23/2825 under Docket No. M0546.70012US01 from which the undersigned is authorized to draw.

Respectfully submitted,

By: /Amy J. McMahon/
Amy J. McMahon, PhD, Reg. No. 73,073
Wolf, Greenfield & Sacks, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2206
Telephone: (617) 646-8000

Docket No.: M0546.70012US01
Date: July 13, 2018

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 15/988,463

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	300
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	660
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	760
TOTAL CLAIMS (37 CFR 1.16(i))	10 minus 20 =	*			OR	x 100 =	0.00
INDEPENDENT CLAIMS (37 CFR 1.16(h))	1 minus 3 =	*				x 460 =	0.00
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 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).						400
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							0.00
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	2120

APPLICATION AS AMENDED - PART II

		(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($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	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	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE($)	ADDITIONAL FEE($)	OR	RATE($)	ADDITIONAL FEE($)
AMENDMENT B	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 found in the appropriate box in column 1.

UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
15/988,463	05/24/2018	1629	2120	M0546.70012US01	10	1

CONFIRMATION NO. 7597

23628
WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206

FILING RECEIPT

OC000000100144928

Date Mailed: 06/11/2018

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**

Inventor(s)

Stuart Alexander Cook, Singapore, SINGAPORE;
Sebastian Schaefer, Singapore, SINGAPORE;

Applicant(s)

Singapore Health Services PTE LTD., Singapore, SINGAPORE;
National University of Singapore, Singapore, SINGAPORE;

Assignment For Published Patent Application

Singapore Health Services PTE LTD., Singapore, SINGAPORE
National University of Singapore, Singapore, SINGAPORE

Power of Attorney: The patent practitioners associated with Customer Number 23628

Domestic Priority data as claimed by applicant

This application is a DIV of 15/381,622 12/16/2016

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.)
UNITED KINGDOM 1522186.4 12/16/2015 No Access Code Provided

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 06/08/2018

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 15/988,463**

Projected Publication Date: 09/20/2018

Non-Publication Request: No

Early Publication Request: No
Title

TREATMENT OF FIBROSIS

Preliminary Class

514

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

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 (1-866-999-4258).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

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To: Patents_eOfficeAction@WolfGreenfield.com,WGS_eOfficeAction@WolfGreenfield.com,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 23628

Jun 11, 2018 04:12:31 AM

Dear PAIR Customer:

WOLF GREENFIELD & SACKS, P.C.
600 ATLANTIC AVENUE
BOSTON, MA 02210-2206
UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 23628 , have new outgoing correspondence. This correspondence is now available for viewing in Private PAIR.

The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

Disclaimer:
The list of documents shown below is provided as a courtesy and is not part of the official file wrapper. The content of the images shown in PAIR is the official record.

Application	Document	Mailroom Date	Attorney Docket No.
15988463	APP.FILE.REC	06/11/2018	M0546.70012US01

To view your correspondence online or update your email addresses, please visit us anytime at https://sportal.uspto.gov/secure/myportal/privatepair.

If you have any questions, please email the Electronic Business Center (EBC) at EBC@uspto.gov with 'e-Office Action' on the subject line or call 1-866-217-9197 during the following hours:

Monday - Friday 6:00 a.m. to 12:00 a.m.

Thank you for prompt attention to this notice,

UNITED STATES PATENT AND TRADEMARK OFFICE

PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

REQUEST FOR PARTICIPATION IN THE GLOBAL/IP5 PATENT PROSECUTION HIGHWAY (PPH) PILOT PROGRAM IN THE USPTO

Application No.:	15/988,463-Conf. #7597	First Named Inventor:	Stuart Alexander Cook
Filing Date:	May 24, 2018	Attorney Docket No.:	M0546.70012US01
Title of the Invention:	TREATMENT OF FIBROSIS		

THIS REQUEST FOR PARTICIPATION IN THE PPH PILOT PROGRAM ALONG WITH THE REQUIRED DOCUMENTS MUST BE SUBMITTED VIA EFS-WEB. INFORMATION REGARDING EFS-WEB IS AVAILABLE AT
HTTPS://WWW.USPTO.GOV/PATENTS-APPLICATION-PROCESS/APPLYING-ONLINE/ABOUT-EFS-WEB

APPLICANT HEREBY REQUESTS PARTICIPATION IN THE PATENT PROSECUTION HIGHWAY (PPH) PILOT PROGRAM AND PETITIONS TO MAKE THE ABOVE-IDENTIFIED APPLICATION SPECIAL UNDER THE PPH PILOT PROGRAM.

Office of earlier examination (OEE): Europe (European Patent Office)

OEE application number: PCT/EP2016/081430

Both the OEE application and the above-identified U.S. application have the following earliest date (filing or priority date): December 16, 2015

Type of OEE work product relied upon: International Preliminary Report on Patentability

Mailing date of OEE work product: November 6, 2017

Supporting Documents

1. OEE Work Product and Translation

A copy of the OEE work product and translation if not already in English:

☐ Attached ☒ Previously submitted ☐ Not required because the decision to grant a patent was the first office action

☐ Applicant requests the USPTO to attempt to obtain the OEE work product from the Dossier Access System or PATENTSCOPE

NOTE: If the applicant requests the USPTO to obtain the OEE work product electronically and such attempt is unsuccessful, the applicant will be required to supply the document. Accordingly, to avoid dismissal of the initial PPH request and potential denial of participation in the PPH program, the applicant should verify that the OEE work product is actually available via the Dossier Access System or PATENTSCOPE before requesting retrieval. If the applicant is unable to verify availability, then the applicant should submit the document with the PPH request.

2. References Cited in OEE Work Product

An information disclosure statement (IDS) listing the references cited in the OEE work product and document copies (except U.S. patents and U.S. published patent applications):

☐ Attached ☒ Previously Submitted ☐ Not required because no references were cited in the OEE work product

Certificate of Electronic Filing under 37 CFR §1.8

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 CFR § 1.6(a)(4).

Dated: June 6, 2018 Electronic Signature for Nancy J. Arsenault: /Nancy J. Arsenault/

REQUEST FOR PARTICIPATION IN THE GLOBAL/IP5 PPH PILOT PROGRAM IN THE USPTO
(continued)

Application No.:	15/988,463-Conf. #7597	First Named Inventor:	Stuart Alexander Cook

3. Claims Correspondence Certification Statement

All of the claims in this application sufficiently correspond to patentable/allowable claims in the OEE application.

4. Claims Correspondence Table

Claims in U.S. Application	Patentable Claims in OEE Application	Explanation Regarding the Correspondence
1	7	Claim 1 sufficiently corresponds to OEE claim 7 because claim 1 is within the scope of OEE claim 7
21	4	Claim 21 sufficiently corresponds to OEE claim 4 because claim 21 is within the scope of OEE claim 4
22	6	Claim 22 sufficiently corresponds to OEE claim 6 because claim 22 is within the scope of OEE claim 6
23	8	Claim 23 sufficiently corresponds to OEE claim 8 because claim 23 is within the scope of OEE claim 8
24	9	Claim 24 sufficiently corresponds to OEE claim 9 because claim 24 is within the scope of OEE claim 9
25	10	Claim 25 sufficiently corresponds to OEE claim 10 because claim 25 is within the scope of OEE claim 10
26	11	Claim 26 sufficiently corresponds to OEE claim 11 because claim 26 is within the scope of OEE claim 11
27	12	Claim 27 sufficiently corresponds to OEE claim 12 because claim 27 is within the scope of OEE claim 12
28	13	Claim 28 sufficiently corresponds to OEE claim 13 because claim 28 is within the scope of OEE claim 13
29	14	Claim 29 sufficiently corresponds to OEE claim 14 because claim 29 is within the scope of OEE claim 14

Signature	/Amy J. McMahon/	Date	June 6, 2018
Name (print or type)	Amy J. McMahon, PhD	Registration Number	73,073

Electronic Acknowledgement Receipt

EFS ID:	32819253
Application Number:	15988463
International Application Number:	
Confirmation Number:	7597
Title of Invention:	TREATMENT OF FIBROSIS
First Named Inventor/Applicant Name:	Stuart Alexander Cook
Customer Number:	23628
Filer:	Amy Jeanette McMahon/Nancy Arsenault
Filer Authorized By:	Amy Jeanette McMahon
Attorney Docket Number:	M0546.70012US01
Receipt Date:	06-JUN-2018
Filing Date:	
Time Stamp:	13:14:15
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	M054670012US01-TRN-AM.pdf	25228 7b7a3272c6e0f6464732cc8d4c135e32ad2ae747	no	1

Warnings:

Information:					
2	Petition to make special under Patent Prosecution Hwy	M054670012US01-PPH-AM.pdf	24504 3bcfb078fd7aa7ef0ef76cc780615f95980ca9e9	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	49732	

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

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Doc Code: TRAN.LET
Document Description: Transmittal Letter

USED IN LIEU OF PTO/SB/21 (07-09)

TRANSMITTAL FORM *(to be used for all correspondence after initial filing)*			
		Application Number	15/988,463-Conf. #7597
		Filing Date	May 24, 2018
		First Named Inventor	Stuart Alexander Cook
		Art Unit	Not Yet Assigned
		Examiner Name	Not Yet Assigned
Total Number of Pages in This Submission		Attorney Docket Number	M0546.70012US01

ENCLOSURES (***Check all that apply***)

☐ Fee Transmittal Form	☐ Drawing(s)	☐ After Allowance Communication to TC
☐ Fee Attached	☐ Licensing-related Papers	☐ Appeal Communication to Board of Appeals and Interferences
☐ Amendment/Reply	☐ Petition	☐ Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
☐ After Final	☐ Petition to Convert to a Provisional Application	☐ Proprietary Information
☐ Affidavits/declaration(s)	☐ Power of Attorney, Revocation Change of Correspondence Address	☐ Status Letter
☐ Extension of Time Request	☐ Terminal Disclaimer	☒ Other Enclosure(s) (please Identify below):
☐ Express Abandonment Request	☐ Request for Refund	Request for Participation in the Global/IP5 Patent Prosecution Highway Pilot Program
☐ Information Disclosure Statement	☐ CD, Number of CD(s) _____	
☐ Certified Copy of Priority Document(s)	☐ Landscape Table on CD	
☐ Reply to Missing Parts/ Incomplete Application	Remarks	
☐ Reply to Missing Parts under 37 CFR 1.52 or 1.53		

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	WOLF, GREENFIELD & SACKS, P.C.		
Signature	/Amy J. McMahon/		
Printed name	Amy J. McMahon, PhD		
Date	June 6, 2018	Reg. No.	73,073

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I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being transmitted via the Office's electronic filing system in accordance with 37 C.F.R. § 1.6(a)(4).

Dated: June 6, 2018 Electronic Signature for Nancy J. Arsenault: /Nancy J. Arsenault/

6362702.1

FORM PTO-1449/A and B (modified PTO/SB/08)	APPLICATION NO.: 15/988,463	ATTY. DOCKET NO.: M0546.70012US01
INFORMATION DISCLOSURE STATEMENT BY APPLICANT	FILING DATE: May 24, 2018	CONFIRMATION NO.: 7597
	FIRST NAMED INVENTOR: Stuart Alexander Cook	
Sheet 1 of 3	GROUP ART UNIT: 1629	EXAMINER: Not Yet Assigned

U.S. PATENT DOCUMENTS

Examiner's Initials #	Cite No.	U.S. Patent Document		Name of Patentee or Applicant of Cited Document	Date of Publication or Issue of Cited Document MM-DD-YYYY
		Number	Kind Code		
		2003-0147849	A1	Warne et al.	08-07-2003
		2004-0126358	A1	Warne et al.	07-01-2004
		2004-0142871	A1	Shaughnessy et al.	07-22-2004
		2006-0062760	A1	Keith et al.	03-23-2006
		2007-0160577	A1	Damle et al.	07-12-2007
		2009-0191147	A1	Keith et al.	07-30-2009
		2010-0062058	A1	Warne et al.	03-11-2010
		2010-0093976	A1	Azuma et al.	04-15-2010
		2010-0183544	A1	Jenkins et al.	07-22-2010
		2013-0302277	A1	Jenkins et al.	11-14-2013
		2014-0219919	A1	Edwards et al.	08-07-2014
		2016-0031999	A1	Edwards et al.	02-04-2016
		2017-0174759	A1	Cook et al.	06-22-2017
		5,679,339	A	Keith et al.	10-21-1997
		6,126,933	A	Warne et al.	10-03-2000
		6,540,993	B1	Warne et al.	04-01-2003
		6,846,907	B1	Shaughnessy et al.	01-25-2005
		6,953,777	B1	Keith et al.	10-11-2005
		6,998,123	B1	Shaughnessy et al.	02-14-2006
		7,993,637	B2	Baca	08-09-2011
		8,182,814	B2	Baca et al.	05-22-2012
		8,361,966	B2	Azuma et al.	01-29-2013
		8,518,888	B2	Jenkins et al.	08-27-2013
		8,540,977	B2	Baca	09-24-2013
		9,340,618	B2	Edwards et al.	05-17-2016

FOREIGN PATENT DOCUMENTS

Examiner's Initials #	Cite No.	Foreign Patent Document			Name of Patentee or Applicant of Cited Document	Date of Publication of Cited Document MM-DD-YYYY	Translation (Y/N)
		Office/ Country	Number	Kind Code			
		WO	1998/36061	A2	The Victoria University of Manchester	08-20-1998	
		WO	2000/078336	A1	Genetics Institute, Inc. et al.	12-28-2000	
		WO	2002/020609	A2	Smithkline Beecham PLC	03-14-2002	
		WO	2005/070446	A1	Scios Inc.	08-04-2005	
		WO	2005/098041	A2	University of Florida Research Foundation, Inc.	10-20-2005	
		WO	2009/052588	A1	CSL Limited	04-30-2009	

EXAMINER:	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.

FORM PTO-1449/A and B (modified PTO/SB/08)				APPLICATION NO.: 15/988,463	ATTY. DOCKET NO.: M0546.70012US01
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				FILING DATE: May 24, 2018	CONFIRMATION NO.: 7597
				FIRST NAMED INVENTOR: Stuart Alexander Cook	
Sheet	2	of	3	GROUP ART UNIT: 1629	EXAMINER: Not Yet Assigned

		WO	2014/121325	A1	CSL Limited	08-14-2014	
		WO	2017/103108	A1	Singapore Health Services PTE, Ltd.	06-22-2017	

OTHER ART – NON PATENT LITERATURE DOCUMENTS

Examiner's Initials #	Cite No	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.	Translation (Y/N)
		International Search Report and Written Opinion for International Patent Application No. PCT/EP2016/081430, mailed April 18, 2017.	
		Chapter II Demand filed August 14, 2017 for International Patent Application No. PCT/EP2016/081430.	
		International Preliminary Report on Patentability (Chapter II) for International Patent Application No. PCT/EP2016/081430, mailed November 6, 2017.	
		Third Party Submission Under 37 C.F.R. § 1.290 for US Application No. 15/381,622 (M0546.70012US00), filed April 30, 2018.	
		ANCEY et al., A fusion protein of the gp130 and interleukin-6Ralpha ligand-binding domains acts as a potent interleukin-6 inhibitor. J Biol Chem. 2003 May 9;278(19):16968-72.	
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FORM PTO-1449/A and B (modified PTO/SB/08)				APPLICATION NO.: 15/988,463	ATTY. DOCKET NO.: M0546.70012US01
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				FILING DATE: May 24, 2018	CONFIRMATION NO.: 7597
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Sheet	3	of	3	GROUP ART UNIT: 1629	EXAMINER: Not Yet Assigned

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First Named Inventor/Applicant Name:	Stuart Alexander Cook
Customer Number:	23628
Filer:	Amy Jeanette McMahon/Nathaniel Taylor
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(54) Title: REDUCING FIBROSIS AND/OR SCARRING BY INHIBITING INTERLEUKIN–6 RECEPTOR–MEDIATED ACTIVITY

(57) Abstract

The present application relates to the use of agents which inhibit Interleukin–6 receptor mediated activity for the treatment of wounds and/or fibrotic disorders such that fibrosis and/or scarring are reduced or prevented. Preferred agents for use in such treatments include Interleukin–6 neutralising antibodies, Interleukin–6 Receptor antagonists and inhibitors of Interleukin–6 Receptor signal transduction.

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REDUCING FIBROSIS AND/OR SCARRING BY INHIBITING INTERLEUKIN-6 RECEPTOR-MEDIATED ACTIVITY

The present invention relates to reducing fibrosis in the healing of wounds and other conditions in which fibrosis is a major mechanism of tissue repair or where excessive fibrosis leads to pathological derangement and malfunctioning of the tissue.

The term "wound" as used herein is exemplified but not limited to injuries to the skin. Other types of wound can involve damage, injury or trauma to an internal tissue or organ such as the lung, kidney, heart, gut, tendons or liver.

Wound healing in adult tissues is a complicated reparative process. The healing process begins with the recruitment of a variety of specialised cells to the site of the wound and involves extracellular matrix and basement membrane deposition, angiogenesis, selective protease activity and re-epithelialisation. An important component of the healing process in adult mammals is the stimulation of fibroblasts to generate the extracellular matrix. This extracellular matrix constitutes a major component of a connective tissue which develops to repair the wounded area and which may develop into a scar.

A scar is an abnormal morphological structure resulting from a previous injury or wound (e.g. an incision, excision or trauma). Scars are composed of a connective tissue which is predominately a matrix of collagen types 1 and 3 and fibronectin. The scar may consist of collagen fibres in an abnormal organisation (as seen in scars of the skin) or it may be an abnormal accumulation of connective tissue (as seen in scars of the central nervous system). Most scars consist of abnormally organised collagen and also excess collagen. In man, in the skin, scars may be depressed below the surface or elevated above the surface of the skin. Hypertrophic scars are a more severe form of normal scarring, are elevated above the normal surface of the skin and contain excessive collagen arranged in an abnormal pattern. A keloid is another form of pathological scarring which is not only elevated above the surface of the skin but also

extends beyond the boundaries of the original injury. In a keloid there is excessive connective tissue which is organised in an abnormal fashion predominately in whirls of collagenous tissue. There are genetic predispositions to forming both hypertrophic scars and keloids. They are particularly common in Africo-Carribean and Mongoloid races.

There are various medical situations for which it is desirable to promote the healing of wounds such that scar formation is regulated. Examples of such situations are scars of the skin where excessive scarring may be detrimental to tissue function and particularly when scar contracture occurs (for instance skin burns and wounds which impair flexibility of a joint). The reduction of scarring to the skin when cosmetic considerations are important is also highly desirable. In the skin, hypertrophic or keloid scars (particularly in Africo-Caribbean and Mongoloid races) can cause functional and cosmetic impairment and there is a need to prevent their occurrence. Scarring resulting from skin grafts in both donor sites and from the application of artificial skin can also be problematic and need to be minimised or prevented.

As well as scars of the skin, internal scarring or fibrosis can be highly detrimental and specific examples include:

(i) Within the central nervous system, glial scarring can prevent neuronal reconnection (e.g. following neuro-surgery or penetrating injuries of the brain).

(ii) Scarring in the eye can be detrimental. In the cornea, scarring can result in abnormal opacity and lead to problems with vision or even blindness. In the retina, scarring can cause buckling or retinal detachment and consequently blindness. Scarring following wound healing in operations to relieve pressure in glaucoma (e.g. glaucoma filtration surgery) results in the failure of the surgery whereby the aqueous humour fails to drain and hence the glaucoma returns.

(iii) Scarring in the heart (e.g. following surgery or myocardial infarction) can give rise to abnormal cardiac function.

(iv) Operations involving the abdomen or pelvis, often result in adhesion between viscera. For instance, adhesions between elements of the gut and the body wall may form and cause twisting in the bowel loop leading to ischaemia, gangrene and the necessity for emergency treatment (untreated they may even be fatal). Likewise, trauma or incisions to the guts can lead to scarring and scar contracture to strictures which cause occlusion of the lumen of the guts which again can be life threatening.

(v) Scarring in the pelvis in the region of the fallopian tubes can lead to infertility.

(vi) Scarring following injury to muscles can result in abnormal contraction and hence poor muscular function.

(vii) Scarring or fibrosis following injury to tendons and ligaments can result in serious loss of function.

Related to the above is the fact that there are a number of medical conditions known as fibrotic disorders in which excessive fibrosis leads to pathological derangement and malfunctioning of tissue. Fibrotic disorders are characterised by the accumulation of fibrous tissue (predominately collagens) in an abnormal fashion within the tissue. Accumulation of such fibrous tissues may result from a variety of disease processes. These diseases do not necessarily have to be caused by surgery, traumatic injury or wounding. Fibrotic disorders are usually chronic. Examples of fibrotic disorders include cirrhosis of the liver, liver fibrosis, glomerulonephritis, pulmonary fibrosis, scleroderma, myocardial fibrosis, fibrosis following myocardial infarction, central nervous system fibrosis following a stroke or neuro-degenerative disorders (e.g. Alzheimer's Disease), proliferative vitreoretinopathy (PVR) and arthritis. There is therefore also a need for medicaments which may be used for the treatment of such conditions by regulating (i.e. preventing, inhibiting or reversing) fibrosis / scarring in these fibrotic disorders.

There are however circumstances where the rate of healing is of primary importance and scar formation or fibrosis is only of secondary consideration. For example, it is often desirable to increase the rate of healing in the case of acute wounds (such as penetrative injuries, burns, nerve damage or even wounds resulting from elective surgery), chronic wounds (such as diabetic, venous and decubitus ulceration) or for generally healing compromised individuals (for example the elderly). In these examples, the wounds can severely influence quality of life or even result in death and therefore the rate of healing often needs to be increased as much as is clinically possible. When the rate of wound healing is increased using many conventional therapies, there is often an associated increase in scar formation but this may be of secondary importance compared to the need to increase the rate of healing. However it will be appreciated that it is desirable to be able to increase that rate of wound healing and to also limit scar formation or fibrosis in these circumstances.

It is known that, following wounding, there is an inflammatory and immune response which is at least partly mediated by a plurality of agents (e.g. chemotactic agents and growth factors) released at the wound site.

Thus, for example, it has been shown that Interleukin-6 (IL-6) increases rapidly upon initial tissue trauma, peaking within the first 12 hours (Mateo *et al.* Am J Physiol 266(6, Part 2): R1840-R1844), probably mediated in part by IL-1α expression (Goretsky *et al.* J Trauma Injury Infection and Critical Care. 40(6):894/899). Neutrophils, macrophages, lymphocytes, epithelial cells, keratinocytes and endothelial cells all produce IL-6 following wounding. Platelets are not a source of IL-6, so local levels are mainly due to release from the immediate influx of neutrophils.

WO-A-93/21771 (University of Utah) discloses that dehydroepiondrosterone (DHEA) congeners (e.g. DHEA and DHEA-S) may be used for the reduction ("down-regulation") of abnormally elevated IL-6 levels in a patient to restore normal IL-6 levels and/or alleviate one or more symptoms of a pathological condition associated

with elevated levels of IL-6. The DHEA may be used for example for the reduction of elevated IL-6 levels caused by trauma or an autoimmune disease.

It is also proposed in WO-A-93/21771 that DHEA may be used for the treatment of individuals who have abnormally elevated IL-6 levels and who are non responsive to growth factors such as PDGF, TGF-β and insulin. Such individuals may be suffering from disorders such as inhibition of wound healing, osteoporosis and diabetes respectively. This suggests that decreasing the level of IL-6 would (in persons who are non-responsive to growth factors) accelerate wound healing and increase fibrosis in scarring since both TGFβ (especially TGFβ1 which is the particular TGFβ which would be understood when TGFβ is referred to generically) and PDGF are pro-scarring, i.e. pro-fibrotic, growth factors.

WO-A-95/00103 discloses that antisense oligonucleotides to fibrogenic cytokines may be used to prevent or reduce fibrosis and/or scarring. More specifically WO-A-95/00103 speculates that an antisense oligonucleotide to IL-6 may have efficacy for preventing or reducing scar formation or fibrosis. However antisense oligonucleotides to IL-6 are not very versatile for use during wound healing and/or fibrosis because they cannot readily cross cell membranes to enter the cell nucleus (where they prevent IL-6 expression). At best these oligonucleotides are only able to enter a cell nucleus at the time of wounding when cells in the wound vicinity become transiently permeable such that the oligonulceotide may cross cellular membranes for entering into the cell nucleus. This means that for optimum effect the oligonucleotides are required to be delivered to the tissue before wounding so that they can be taken up into cells at the time of wounding. They are therefore most suitable for use in wounds incurred by elective surgery when the oligonucleotide may be administered in advance of wounding.

We have however now surprisingly found, and this forms the basis of the present invention, that agents which inhibit IL-6 receptor mediated activity are particularly effective for preventing or reducing fibrosis and/or scarring.

According to a first aspect of the present invention there is provided the use of an agent that inhibits Interleukin-6 receptor mediated activity for the manufacture of a medicament for preventing or reducing fibrosis and/or scarring.

According to a second aspect of the present invention there is provided a method of treating a patient to prevent or reduce fibrosis and/or scarring comprising administering to the patient a therapeutically effective amount of an agent that inhibits Interleukin-6 receptor mediated activity.

According to a third aspect of the present invention there is provided a composition for preventing or reducing fibrosis and/or scarring which comprises a therapeutically effective amount of an agent that inhibits Interleukin-6 receptor mediated activity and a pharmaceutically acceptable vehicle.

By "inhibits Interleukin-6 receptor mediated activity" we mean that the agent inhibits physiological effects mediated by Interleukin-6 present at the site of wounding, fibrosis or scarring. The inhibition of these physiological effects may be achieved by use of, for example, an agent which prevents or limits the interaction of IL-6 (present at the site of wounding, fibrosis or scarring) with its receptor, one which prevents activation of the IL-6 receptor by endogenous IL-6, one which reduces IL-6 receptor expression in cells at the site of wounding, fibrosis or scarring, one which increases IL-6 receptor degradation or one which prevents or inhibits post-receptor signal transduction mechanisms. Examples of suitable agents are given below.

Compared to the antisense oligonucleotides disclosed in WO-A-95/00103, the agents used according to the present invention are surprisingly effective for preventing

or reducing scar formation or fibrosis. A complex interacting cascade of cytokines (particularly the Interleukins) is thought to lead to the development of scars and/or fibrosis and it would be thought that the prevention of the expression of IL-6 (e.g. by the oligonucleotides disclosed by WO-A-95/00103) would be necessary to prevent the formation of this cascade and thereby prevent or reduce scarring and/or fibrosis. Agents which effect IL-6 receptor mediated activity (without modulating IL-6 expression) would have previously been expected to be less effective for preventing or reducing scarring and/or fibrosis because IL-6 would still be present at the wound site and would be able to recruit further cytokines into the cascade which leads to fibrosis and scarring. However we have found that the agents used according to the present invention are effective for preventing or reducing fibrosis and scarring despite the fact that IL-6 may be present in the wound or at the site of fibrosis.

Furthermore, agents which inhibit IL-6 receptor mediated activity according to the invention are not hindered by the permeability problems faced by antisense oligonucleotides which need to enter the cell nucleus. This is because many of the agents exert their effect by acting extracellularly (e.g. by combining with the IL-6 receptor which is a cell surface receptor).

Agents which inhibit Interleukin-6 receptor mediated activity are useful in situations or conditions where scarring needs to be prevented or reduced such as:

(i) where scars of the skin may be excessive and/or detrimental to tissue function and particularly when scar contracture occurs or may occur (for instance skin burns and wounds which impair flexibility of a joint and particularly scarring in children);

(ii) scarring to the skin when cosmetic considerations are important;

(iii) when hypertrophic or keloid scars (particularly in Africo-Caribbean and Mongoloid races) may occur which can cause functional and cosmetic impairment;

(iv) scarring resulting from skin grafts in both donor sites and from the application of artificial skin;

(v) scarring within the central nervous system (e.g. following neuro-surgery or penetrating injuries of the brain), for example glial scarring can prevent reconnection of severed neurons;

(vi) scarring in the eye and particularly of the cornea (scarring can result in abnormal opacity and lead to problems with vision or even blindness), in the retina (scarring can cause buckling or retinal detachment and consequently blindness) and scarring following wound healing in operations to relieve pressure in glaucoma (e.g. glaucoma filtration surgery) which can result in the failure of the surgery whereby the aqueous humour fails to drain and hence the glaucoma returns;

(vii) scarring in the heart (e.g. following surgery or myocardial infarction) which can give rise to abnormal cardiac function;

(viii) scarring of the gut such as may occur following operations involving the abdomen or pelvis that result in adhesion between viscera (adhesions between elements of the gut and the body wall can form and cause twisting in the bowel loop leading to ischaemia, gangrene and the necessity for emergency treatment -untreated they may even be fatal); likewise, trauma or incisions to the guts can lead to scarring and scar contracture or strictures which cause occlusion of the lumen of the guts which again can be life threatening;

(ix) scarring in the pelvis in the region of the fallopian tubes which can lead to infertility;

(x) scarring following injury to muscles which can result in abnormal contraction and hence poor muscular function;

(xi) scarring or fibrosis following injury to tendons and ligaments which can result in serious loss of function.

Inhibitors of IL-6 activity are also useful for treating fibrotic disorders such as cirrhosis of the liver, liver fibrosis, glomerulonephritis, pulmonary fibrosis, scleroderma, myocardial hibernation, fibrosis following myocardial infarction, central nervous system fibrosis following a stroke or neuro-degenerative disorders (e.g. Alzheimer's Disease), proliferative vitreoretinopathy (PVR) and arthritis.

A preferred use for inhibitors of IL-6 receptor mediated activity is in the prevention of inappropriate scar formation following a dermal wound.

It is an advantage of the present invention that the agents improve scar quality without any significant detriment to the rate of wound healing. Thus the agents may be used in circumstances where the rate of healing is of primary importance. For example, the agents may be used in the treatment of acute wounds (such as penetrative injuries, burns, nerve damage or even wounds resulting from elective surgery), chronic wounds (such as diabetic, venous and decubitus ulceration), for generally healing compromised individuals (for example the elderly) or for any other circumstance where the rate of healing needs to be increased as much as is clinically possible and an anti-scarring or anti-fibrotic agent would normally be contra-indicated. Thus for example an agent which inhibits IL-6 receptor mediated activity may be used with an agent which increases the rate of wound healing to also improve scar quality.

The agents are suited for use in subjects (human or animal) in which the amount of IL-6 associated with the wound is within the normal range associated with a healing wound.

Suitable inhibitors of IL-6 activity and thereby preferred compounds for use according to the invention include IL-6 Receptor antagonists (compounds which inhibit receptor activation by IL-6); compounds that disrupt signalling mediated by the activated IL-6 receptor (e.g. inhibitors of second messenger production, kinase inhibitors); enzymes that specifically degrade IL-6 (thus preventing IL-6 combining with its receptor), neutralising antibodies to IL-6 or its receptor, agents which increase IL-6 receptor degradation or sequestration from the cell surface, oligonucleotide aptmers which bind to and neutralise IL-6 or its receptor and molecules which bind to IL-6 and increase its clearance from a wound site.

Preferred agents are neutralising antibodies for IL-6 which prevent IL-6 from associating with its receptor. Such antibodies may be high affinity antibodies used at a high concentration because low affinity/ low concentrations of neutralising antibody are known to act as carrier protective agents and so potentiate the activity of IL-6 (Heremans et al. Eur. J. Immunol. 22 p2395-2401, 1992)). Examples of these preferred neutralising antibodies include the anti-human IL-6 antibody designated AF 206 NA and the anti-mouse IL-6 antibody designated AF 406 NA (both available from R & D Systems Inc., Minneapolis , USA). It will be appreciated that these antibodies are most suitable for inhibiting or preventing scarring and/or fibrosis in humans and mice respectively and that species specific antibodies may be easily developed for use in other animals.

Another preferred type of neutralising antibody is one raised against the IL-6 receptor. Such antibodies prevent IL-6 from activating the receptor. An example of this type of antibody is the anti-human antibody AF 227 NA (also available from R & D Systems Inc., Minneapolis , USA). Another example of such an antibody is a anti-GP130 antibody (GP130 being the second subunit of the IL-6 Receptor which also functions as a receptor subunit for LIF, OSM, CNTF and IL-11).

Other preferred compounds for use according to the invention are IL-6 Receptor antagonists and agents which prevent or inhibit post-receptor signal transduction mechanisms.

The agent for inhibiting the activity of IL-6 used according to the invention may be a protein or derivatives thereof (e.g analogues of IL-6 that act as IL-6 receptor antagonists). Alternatively agents that are non-proteins, but which nevertheless are pharmacologically active as inhibitors of IL-6 activity, may also be used.

The agent which is, or which is to be, administered to the patient may be one which *per se* inhibits Interleukin-6 (IL-6) receptor mediated activity or one which generates, or is converted to, an "active" agent within the body of the patient which in turn inhibits IL-6 receptor activity.

The medicaments and compositions of the invention may take a number of different forms depending, in particular on the manner in which the inhibitor of IL-6 activity is to be used. Thus, for example, the medicament or composition may be in the form of a liquid, ointment, cream, gel, hydrogel, powder or aerosol. It will be appreciated that the vehicle for the inhibitor of IL-6 activity should be one which is well tolerated by the patient and allows release of the active agent to the wound. Such a vehicle is preferably biodegradeable, biocompatable, bioresorbable, non-inflammatory, non-immunogenic. For instance,. the vehicle may comprise solutions or polymers of hyaluronic acid.

A medicament comprising an agent which inhibits IL-6 activity may be used in a number of ways. Thus, for example, a medicament in accordance with the first aspect of the invention may be applied in and/or around a wound of a patient to provide the desired reduction in fibrosis or scarring. Such a medicament may be provided on a sterile dressing or patch which may be used to cover or even pack a wound to be treated.

If the medicament is to be applied directly to an actual wound, trauma or injury, then the pharmaceutically acceptable vehicle will be one which does not cause an inflammatory response or is toxic to the tissue.

Topical application is a preferred means of administering agents which inhibit IL-6 activity to a subject (person or animal) in need of treatment.

It is possible to use medicaments in accordance with the invention as a prophylactic. For instance, prior to surgery (particularly elective surgery) it may be desirable to provide an agents which inhibits IL-6 activity for regulation of healing of the subsequently formed surgical wound so as to reduce scarring and/or treat a fibrotic disorder. In this case the vehicle of the composition will need to be one capable of delivering the agent to the target tissue. For example the vehicle may need to be suitable for carrying the agent across the keratinous layer of the skin. Examples of suitable vehicles for this purpose include dimethyl sulphoxide and acetic acid.

A further important application of agents which inhibit IL-6 activity relates to wound healing in the eye. For example, medicaments may be used to reduce or control scarring resulting from surgical operations on the eye, e.g. laser surgery on the cornea. In this case, the medicament of the invention may be in the form of eye drops. Alternatively the composition of the invention may be an injectable solution.

Medicaments in accordance with the invention may be used in a range of internal wound healing applications (in addition to that mentioned above for the eye). Thus for example, the composition may be formulated for inhalation (e.g. as an aerosol or spray) for use in wound healing of the lungs or for the prevention or treatment of fibrosis, adhesions and strictures in the lung. The medicaments may also be applied to internal organs of the abdomen and pelvis to prevent adhesions or strictures following surgery or arising from inflammatory conditions.

The medicaments and compositions may be administered by release from an implantable device (e.g. a biopolymer implant) Such release may be biological or externally triggered (e.g. by ultrasound).

It will be appreciated that the amount of an agent which inhibits IL-6 activity to be incorporated in a medicament and/or the amount of the agent to be applied to a wound site depends on a number of factors such as the biological activity and

bioavailability of the agent, which in turn depends on the mode of administration and the its physicochemical properties. Other factors include:

A) The half-life of the agent in the subject being treated.

B) The specific condition to be treated.

C) The age of the subject.

D) The sex of the subject

The frequency of administration will also be influenced by the above mentioned factors and particularly the half-life of the agent within the subject being treated.

Generally when used to treat existing wounds or fibrotic disorders it is usually advantageous to administer a medicament containing an agent which inhibits IL-6 receptor mediated activity as soon as the wound has occurred or the disorder has been diagnosed.

The medicaments are preferably applied within 48 hours post wounding and more preferably within 12 hours post wounding to realise the best anti-scarring results. It is particularly preferred that the medicament is applied at the time of, or shortly after (e.g. within 3 hours) post-wounding. However, fibrosis and scarring can develop over days or even weeks. Therefore the subject being treated may well benefit by commencing administration of an agent which inhibits IL-6 activity even if the medicament is first administered days or even weeks after the original wound occurred or the disorder developed (or was diagnosed).

Therapy with the agent should continue until the wound has healed to a clinicians satisfaction or, in the case of a fibrotic disorder, the risk or cause of abnormal fibrous tissue formation has been removed.

When used as a prophylactic (e.g. before surgery or when there is a risk of developing a fibrotic disorder) the medicament should be administered as soon as the risk of undesirable fibrosis has been recognised (as may be the case in people prone to develop keloid or hypertrophic scarring). For instance, a cream or ointment containing an IL-6 antagonist may be applied to a site on the skin of a subject where elective surgery is to be performed and reduced scarring of the subsequent wound is subsequently desired (e.g. surgery of the face or other cosmetically sensitive areas). In this case, the IL-6 antagonist may be applied during the preoperative preparation of the subject or it may even be desirable to apply the agent in the hours or days preceding the surgery (depending upon the health status and age of subject as well as the size of the wound to be formed).

Frequency of administration will depend upon the biological half-life of the agent used. Typically a cream or ointment containing a compound should be administered to a target tissue such that the concentration of the agent at the wound site or tissue affected by a fibrotic disorder is maintained at a level suitable for having a therapeutic effect. This may require administration daily or even several times daily.

Known procedures, such as those conventionally employed by the pharmaceutical industry (e.g. *in vivo* experimentation, clinical trials etc), may be used to establish specific formulations of medicaments and compositions and precise therapeutic regimes (such as daily doses of the compounds and the frequency of administration).

By way of example only a neutralising antibody may be administered to a wound as soon as the wound occurs. Such antibodies may be given twice daily and may be used for up to 5 days post-wounding.

Generally, medicaments and compositions in accordance with the invention will contain 0.001% to 10% by weight of the agent which inhibits IL-6 activity, preferably 0.0025% to 5% and more preferably 0.05% to 2.5%.

A suitable daily dose of an agent which inhibits IL-6 activity will depend upon the factors discussed above as well as upon the size of the wound, or extent of the fibrotic disorder, to be treated. Typically the amount of a compound required for the treatment of wounds or fibrotic disorders will be within the range of 1ng to 100g of the active compound/ 24 hours depending upon the size of the wound or extent of fibrosis amongst several other factors. By way of example, we have found that 0.3μg - 3mg/ 24 hour is a suitable quantity of a neutralising antibody for IL-6 (e.g. AF 206 NA for human wounds) to apply per linear centimetre of an incisional wound of the skin and more preferably 3μg - 300μg/ 24 hours.

A preferred means of using protein or peptide agents which inhibit Interleukin-6 receptor mediated activity is to deliver the agent to the wound, or other target tissue, by means of gene therapy. For instance, gene therapy could be used to increase expression of peptide antagonists of IL-6 receptors. Alternatively gene therapy may be used to express a neutralising antibody against IL-6 or its receptor. Therefore according to a fourth aspect of the present invention there is provided a delivery system for use in a gene therapy technique, said delivery system comprising a DNA molecule encoding for a protein which directly or indirectly prevents or reduces fibrosis and/or scarring by inhibiting Interleukin 6 receptor mediated activity, said DNA molecule being capable of being transcribed to lead to the expression of said protein.

According to a fifth aspect of the present invention there is provided the use of a delivery system as defined in the preceding paragraph for use in the manufacture of a medicament for preventing or reducing fibrosis and/or scarring.

According to a sixth aspect of the present invention there is provided a method of preventing or reducing fibrosis and/or scarring comprising administering to a patient in need of treatment a therapeutically effective amount of a delivery system as defined for the fourth aspect of the invention.

The delivery systems are highly suitable for achieving sustained levels of an active agent at a wound site or site of fibrosis over a longer period of time than is possible for most conventional delivery systems. Protein may be continuously expressed from cells at the wound site or site of fibrosis that have been transformed with the DNA molecule of the fourth aspect of the invention. Therefore, even if the protein has a very short half-life as an agent *in vivo*, therapeutically effective amounts of the protein may be continuously expressed from the treated tissue.

Furthermore, the delivery system of the invention may be used to provide the DNA molecule (and thereby the protein which is an active therapeutic agent) without the need to use conventional pharmaceutical vehicles such as those required in ointments or creams that are contacted with the wound. This is particularly beneficial as it can often be difficult to provide a satisfactory vehicle for a compound for use in wound healing (which are required to be non-inflammatory, biocompatible, bioresorbable and must not degrade or inactivate the active agent (in storage or in use)).

The delivery system is such that the DNA molecule is capable of being expressed (when the delivery system is administered to a patient) to produce a protein which directly or indirectly has activity for preventing or reducing fibrosis and/or scarring. By "directly" we mean that the product of gene expression *per se* has the required activity for regulating fibrosis or scarring. By "indirectly" we mean that the product of gene expression undergoes or mediates (e.g. as an enzyme) at least one further reaction to provide an agent effective for regulating fibrosis or scarring.

The DNA molecule may be contained within a suitable vector to form a recombinant vector. The vector may for example be a plasmid, cosmid or phage. Such recombinant vectors are highly useful in the delivery systems of the invention for transforming cells with the DNA molecule.

Recombinant vectors may also include other functional elements. For instance, recombinant vectors can be designed such that the vector will autonomously replicate in the nucleus of the cell. In this case, elements which induce DNA replication may be required in the recombinant vector. Alternatively the recombinant vector may be designed such that the vector and recombinant DNA molecule integrates into the genome of a cell. In this case DNA sequences which favour targeted integration (e.g. by homologous recombination) are desirable. Recombinant vectors may also have DNA coding for genes that may be used as selectable markers in the cloning process.

The recombinant vector may also further comprise a promoter or regulator to control expression of the gene as required.

The DNA molecule may (but not necessarily) be one which becomes incorporated in the DNA of cells of the subject being treated. Undifferentiated cells may be stably transformed leading to the production of genetically modified daughter cells (in which case regulation of expression in the subject may be required e.g. with specific transcription factors or gene activators). Alternatively, the delivery system may be designed to favour unstable or transient transformation of differentiated cells in the subject being treated. When this is the case, regulation of expression may be less important because expression of the DNA molecule will stop when the transformed cells die or stop expressing the protein (ideally when the fibrosis or scarring has been treated or prevented).

The delivery system may provide the DNA molecule to the subject without it being incorporated in a vector. For instance, the DNA molecule may be incorporated

within a liposome or virus particle. Alternatively the "naked" DNA molecule may be inserted into a subject's cells by a suitable means e.g. direct endocytotic uptake.

The DNA molecule may be transferred to the cells of a subject to be treated by transfection, infection, microinjection, cell fusion, protoplast fusion or ballistic bombardment. For example, transfer may be by ballistic transfection with coated gold particles, liposomes containing the DNA molecule, viral vectors (e.g. adenovirus) and means of providing direct DNA uptake (e.g. endocytosis) by application of plasmid DNA directly to the wounded area topically or by injection.

Whilst the above considerations mainly apply to wounds and fibrotic disorders of man it will be appreciated that wound healing, scarring and fibrosis can also be problematic in other animals (especially veterinary and domestic animals such as cattle, horses, dogs, cats etc). For instance tendon and ligament damage leading to scarring or fibrosis are a major reason for having to put down horses. The agents, medicaments, compositions and delivery systems discussed above are suitable for use in the healing of such animals.

The present invention will further be described in the following non-limiting Examples.

EXAMPLE 1

The inventors examined the influence of IL-6 on scarring during wound healing by assessing wound healing in IL-6 "knockout mice" (i.e. mice genetically engineered so as not to express IL-6) and normal "wild type" control mice. IL-6 knockout mice generated according to the experimental procedures disclosed by Kopf et al (Nature, 368 (6469) 239-342, 1994) were used in the present Example.

1.1. Methods

1.1.1 Treatments

A total of 55 mice were used (29 knockout and 26 wild type). The mice were anaesthetised using IP injection of Avertine, the dorsal surface shaved and two 1cm incisions made through the skin down to and including the panniculus carnosus muscle at specific anatomical positions. The wounds were left unsutured and the animals returned to individual cages. One group of animals was killed and the wounds harvested after 1 day(d), 3d, 5d, 7d, 14 and 70d post-wounding. Half the wound was fixed in formal saline and half embedded in OCT medium and frozen over liquid nitrogen. Photographic records were kept of the wounds at each time point, to enable comparison of microscopic and macroscopic results.

1.1.2 Histological Assessment.

Haematoxylin and Eosin (H&E) and Masson's Trichrome stains were used to determine the cellularity and connective tissue content of the wounds respectively (using 7μm wax sections of the harvested wounds).

1.1.3 Scoring of Scars 70 Days Post Wounding.

The histology slides were scored using a Visual Analogue Scale (VAS) consisting of a 10cm line where 0 represents normal skin and 10 a hypertrophic scar/keloid, and separately using a 0-5 rank scale, where 0 represents normal skin and 5 a hypertrophic scar/keloid and 3 is the score for a control scar.

1.2. Results

The IL-6 knockout mice had a decreased inflammatory reaction at 1 and 3 days. The wounds of the knockout mice contained fewer inflammatory cells at 1 and 3 days than control wounds. At 5 days the wounds of the IL-6 knockout mice had re-epithelialised and contained some inflammatory cells along with numerous fibroblasts. There was a greater amount of new collagen deposited within the knockout mice wounds compared to controls. At 7 and 14 days the wounds of the knockout mice contained a lot of newly synthesised collagen and numerous fibroblasts, suggesting fibroblast proliferation and migration at earlier stages had been increased. The scars at 40 and 70 days post-wounding contained more mature collagen fibres which were arranged in a more normal random orientation rather than aligned as observed in control wild type scars. Macroscopically, the scars were much finer and hardly visible in the knockout mice, compared to the control scars.

Scores for 70 Day Scars in Wild Type (WT) and Interleukin-6 Knockout (KO) Animals.

Slide	**Score (VAS)**	**Score (0-5)**
WT1	7.15	4
WT2	3.7	2
WT3	7.2	3
WT4	8.25	4
	Average=6.575	Average=3.25
KO1	5.1	3
KO2	1.4	1
KO3	3.45	2
KO4	2.1	1
	Average=3.01	Average=1.75

1.3. Summary

These results demonstrate that IL-6 deficient mice have superior wound healing such that there is decreased scar formation compared to control animals and show that agents which inhibit Interleukin-6 receptor mediated activity may be used to prevent or reduce scar formation. This is surprising because it is the opposite of the effect IL-6 would be expected to have in the light of the disclosure in WO-A-93/21771 (which teaches that a reduction in IL-6 activity causes an increase in the rate of wound healing and therefore suggests that inhibitors of IL-6 activity would be expected to increase scar formation).

The inventors further established that in IL-6 knockout mice there is not only significantly reduced scar formation following wounding but there is also only a slight retardation in the rate of wound healing.

EXAMPLE 2

A second set of experiments was performed using IL-6 knockout mice in which excisional wounds were made instead of incisional wounds. The same methods as used in Example 1 were employed except as indicated below.

A total of 11 mice were used (5 knockout and 6 wild type). Excisional wounds were created on the shaved dorsal surfaces of the mice. Two wounds of 5mm x 5mm were made at a distance of 2cm from the base of the skull and 1cm out from the spine. The wounds were harvested at 7 and 70 days post-wounding only. Photographic records of the wounds at 40 days were kept.

At 7d post-wounding, the wounds on the wild type mice were still very wide, had eschars and were not fully re-epithelialised. There was not much new collagen and few fibroblasts in the wounds. The excisional wounds from the IL-6 null mice

were quite cellular, some had re-epithelialised and most still had eschars, similar to the wild type wounds. At 70 days post-wounding, there were large visible scars on the backs of the control mice. The IL-6 knockout mice had mostly small, narrower scars which was also evident histologically. In some scars the collagen fibres were thicker, and not as aligned as in the scars from control mice. Macroscopically, it appeared that the square excisions had contracted in a linear manner in the IL-6 null mice, as the scars were narrow and linear at 70 days post-wounding.

These results further demonstrate that agents which inhibit Interleukin-6 receptor mediated activity may be used to prevent or reduce scar formation.

EXAMPLE 3

Experiments was performed using wild type mice (which express IL-6) to examine the effect of a neutralising antibody for IL-6 (an agent used according to the invention) on scar formation following incisional wounding. The same methods as used in Example 1 were employed except as indicated below.

3.1 Methods

2 incisional wounds were made on the back of a mouse as described in method 1.1.1. At the time of wounding, the first wound received 100µl (50µl down each wound margin) of 100µg/ml of AF 406 NA (an anti-mouse IL-6 antibody from R & D Systems Inc., Minneapolis, USA) and the second wound acted as a control and received 100µl of vehicle for the antibody only (Phosphate Buffered Saline - PBS). The first and second wounds received a further 100µl of antibody and vehicle respectively at both 12 hours and 24 hours post-wounding.

7 days post-wounding the wounds were harvested and histological assessment carried out as described in method 1.1.2.

3.2 Results

We found that after 7 days there was less fibronectin, inflammatory cells and blood vessels in the wound area of the IL-6 neutralising antibody treated wound compared to the control wound (PBS treated). This indicated that there is less fibrosis (and that scar formation is reduced) in the antibody treated wound than was observed in control wounds. This illustrates that agents used according to the invention are effective for reducing fibrosis and scarring.

CLAIMS

1. The use of an agent that inhibits Interleukin-6 receptor mediated activity for the manufacture of a medicament for preventing or reducing fibrosis and/or scarring.

2. The use according to claim 1 wherein the agent is one which prevents or limits the interaction of IL-6 with its receptor.

3. The use according to claim 2 wherein the agent is an anti-IL-6 neutralising antibody.

4. The use according to claim 2 wherein the agent is an anti-IL-6 Receptor neutralising antibody.

5. The use according to claim 2 wherein the agent is an IL-6 receptor antagonist.

6. The use according to claim 1 wherein the agent is one which prevents or inhibits post-receptor signal transduction mechanisms.

7. The use according to any one of claims 1 to 6 for the healing of wounds.

8. The use according to claim 7 for the healing of wounds where excessive scarring may be detrimental to tissue function.

9. The use according to claim 7 or 8 for wounds of the eye, nervous tissue, skin, internal organs, burns or acute wounds.

10. The use according to claim 7 or 8 for skin wounds to the face.

11. The use according to claim 7 or 8 for preventing or reducing the formation of hypertrophic scars or keloids.

12. The use according to any one of claims 1 to 6 for preventing or reducing connective tissue formation in fibrotic diseases.

13. The use according to claim 12, wherein the fibrotic disease is one of glomerulonephritis, liver cirrhosis, pulmonary fibrosis and scleroderma.

14. The use according to any one of claims 1 to 13 wherein the agent is provided in a composition which is a liquid, ointment, cream, gel, hydrogel, powder, aerosol or implantable device.

15. A method of treating a patient to prevent or reduce fibrosis and/or scarring comprising administering to the patient a therapeutically effective amount of an agent that inhibits Interleukin-6 receptor mediated activity.

16. A composition for preventing or reducing fibrosis and/or scarring which comprises a therapeutically effective amount of an agent that inhibits Interleukin-6 receptor mediated activity and a pharmaceutically acceptable vehicle.

17. A DNA molecule encoding for a protein which directly or indirectly prevents or reduces fibrosis and/or scarring by inhibiting Interleukin 6 receptor mediated activity, said DNA molecule being capable of being transcribed to lead to the expression of said protein.

18. The use of a delivery system according to claim 17 for use in the manufacture of a medicament for preventing or reducing fibrosis and/or scarring.

19. A method of preventing or reducing fibrosis and/or scarring comprising administering to a patient in need of treatment a therapeutically effective amount of a delivery system as defined in claim 17.

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(54) Title: TREATMENT OF FIBROSIS BY ANTAGONISM OF IL-13 AND IL-13 RECEPTOR CHAINS

(57) Abstract: Methods are provided for treating or inhibiting the formation of tissue fibrosis using IL-13 antagonists, including without limitation soluble forms of the IL-13 receptor.

TREATMENT OF FIBROSIS BY ANTAGONISM OF IL-13 AND IL-13 RECEPTOR CHAINS

This application is a continuation-in-part of application Ser. No. 09/301,808, filed April 28, 1999; which is a continuation-in-part of application Ser. No. 08/841,751, filed April 30, 1997; which is a divisional application of application Ser. No. 08/609,572, filed March 1, 1996 and issued as U.S. Patent No. 5,710,023 on January 20, 1998; all of which are incorporated by reference herein.

Field of the Invention

The present invention relates to the treatment and inhibition of fibrosis by antagonism of the interaction of IL-13 with its receptor and receptor components.

Background of the Invention

A variety of regulatory molecules, known as cytokines, have been identified including interleukin-13 (IL-13). Various protein forms of IL-13 and DNA encoding various forms of IL-13 activity are described in McKenzie et al., Proc. Natl. Acad. Sci. USA 90:3735 (1993); Minty et al., Nature 362:248 (1993); and Aversa et al., WO94/04680. Thus, the term "IL-13" includes proteins having the sequence and/or biological activity described in these documents, whether produced by recombinant genetic engineering techniques; purified from cell sources producing the factor naturally or upon induction with other factors; or synthesized by chemical techniques; or a combination of the foregoing.

IL-13 is a cytokine that has been implicated in production of several biological activities including: induction of IgG4 and IgE switching, including in human immature B cells (Punnonen et al., J. Immunol. 152:1094 (1994)); induction of germ line IgE heavy chain (ϵ) transcription and CD23 expression in normal human B cells (Punnonen et al., Proc. Natl. Acad. Sci. USA 90:3730 (1993)); and induction of B cell proliferation in the presence of CD40L or anti-CD40 mAb (Cocks et al., Int. Immunol. 5:657 (1993)). Although many activities of IL-13 are similar to those of IL-4, in contrast to IL-4, IL-13

does not have growth promoting effects on activated T cells or T cell clones (Zurawski et al., EMBO J. 12:2663 (1993)).

Like most cytokines, IL-13 exhibits certain biological activities by interacting with an IL-13 receptor ("IL-13R") on the surface of target cells. IL-13R and the IL-4 receptor ("IL-4R") sharing a common component, which is required for receptor activation; however, IL-13 does not bind to cells transfected with the 130 kD IL-4R (Zurawski et al., *supra*). Thus, the IL-13R must contain at least one other ligand binding chain. Cytokine receptors are commonly composed or two or three chains. The cloning of one ligand binding chain for IL-13 has been recently reported (Hilton et al., Proc. Natl. Acad. Sci. 93:497-501).

It would be desirable to identify and clone the sequence for any other IL-13 binding chain of IL-13R so that IL-13R proteins can be produced for various reasons, including production of therapeutics and screening for inhibitors of IL-13 binding to the receptor and receptor signaling.

Summary of the Invention

In accordance with the present invention, polynucleotides encoding the IL-13 binding chains of the interleukin-13 receptor are disclosed, including without limitation those from the murine and human receptors. In certain embodiments, the invention provides an isolated polynucleotide comprising a nucleotide sequence selected from the group consisting of:

(a) the nucleotide sequence of SEQ ID NO:1 from nucleotide 256 to nucleotide 1404;

(b) the nucleotide sequence of SEQ ID NO:3 from nucleotide 103 to nucleotide 1242;

(c) a nucleotide sequence varying from the sequence of the nucleotide sequence specified in (a) or (b) as a result of degeneracy of the genetic code;

(d) a nucleotide sequence capable of hybridizing under stringent conditions to the nucleotide specified in (a) or (b);

(e) a nucleotide sequence encoding a species homologue of the sequence specified in (a) or (b); and

(f) an allelic variant of the nucleotide sequence specified in (a) or (b).

Preferably, the nucleotide sequence encodes a protein having a biological activity of the human IL-13 receptor. The nucleotide sequence may be operably linked to an expression control sequence. In preferred embodiments, the polynucleotide comprises the nucleotide sequence of SEQ ID NO:1 from nucleotide 256 to nucleotide 1404; the nucleotide sequence of SEQ ID NO:1 from nucleotide 319 to nucleotide 1257; the nucleotide sequence of SEQ ID NO:1 from nucleotide 1324 to nucleotide 1404; the nucleotide sequence of SEQ ID NO:3 from nucleotide 103 to nucleotide 1242; the nucleotide sequence of SEQ ID NO:3 from nucleotide 178 to nucleotide 1125; or the nucleotide sequence of SEQ ID NO:3 from nucleotide 1189 to nucleotide 1242.

The invention also provides isolated polynucleotides comprising a nucleotide sequence encoding a peptide or protein comprising an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence of SEQ ID NO:2;

(b) the amino acid sequence of SEQ ID NO:2 from amino acids 22 to 334;

(c) the amino acid sequence of SEQ ID NO:2 from amino acids 357 to 383;

(d) the amino acid sequence of SEQ ID NO:4;

(e) the amino acid sequence of SEQ ID NO:4 from amino acids 26 to 341;

(f) the amino acid sequence of SEQ ID NO:4 from amino acids 363 to 380; and

(g) fragments of (a)-(f) having a biological activity of the IL-13 receptor binding chain. Other preferred embodiments encode the amino acid sequence of SEQ ID NO:2 from amino acids 1 to 331 and the amino acid sequence of SEQ ID NO:2 from amino acids 26 to 331.

Host cells, preferably mammalian cells, transformed with the polynucleotides are also provided.

In other embodiments, the invention provides a process for producing a IL-13bc protein. The process comprises:

(a) growing a culture of the host cell of the present invention in a suitable culture medium; and

(b) purifying the human IL-13bc protein from the culture.

Proteins produced according to these methods are also provided.

The present invention also provides for an isolated IL-13bc protein comprising an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence of SEQ ID NO:2;

(b) the amino acid sequence of SEQ ID NO:2 from amino acids 22 to 334;

(c) the amino acid sequence of SEQ ID NO:2 from amino acids 357 to 383;

(d) the amino acid sequence of SEQ ID NO:4;

(e) the amino acid sequence of SEQ ID NO:4 from amino acids 26 to 341;

(f) the amino acid sequence of SEQ ID NO:4 from amino acids 363 to 380; and

(g) fragments of (a)-(f) having a biological activity of the IL-13 receptor binding chain

Preferably the protein comprises the amino acid sequence of SEQ ID NO:2; the sequence from amino acid 22 to 334 of SEQ ID NO:2; the sequence of SEQ ID NO:4; or the sequence from amino acid 26 to 341 of SEQ ID NO:4. In other preferred embodiments, the specified amino acid sequence is part of a fusion protein (with an additional amino acid sequence not derived from IL-13bc). Preferred fusion proteins comprise an antibody fragment, such as an Fc fragment. Particularly preferred embodiments comprise the amino acid sequence of SEQ ID NO:2 from amino acids 1 to 331 and the amino acid sequence of SEQ ID NO:2 from amino acids 26 to 331.

Pharmaceutical compositions comprising a protein of the present invention and a pharmaceutically acceptable carrier are also provided.

The present invention further provides for compositions comprising an antibody which specifically reacts with a protein of the present invention.

Methods of identifying an inhibitor of IL-13 binding to the IL-13bc or IL-13 receptor are also provided. These methods comprise:

(a) combining an IL-13bc protein or a fragment thereof with IL-13 or a fragment thereof, said combination forming a first binding mixture;

(b) measuring the amount of binding between the protein and the IL-13 or fragment in the first binding mixture;

(c) combining a compound with the protein and the IL-13 or fragment to form a second binding mixture;

(d) measuring the amount of binding in the second binding mixture; and

(e) comparing the amount of binding in the first binding mixture with the amount of binding in the second binding mixture;

wherein the compound is capable of inhibiting IL-13 binding to the IL-13bc protein or IL-13 receptor when a decrease in the amount of binding of the second binding mixture occurs. Inhibitors of IL-13R identified by these methods and pharmaceutical compositions containing them are also provided.

Methods of inhibiting binding of IL-13 to the IL-13bc proteins or IL-13 receptor in a mammalian subject are also disclosed which comprise administering a therapeutically effective amount of a composition containing an IL-13bc protein, an IL-13bc or IL-13R inhibitor or an antibody to an IL-13bc protein.

Methods are also provided for potentiating IL-13 activity, which comprise combining a protein having IL-13 activity with a protein of the present invention and contacting such combination with a cell expressing at least one chain of IL-13R other than IL-13bc. Preferably, the contacting step is performed by administering a therapeutically effective amount of such combination to a mammalian subject.

Further methods are provided for treating an IL-13-related condition in a mammalian subject, said method comprising administering a therapeutically effective amount of a composition comprising an IL-13 antagonist and a pharmaceutically acceptable carrier. Other methods provide for a method of inhibiting the interaction of IL-13 with an IL-13bc protein in a mammalian subject comprising administering a therapeutically effective amount of a composition comprising an IL-13 antagonist and a pharmaceutically acceptable carrier. Preferably, the antagonist is selected from the group consisting of an IL-13bc protein, a soluble form of IL-13Rα1, an antibody to IL-13 or an IL-13-binding fragment thereof, an antibody to IL-13bc or an IL-13bc-binding fragment thereof, an antibody to IL-13Rα1 or an IL-13Rα1-binding fragment thereof, IL-13R-binding mutants of IL-4, a small molecule capable of inhibiting the interaction of IL-13 with IL-13bc and a small molecule capable of inhibiting the interaction of IL-13 with IL-13Rα1.

In yet other embodiments, the invention provides for a method of treating tissue fibrosis in a mammalian subject. The method comprises administering a therapeutically effective amount of a pharmaceutical composition comprising a protein and a pharmaceutically acceptable carrier, wherein the protein comprises an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence of SEQ ID NO:2;

(b) the amino acid sequence of SEQ ID NO:2 from amino acids 22 to 334;

(c) the amino acid sequence of SEQ ID NO:2 from amino acids 357 to 383;

(d) the amino acid sequence of SEQ ID NO:4;

(e) the amino acid sequence of SEQ ID NO:4 from amino acids 26 to 341;

(f) the amino acid sequence of SEQ ID NO:4 from amino acids 363 to 380; and

(g) fragments of (a)-(f) having a biological activity of the IL-13 receptor binding chain.

The invention also provides for a method of inhibiting formation of tissue fibrosis in a mammalian subject. The method comprises administering a therapeutically effective amount of a pharmaceutical composition comprising a protein and a pharmaceutically acceptable carrier, wherein the protein comprises an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence of SEQ ID NO:2;

(b) the amino acid sequence of SEQ ID NO:2 from amino acids 22 to 334;

(c) the amino acid sequence of SEQ ID NO:2 from amino acids 357 to 383;

(d) the amino acid sequence of SEQ ID NO:4;

(e) the amino acid sequence of SEQ ID NO:4 from amino acids 26 to 341;

(f) the amino acid sequence of SEQ ID NO:4 from amino acids 363 to 380; and

(g) fragments of (a)-(f) having a biological activity of the IL-13 receptor binding chain.

Other embodiments of the invention provide for a method of treating or inhibiting tissue fibrosis in a mammalian subject. The method comprises administering a therapeutically effective amount of a composition comprising (a) a molecule selected from the group consisting of an IL-13 antagonist and an IL-4 antagonist, and (b) a pharmaceutically acceptable carrier.

In practicing such methods of treating or inhibiting fibrosis, preferably the tissue fibrosis affects a tissue selected from the group consisting of liver, skin epidermis, skin endodermis, muscle, tendon, cartilage, cardiac tissue, pancreatic tissue, lung tissue, uterine tissue, neural tissue, testis, ovary, adrenal gland, artery, vein, colon, small intestine, biliary tract and gut; most preferably, liver tissue (including tissue infected with schistosoma). In certain embodiments, the fibrois results from the healing of a wound (including a surgical incision).

In practicing such methods of treating or inhibiting fibrosis using an antagonist, preferably such antagonist is selected from the group consisting of an IL-13bc protein, a soluble form of IL-13Rα1, an antibody to IL-13 or an IL-13-binding fragment thereof, an antibody to IL-13bc or an IL-13bc-binding fragment thereof, an antibody to IL-13Rα1 or an IL-13Rα1-binding fragment thereof, IL-13R-binding mutants of IL-4, a small molecule capable of inhibiting the interaction of IL-13 with IL-13bc and a small molecule capable of inhibiting the interaction of IL-13 with IL-13Rα1. In particularly preferred embodiments, the antagonist is an IL-13bc protein comprising an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence of SEQ ID NO:2;

(b) the amino acid sequence of SEQ ID NO:2 from amino acids 22 to 334;

(c) the amino acid sequence of SEQ ID NO:2 from amino acids 357 to 383;

(d) the amino acid sequence of SEQ ID NO:4;

(e) the amino acid sequence of SEQ ID NO:4 from amino acids 26 to 341;

(f) the amino acid sequence of SEQ ID NO:4 from amino acids 363 to 380; and

(g) fragments of (a)-(f) having a biological activity of the IL-13 receptor binding chain.

In other preferred methods of practicing such methods using an antagonist the antagonist is selected from the group consisting of a soluble form of IL-4R, an antibody to IL-4 or an IL-4-binding fragment thereof, an antibody to IL-4R or an IL-4R-binding fragment thereof, and a small molecule capable of inhibiting the interaction of IL-4 with IL-4R.

Brief Description of the Drawings

Fig. 1: The figure presents photographs of IL-13, IL-4, IL-11 and mock transfected COS cells after exposure to IL-13bc-Fc as described in Example 4 below.

Fig. 2: Characterization of the roles of IL-4 and IL-13 in schistosomiasis pathogenesis. C57BL/6 WT and IL-4-deficient (4KO) mice were infected with 25 cercariae of *Schistosoma mansoni* and then sacrificed at week 8 post-infection to evaluate the size of liver granulomas (panel A), tissue eosinophilia (panel B), and hepatic fibrosis (panel C). Separate groups of mice were treated with control-Fc or sIL-13Rα2-Fc as described in the Methods section. The data shown are measurements from individual mice and the lines

designate the means for each group. Statistical comparisons were made by Student's t-test (panels A and B) and by Analysis of Covariance (panel C). Significant comparisons and their p values are indicated in the figure. All data were reproduced in a second study.

Fig. 3: Liver collagen is reduced in sIL-13Rα2-Fc-treated /infected mice. Liver sections were prepared 8 weeks after challenge infection. Sections from control Fc-treated (panels A and B) and sIL-13Rα2-Fc-treated WT infected mice that contained nearly identical tissue egg burdens were stained with picrosirius red (panels A and C) and illuminated using polarized light to highlight the areas rich in collagen (panels B and D). Birefringent areas indicate positive collagen staining and the areas shown are representative for each liver (magnification, X 40). Liver sections from sIL-13Rα2-Fc-treated mice showed only very slight granuloma and portal tract-associated collagen, in comparison with control animals.

Fig. 4: The Th1/Th2-type cytokine profile is unaffected by sIL-13Rα2-Fc treatment. C57BL/6 WT and IL-4-deficient (4KO) mice were infected with 25 cercariae of *Schistosoma mansoni* and separate groups of mice were treated with control-Fc or sIL-13Rα2-Fc as described in the Methods section. Mesenteric lymph node cells were isolated from individual mice and single cell suspensions were prepared (3×10^6 cells/well in 24 well plates) and stimulated with medium alone (squares), SEA at 20 ug/ml (circles), or with SEA and 50 ug/ml of anti-CD4 mAb (triangles). All cytokines were assayed in culture supernatants by ELISA 72 hrs post-stimulation as described in the Methods section. The symbols represent values for individual mice and the bars indicate the means within each group.

Fig. 5: Th2-type cytokine mRNA expression is reduced in the livers of infected IL-4-deficient mice but unaffected by IL-13 blockade. C57BL/6 WT and IL-4-deficient (4KO) mice were infected with 25 cercariae of *Schistosoma mansoni* and separate groups of mice were treated with control-Fc or sIL-13Rα2-Fc as described in the Methods section. All animals were sacrificed on wk 8 postinfection and liver specimens were prepared for RT-PCR analysis as described in the Methods section. The data shown are the individual values of 9 to 10 animals per group and the bar indicates the average within each group. The * symbol indicates that the data are significantly different from the WT control-Fc group as determined by Student's t-test ($p<.05$). The average values from five uninfected

WT (black circle) and five uninfected IL-4-deficient mice (open circle) are shown on the Y-axis for each cytokine. All data were reproduced in a second study.

Fig. 6: Collagen I and Collagen III mRNA expression is reduced in the livers of infected sIL-13Rα2-Fc treated mice, but unaffected by IL-4-deficiency. C57BL/6 WT and IL-4-deficient (4KO) mice were infected with 25 cercariae of *Schistosoma mansoni* and separate groups of mice were treated with control-Fc or sIL-13Rα2-Fc as described in the Methods section. All animals were sacrificed on wk 8 postinfection and liver specimens were prepared for RT-PCR analysis as described in the Methods section. The data shown are the individual values of 9 to 10 animals per group and the bar indicates the average within each group. The * symbol indicates that the data are significantly different from the WT and IL-4-deficient control-Fc groups as determined by Student's t-test ($p<.05$). The average values from five uninfected WT (black circle) and five uninfected IL-4-deficient mice (open circle) are shown on the Y-axis for each cytokine. These data were reproduced in a separate study.

Fig. 7: IL-13 induces type I collagen synthesis in murine 3T3 fibroblasts. Cells were stimulated with media (lane 1), rIL-4 at 1000 Units/ml (lane 2) or rIL-13 at 20 ng/ml (lanes 3 and 4, from R&D Systems and Genetics Institute, respectively) for 48 h. Total cell lysates were separated on 6% SDS-PAGE under reducing conditions, transferred to nitrocellulose membrane and probed with rabbit IgG anti-mouse type I collagen. The top doublet and bottom band (arrows) correspond to the purified rat collagen type I separated in lane 5 (panel A). The bottom figure (panel B) is the densitometric values (arbitrary pixel units).

Detailed Description of Preferred Embodiments

The inventors of the present application have for the first time identified and provided polynucleotides encoding the IL-13 binding chain of IL-13R (hereinafter "IL-13bc"), including without limitation polynucleotides encoding murine and human IL-13bc.

SEQ ID NO:1 provides the nucleotide sequence of a cDNA encoding the murine IL-13bc. SEQ ID NO:2 provides predicted the amino acid sequence of the receptor chain, including a putative signal sequence from amino acids 1-21. The mature murine IL-13bc is believed to have the sequence of amino acids 22-383 of SEQ ID NO:2. The mature murine receptor chain has at least three distinct domains: an extracellular domain

(comprising approximately amino acids 22-334 of SEQ ID NO:2), a transmembrane domain (comprising approximately amino acids 335-356 of SEQ ID NO:2) and an intracellular domain (comprising approximately amino acids 357-383 of SEQ ID NO:2).

SEQ ID NO:3 provides the nucleotide sequence of a cDNA encoding the human IL-13bc. SEQ ID NO:4 provides predicted the amino acid sequence of the receptor chain, including a putative signal sequence from amino acids 1-25. The mature human IL-13bc is believed to have the sequence of amino acids 26-380 of SEQ ID NO:4. The mature human receptor chain has at least three distinct domains: an extracellular domain (comprising approximately amino acids 26-341 of SEQ ID NO:4), a transmembrane domain (comprising approximately amino acids 342-362 of SEQ ID NO:4) and an intracellular domain (comprising approximately amino acids 363-380 of SEQ ID NO:4).

The first 81 amino acids of the human IL-13bc sequence are identical to the translated sequence of an expressed sequence tag (EST) identified as "yg99f10.r1 Homo sapiens cDNA clone 41648 5'" and assigned database accession number R52795.gb_est2. There are no homologies or sequence motifs in this EST sequence which would lead those skilled in the art to identify the encoded protein as a cytokine receptor. A cDNA clone corresponding to this database entry is publicly-available from the I.M.A.G.E. Consortium. Subsequent to the priority date of the present application, such clone was ordered by applicants and sequenced. The sequence of such clone was determined to be the sequence previously reported by applicants as SEQ ID NO:3 herein.

Soluble forms of IL-13bc protein can also be produced. Such soluble forms include without limitation proteins comprising amino acids 1-334 or 22-334 of SEQ ID NO:2 or amino acids 1-341 or 26-341 of SEQ ID NO:4. The soluble forms of the IL-13bc are further characterized by being soluble in aqueous solution, preferably at room temperature. IL-13bc proteins comprising only the intracellular domain or a portion thereof may also be produced. Any forms of IL-13bc of less than full length are encompassed within the present invention and are referred to herein collectively with full length and mature forms as "IL-13bc" or "IL-13bc proteins." IL-13bc proteins of less than full length may be produced by expressing a corresponding fragment of the polynucleotide encoding the full-length IL-13bc protein (SEQ ID NO:1 or SEQ ID NO:3). These corresponding polynucleotide fragments are also part of the present invention. Modified polynucleotides as described above may be made by standard molecular biology

techniques, including construction of appropriate desired deletion mutants, site-directed mutagenesis methods or by the polymerase chain reaction using appropriate oligonucleotide primers.

For the purposes of the present invention, a protein has "a biological activity of the IL-13 receptor binding chain" if it possess one or more of the following characteristics: (1) the ability to bind IL-13 or a fragment thereof (preferably a biologically active fragment thereof); and/or (2) the ability to interact with the second non-IL-13-binding chain of IL-13R to produce a signal characteristic of the binding of IL-13 to IL-13R. Preferably, the biological activity possessed by the protein is the ability to bind IL-13 or a fragment hereof, more preferably with a K_D of about 0.1 to about 100 nM. Methods for determining whether a particular protein or peptide has such activity include without limitation the methods described in the examples provided herein.

IL-13bc or active fragments thereof (IL-13bc proteins) may be fused to carrier molecules such as immunoglobulins. For example, soluble forms of the IL-13bc may be fused through "linker" sequences to the Fc portion of an immunoglobulin. Other fusions proteins, such as those with GST, Lex-A or MBP, may also be used.

The invention also encompasses allelic variants of the nucleotide sequences as set forth in SEQ ID NO:1 or SEQ ID NO:3, that is, naturally-occurring alternative forms of the isolated polynucleotide of SEQ ID NO:1 or SEQ ID NO:3 which also encode IL-13bc proteins, preferably those proteins having a biological activity of IL-13bc. Also included in the invention are isolated polynucleotides which hybridize to the nucleotide sequence set forth in SEQ ID NO:1 or SEQ ID NO:3 under highly stringent conditions (for example, 0.1xSSC at 65°C). Isolated polynucleotides which encode IL-13bc proteins but which differ from the nucleotide sequence set forth in SEQ ID NO:1 or SEQ ID NO:3 by virtue of the degeneracy of the genetic code are also encompassed by the present invention. Variations in the nucleotide sequence as set forth in SEQ ID NO:1 or SEQ ID NO:3 which are caused by point mutations or by induced modifications are also included in the invention.

The present invention also provides polynucleotides encoding homologues of the murine and human IL-13bc from other animal species, particularly other mammalian species. Species homologues can be identified and isolated by making probes or primers from the murine or human sequences disclosed herein and screening a library from an

appropriate species, such as for example libraries constructed from PBMCs, thymus or testis of the relevant species.

The isolated polynucleotides of the invention may be operably linked to an expression control sequence such as the pMT2 or pED expression vectors disclosed in Kaufman et al., Nucleic Acids Res. 19, 4485-4490 (1991), in order to produce the IL-13bc protein recombinantly. Many suitable expression control sequences are known in the art. General methods of expressing recombinant proteins are also known and are exemplified in R. Kaufman, Methods in Enzymology 185, 537-566 (1990). As defined herein "operably linked" means enzymatically or chemically ligated to form a covalent bond between the isolated polynucleotide of the invention and the expression control sequence, in such a way that the IL-13bc protein is expressed by a host cell which has been transformed (transfected) with the ligated polynucleotide/expression control sequence.

A number of types of cells may act as suitable host cells for expression of the IL-13bc protein. Any cell type capable of expressing functional IL-13bc protein may be used. Suitable mammalian host cells include, for example, monkey COS cells, Chinese Hamster Ovary (CHO) cells, human kidney 293 cells, human epidermal A431 cells, human Colo205 cells, 3T3 cells, CV-1 cells, other transformed primate cell lines, normal diploid cells, cell strains derived from in vitro culture of primary tissue, primary explants, HeLa cells, mouse L cells, BHK, HL-60, U937, HaK, Rat2, BaF3, 32D, FDCP-1, PC12, M1x or C2C12 cells.

The IL-13bc protein may also be produced by operably linking the isolated polynucleotide of the invention to suitable control sequences in one or more insect expression vectors, and employing an insect expression system. Materials and methods for baculovirus/insect cell expression systems are commercially available in kit form from, e.g., Invitrogen, San Diego, California, U.S.A. (the MaxBac® kit), and such methods are well known in the art, as described in Summers and Smith, Texas Agricultural Experiment Station Bulletin No. 1555 (1987), incorporated herein by reference. Soluble forms of the IL-13bc protein may also be produced in insect cells using appropriate isolated polynucleotides as described above.

Alternatively, the IL-13bc protein may be produced in lower eukaryotes such as yeast or in prokaryotes such as bacteria. Suitable yeast strains include Saccharomyces cerevisiae, Schizosaccharomyces pombe, Kluyveromyces strains, *Candida,* or any yeast strain capable of expressing heterologous proteins. Suitable bacterial strains include *Escherichia coli, Bacillus subtilis, Salmonella typhimurium,* or any bacterial strain capable of expressing heterologous proteins.

Expression in bacteria may result in formation of inclusion bodies incorporating the recombinant protein. Thus, refolding of the recombinant protein may be required in order to produce active or more active material. Several methods for obtaining correctly folded heterologous proteins from bacterial inclusion bodies are known in the art. These methods generally involve solubilizing the protein from the inclusion bodies, then denaturing the protein completely using a chaotropic agent. When cysteine residues are present in the primary amino acid sequence of the protein, it is often necessary to accomplish the refolding in an environment which allows correct formation of disulfide bonds (a redox system). General methods of refolding are disclosed in Kohno, Meth. Enzym., 185:187-195 (1990). EP 0433225 and copending application USSN 08/163,877 describe other appropriate methods.

The IL-13bc protein of the invention may also be expressed as a product of transgenic animals, e.g., as a component of the milk of transgenic cows, goats, pigs, or sheep which are characterized by somatic or germ cells containing a polynucleotide sequence encoding the IL-13bc protein.

The IL-13bc protein of the invention may be prepared by growing a culture transformed host cells under culture conditions necessary to express the desired protein. The resulting expressed protein may then be purified from the culture medium or cell extracts. Soluble forms of the IL-13bc protein of the invention can be purified from conditioned media. Membrane-bound forms of IL-13bc protein of the invention can be purified by preparing a total membrane fraction from the expressing cell and extracting the membranes with a non-ionic detergent such as Triton X-100.

The IL-13bc protein can be purified using methods known to those skilled in the art. For example, the IL-13bc protein of the invention can be concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate can be

applied to a purification matrix such as a gel filtration medium. Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) or polyetheyleneimine (PEI) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are preferred (e.g., S-Sepharose® columns). The purification of the IL-13bc protein from culture supernatant may also include one or more column steps over such affinity resins as concanavalin A-agarose, heparin-toyopearl® or Cibacrom blue 3GA Sepharose®; or by hydrophobic interaction chromatography using such resins as phenyl ether, butyl ether, or propyl ether; or by immunoaffinity chromatography. Finally, one or more reverse-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, e.g., silica gel having pendant methyl or other aliphatic groups, can be employed to further purify the IL-13bc protein. Affinity columns including IL-13 or fragments thereof or including antibodies to the IL-13bc protein can also be used in purification in accordance with known methods. Some or all of the foregoing purification steps, in various combinations or with other known methods, can also be employed to provide a substantially purified isolated recombinant protein. Preferably, the isolated IL-13bc protein is purified so that it is substantially free of other mammalian proteins.

IL-13bc proteins of the invention may also be used to screen for agents which are capable of binding to IL-13bc or IL-13R or which interfere with the binding of IL-13 to the IL-13 or IL-13bc (either the extracellular or intracellular domains) and thus may act as inhibitors of normal binding and cytokine action ("IL-13R inhibitors"). Binding assays using a desired binding protein, immobilized or not, are well known in the art and may be used for this purpose using the IL-13bc protein of the invention. Purified cell based or protein based (cell free) screening assays may be used to identify such agents. For example, IL-13bc protein may be immobilized in purified form on a carrier and binding to purified IL-13bc protein may be measured in the presence and in the absence of potential inhibiting agents. A suitable binding assay may alternatively employ a soluble form of IL-13bc of the invention. Another example of a system in which inhibitors may be screened is described in Example 2 below.

In such a screening assay, a first binding mixture is formed by combining IL-13 or a fragment thereof and IL-13bc protein, and the amount of binding in the first binding mixture (B_o) is measured. A second binding mixture is also formed by combining IL-13 or a fragment thereof, IL-13bc protein, and the compound or agent to be screened, and the amount of binding in the second binding mixture (B) is measured. The amounts of binding in the first and second binding mixtures are compared, for example, by performing a calculation of the ratio B/B_o. A compound or agent is considered to be capable of inhibiting binding if a decrease in binding in the second binding mixture as compared to the first binding mixture is observed. Optionally, the second chain of IL-13R can be added to one or both of the binding mixtures. The formulation and optimization of binding mixtures is within the level of skill in the art, such binding mixtures may also contain buffers and salts necessary to enhance or to optimize binding, and additional control assays may be included in the screening assay of the invention.

Compounds found to reduce the binding activity of IL-13bc protein to IL-13 or its fragment to any degree, preferably by at least about 10%, more preferably greater than about 50% or more, may thus be identified and then secondarily screened in other binding assays and in vivo assays. By these means compounds having inhibitory activity for IL-13bc binding which may be suitable as therapeutic agents may be identified.

IL-13bc proteins, and polynucleotides encoding them, may also be used as diagnostic agents for detecting the expression or presence of IL-13bc, IL-13R, IL-13 or cells expressing IL-13bc, IL-13R or IL-13. The proteins or polynucleotides may be employed for such purpose in standard procedures for diagnostics assays using these types of materials. Suitable methods are well known to those skilled in the art.

As used herein "IL-13R" refers to IL-13bc and/or a second IL-13 receptor chain known as "IL-13Rα1" or "NR4" (see: murine receptor chain, Hilton et al., Proc. Natl. Acad. Sci. USA 1996, 93:497-501; human receptor chain, Aman et al., J. Biol. Chem. 1996, 271:29265-70, and Gauchat et al., Eur. J. Immunol. 1997, 27:971-8).

IL-13bc acts as a mediator of the known biological activities of IL-13. As a result, IL-13bc protein (particularly, soluble IL-13bc proteins), IL-13R inhibitors (i.e., antagonists of interaction of IL-13 with IL-13R (such as, for example, antibodies to IL-13R (including particularly to IL-13bc or to IL-13Rα1) and fragments thereof, antibodies to IL-13 and fragments thereof, soluble IL-13Rα1 proteins, and small molecule and other inhibitors of

the interaction of IL-13 with IL-13R (including with IL-13bc and/or with IL-13Rα1)) may be useful in treatment or modulation of various medical conditions in which IL-13 is implicated or which are effected by the activity (or lack thereof) of IL-13 (collectively "IL-13-related conditions"). Mutated forms of IL-4 which bind to IL-13R can also be used as IL-13 antagonists (see, for example, those disclosed in Shanafelt et al., Proc. Natl. Acad. Sci. USA 1998, 95:9454-8; Aversa et al., J. Exp. Med. 1993, 178:2213-8; and Grunewald et al., J. Immunol. 1998, 160:4004-9).

IL-13-related conditions include without limitation Ig-mediated conditions and diseases, particularly IgE-mediated conditions (including without limitation atopy, allergic conditions, asthma, immune complex diseases (such as, for example, lupus, nephrotic syndrome, nephritis, glomerulonephritis, thyroiditis and Grave's disease)); inflammatory conditions of the lungs; immune deficiencies, specifically deficiencies in hematopoietic progenitor cells, or disorders relating thereto; cancer and other disease. Such pathological states may result from disease, exposure to radiation or drugs, and include, for example, leukopenia, bacterial and viral infections, anemia, B cell or T cell deficiencies such as immune cell or hematopoietic cell deficiency following a bone marrow transplantation. Since IL-13 inhibits macrophage activation, IL-13bc proteins may also be useful to enhance macrophage activation (i.e., in vaccination, treatment of mycobacterial or intracellular organisms, or parasitic infections).

IL-13bc proteins may also be used to potentiate the effects of IL-13 in vitro and in vivo. For example, an IL-13bc protein can be combined with a protein having IL-13 activity (preferably IL-13) and the resulting combination can be contacted with a cell expressing at least one chain of IL-13R other than IL-13bc (preferably all chains of IL-13R other than IL-13bc, such as IL-13Rα1). Preferably, the contacting step is performed by administering a therapeutically effective amount of such combination to a mammalian subject in vivo. The pre-established association of the IL-13 protein with the IL-13bc protein will aid in formation of the complete IL-13/IL-13R complex necessary for proper signaling. See for example the methods described by Economides et al., Science 270:1351 (1995).

IL-13bc protein and IL-13R inhibitors, purified from cells or recombinantly produced, may be used as a pharmaceutical composition when combined with a pharmaceutically acceptable carrier. Such a composition may contain, in addition to IL-

13bc or inhibitor and carrier, various diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials well known in the art. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredient(s). The characteristics of the carrier will depend on the route of administration.

The pharmaceutical composition of the invention may also contain cytokines, lymphokines, or other hematopoietic factors such as M-CSF, GM-CSF, interleukins (such as, IL-1, IL-2, IL-3, IL-4 . . . IL-24, IL-25), G-CSF, stem cell factor, and erythropoietin. The pharmaceutical composition may also include anti-cytokine antibodies. The pharmaceutical composition may further contain other anti-inflammatory agents. Such additional factors and/or agents may be included in the pharmaceutical composition to produce a synergistic effect with isolated IL-13bc protein or IL-13bc inhibitor, or to minimize side effects caused by the isolated IL-13bc or IL-13bc inhibitor. Conversely, isolated IL-13bc or IL-13bc inhibitor may be included in formulations of the particular cytokine, lymphokine, other hematopoietic factor, thrombolytic or anti-thrombotic factor, or anti-inflammatory agent to minimize side effects of the cytokine, lymphokine, other hematopoietic factor, thrombolytic or anti-thrombotic factor, or anti-inflammatory agent.

The pharmaceutical composition of the invention may be in the form of a liposome in which isolated IL-13bc protein or IL-13bc inhibitor is combined, in addition to other pharmaceutically acceptable carriers, with amphipathic agents such as lipids which exist in aggregated form as micelles, insoluble monolayers, liquid crystals, or lamellar layers which in aqueous solution. Suitable lipids for liposomal formulation include, without limitation, monoglycerides, diglycerides, sulfatides, lysolecithin, phospholipids, saponin, bile acids, and the like. Preparation of such liposomal formulations is within the level of skill in the art, as disclosed, for example, in U.S. Patent No. 4,235,871; U.S. Patent No. 4,501,728; U.S. Patent No. 4,837,028; and U.S. Patent No. 4,737,323, all of which are incorporated herein by reference.

As used herein, the term "therapeutically effective amount" means the total amount of each active component of the pharmaceutical composition or method that is sufficient to show a meaningful patient benefit, e.g., amelioration of symptoms of, healing of, or increase in rate of healing of such conditions. When applied to an individual active ingredient, administered alone, the term refers to that ingredient alone. When applied to

a combination, the term refers to combined amounts of the active ingredients that result in the therapeutic effect, whether administered in combination, serially or simultaneously.

In practicing the method of treatment or use of the present invention, a therapeutically effective amount of isolated IL-13bc protein or IL-13bc inhibitor is administered to a mammal. Isolated IL-13bc protein or IL-13bc inhibitor may be administered in accordance with the method of the invention either alone or in combination with other therapies such as treatments employing cytokines, lymphokines or other hematopoietic factors. When co-administered with one or more cytokines, lymphokines or other hematopoietic factors, IL-13bc protein or IL-13bc inhibitor may be administered either simultaneously with the cytokine(s), lymphokine(s), other hematopoietic factor(s), thrombolytic or anti-thrombotic factors, or sequentially. If administered sequentially, the attending physician will decide on the appropriate sequence of administering IL-13bc protein or IL-13bc inhibitor in combination with cytokine(s), lymphokine(s), other hematopoietic factor(s), thrombolytic or anti-thrombotic factors.

Administration of IL-13bc protein or IL-13bc inhibitor used in the pharmaceutical composition or to practice the method of the present invention can be carried out in a variety of conventional ways, such as oral ingestion, inhalation, or cutaneous, subcutaneous, or intravenous injection. Intravenous administration to the patient is preferred.

When a therapeutically effective amount of IL-13bc protein or IL-13bc inhibitor is administered orally, IL-13bc protein or IL-13bc inhibitor will be in the form of a tablet, capsule, powder, solution or elixir. When administered in tablet form, the pharmaceutical composition of the invention may additionally contain a solid carrier such as a gelatin or an adjuvant. The tablet, capsule, and powder contain from about 5 to 95% IL-13bc protein or IL-13bc inhibitor, and preferably from about 25 to 90% IL-13bc protein or IL-13bc inhibitor. When administered in liquid form, a liquid carrier such as water, petroleum, oils of animal or plant origin such as peanut oil, mineral oil, soybean oil, or sesame oil, or synthetic oils may be added. The liquid form of the pharmaceutical composition may further contain physiological saline solution, dextrose or other saccharide solution, or glycols such as ethylene glycol, propylene glycol or polyethylene glycol. When administered in liquid form, the pharmaceutical composition contains from about 0.5 to

90% by weight of IL-13bc protein or IL-13bc inhibitor, and preferably from about 1 to 50% IL-13bc protein or IL-13bc inhibitor.

When a therapeutically effective amount of IL-13bc protein or IL-13bc inhibitor is administered by intravenous, cutaneous or subcutaneous injection, IL-13bc protein or IL-13bc inhibitor will be in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such parenterally acceptable protein solutions, having due regard to pH, isotonicity, stability, and the like, is within the skill in the art. A preferred pharmaceutical composition for intravenous, cutaneous, or subcutaneous injection should contain, in addition to IL-13bc protein or IL-13bc inhibitor an isotonic vehicle such as Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, Lactated Ringer's Injection, or other vehicle as known in the art. The pharmaceutical composition of the present invention may also contain stabilizers, preservatives, buffers, antioxidants, or other additive known to those of skill in the art.

The amount of IL-13bc protein or IL-13bc inhibitor in the pharmaceutical composition of the present invention will depend upon the nature and severity of the condition being treated, and on the nature of prior treatments which the patient has undergone. Ultimately, the attending physician will decide the amount of IL-13bc protein or IL-13bc inhibitor with which to treat each individual patient. Initially, the attending physician will administer low doses of IL-13bc protein or IL-13bc inhibitor and observe the patient's response. Larger doses of IL-13bc protein or IL-13bc inhibitor may be administered until the optimal therapeutic effect is obtained for the patient, and at that point the dosage is not generally increased further. It is contemplated that the various pharmaceutical compositions used to practice the method of the present invention should contain about 0.1 μg to about 100 mg (preferably about 20 μg to about 500 μg) of IL-13bc protein or IL-13bc inhibitor per kg body weight.

The duration of intravenous therapy using the pharmaceutical composition of the present invention will vary, depending on the severity of the disease being treated and the condition and potential idiosyncratic response of each individual patient. It is contemplated that the duration of each application of the IL-13bc protein or IL-13bc inhibitor will be in the range of 12 to 24 hours of continuous intravenous administration. Ultimately the attending physician will decide on the appropriate duration of intravenous therapy using the pharmaceutical composition of the present invention.

IL-13bc proteins of the invention may also be used to immunize animals to obtain polyclonal and monoclonal antibodies which specifically react with the IL-13bc protein and which may inhibit binding of IL-13 or fragments thereof to the receptor. Such antibodies may be obtained using the entire IL-13bc as an immunogen, or by using fragments of IL-13bc, such as the soluble mature IL-13bc. Smaller fragments of the IL-13bc may also be used to immunize animals. The peptide immunogens additionally may contain a cysteine residue at the carboxyl terminus, and are conjugated to a hapten such as keyhole limpet hemocyanin (KLH). Additional peptide immunogens may be generated by replacing tyrosine residues with sulfated tyrosine residues. Methods for synthesizing such peptides are known in the art, for example, as in R.P. Merrifield, J.Amer.Chem.Soc. 85, 2149-2154 (1963); J.L. Krstenansky, et al., FEBS Lett. 211, 10 (1987).

Neutralizing or non-neutralizing antibodies (preferably monoclonal antibodies) binding to IL-13bc protein may also be useful therapeutics for certain tumors and also in the treatment of conditions described above. These neutralizing monoclonal antibodies may be capable of blocking IL-13 binding to the IL-13bc.

Example 1

Isolation of IL-13bc cDNAs

Isolation of the murine IL-13 receptor chain.

5 ug of polyA+ RNA was prepared from the thymuses of 6-8 week old C3H/HeJ mice. Double stranded, hemimethylated cDNA was prepared using Stratagene's cDNA synthesis kit according to manufacturers instructions. Briefly, the first strand was primed with an oligodT-Xho primer, and after second strand synthesis, EcoRI adapters were added, and the cDNA was digested with XhoI, and purified. The cDNA was ligated to the XhoI-EcoRI sites of the Zap Express (Stratagene) lambda vector, and packaged using Gigapak II Gold packaging extracts (Stratagene) according to the manufacturers instructions. A library of 1.5×10^6 resulting recombinant phage was amplified following manufacturer's instructions. This library was screened with a degenerate 17mer oligonucleotide probe of the sequence KSRCTCCABK CRCTCCA (SEQ ID NO:5) (K = G+T; S= C+G; R=A+G; B=C+G+T) using standard TMAC hybridization conditions as described (Current Protocols in Molecular Biology, Ausubel, et al., editors., John Wiley and Sons, 1995, section 6.4.3). Clone A25 was identified because it hybridized to the

17mer probe, but not to probes derived from known hematopoietin receptors. This clone was isolated in plasmid form from the ZapExpress vector as per manufacturers instruction, and the DNA sequence was determined. The DNA sequence encoded a novel member of the hematopoietin receptor family.

Clone A25 containing the polynucleotide having the sequence of SEQ ID NO:1 was deposited with ATCC as pA25pBKCMV at accession number 69997 on February 22, 1996.

Isolation of the human IL-13 receptor chain.

A partial fragment of the human homolog of the murine receptor was isolated by PCR using oligonucleotides derived from the murine sequence. cDNA was prepared from human testis polyA+ RNA that was obtained from Clontech. A DNA fragment of 274 base pairs was amplified from this cDNA by PCR with the following oligonucleotides: ATAGTTAAACCATTGCCACC (SEQ ID NO:6) and CTCCATTCGCTCCAAATTCC (SEQ ID NO:7) using AmpliTaq polymerase (Promega) in 1X Taq buffer containing 1.5 mM MgCl2 for 30 cycles of incubation (94°C x 1 minute, 42°C for 1 minute, and 72°C for 1 minute). The DNA sequence of this fragment was determined, and two oligonucleotides were prepared from an internal portion of this fragment with the following sequence: AGTCTATCTTACTTTTACTCG (SEQ ID NO:8) and CATCTGAGCAATAAATATTCAC (SEQ ID NO:9). These oligonucleotides were used as probes to screen a human testis cDNA library purchased from CLONTECH (cat #HL1161) . Filters were hybridized at 52°C using standard 5XSSC hybridization conditions and washed in 2X SSC at 52°C . Twenty two clones were isolated that hybridized to both oligonucleotides in a screen of 400,000 clones. DNA sequence was determined from four of the cDNA clones, and all encoded the same novel hematopoietin receptor. The predicted DNA sequence of the full length human receptor chain is shown as SEQ ID NO:3.

The human clone was deposited with ATCC as phA25#11pDR2 at accession number 69998 on February 22, 1996.

Example 2

Expression of Soluble IL-13bc Protein and

Assay of Activity

Production and purification of soluble IL-13bc-Ig.

DNA encoding amino acids 1-331 of the extracellular domain of murine IL-13bc was fused to a spacer sequence encoding gly-ser-gly by PCR and ligated in frame with sequences encoding the hinge CH2 CH3 regions of human IgG1 of the COS-1 expression vector pED.Fc . IL-13bc-Ig was produced from DEAE-dextran transfected COS-1 cells and purified via protein A sepharose chromatography (Pharmacia).

B9 proliferation assay

Stimulation of proliferation of B9 cells (Aarden et al. Eur. J. Immunol. 1987. 17:1411-1416) in response to IL-13 or IL-4 was measured by 3H-thymidine incorporation into DNA. Cells (5 x 103/well) were seeded into 96 well plates with media containing growth factors at varying concentrations in the presence or absence of IL-13bc-Ig at 1ug/ml. After incubation for 3 days 1uCi/well of 3H-thymidine was added and the cells incubated for an additional 4 hrs. Incorporated radioactivity was determined using a LKB 1205 Plate reader.

The B9 cell line proliferated in response to IL-13, IL-4 or IL-6. Only responses to IL-13 were inhibited by the soluble IL-13bc-Ig, indicating that this receptor binds IL-13 specifically, but not IL-4 or IL-6. The tables show cpm. Two separate experiments are shown.

Table I

cytokine dilution	IL-13 (3ng/ml)	IL-13 plus A25-Fc (1ug/ml)	IL-4 (20 ng/ml)	IL-4 plus A25-Fc (1 ug/ml)	Cos IL-6 (1/10,000)
1	37734	1943	6443	6945	37887
1/3	30398	1571	2680	2442	36500
1/10	16101	1461	1767	1771	33335
1/30	2148	1567	1619	1783	27271
1/100	1574	1419	1522	1576	18831
1/300	1512	1531	1373	1577	7768

1/1000	1316	1392	1190	1474	2760
1/3000	1834	1994	1482	1819	1672

Table II

cytokine dilution	IL-13 (3ng/ml)	IL-13 plus A25-Fc (5ug/ml)	IL-4 (20 ng/ml)	IL-4 plus A25-Fc (5ug/ml)	Cos IL-6 (1/10,000)	Cos IL-6 plus A25-Fc (5ug/ml)
1	6413	295	1216	1158	6969	7703
1/3	5432	281	518	656	7827	8804
1/10	2051	281	489	520	8345	10027
1/30	506	319	279	476	8680	9114
1/100	430	372	288	423	7426	10364
1/300	330	287	323	420	5531	6254
1/1000	326	389	348	nt	2524	nt
no cytokine	339	279	404	394	326	279

Example 3

Direct Binding of Soluble IL-13bc to IL-13 Measured by Surface Plasmon Resonance (Biacore Analysis).

A Biacore biosensor was used to measure directly the specific binding of IL-13 to purified IL-13bc-Ig (Pharmacia, Johnsson et al., 1991). Approximately 10,000 to 17,000 resonance units (RU) of purified IL-13bc-Ig , human IgG1 or irrelevant receptor were each covalently immobilized to different flow cells on the sensor chip as recommended by the manufacturer. (RU's are a refelction of the mass of protein bound to the sensor chip surface.) Purified IL-13 was injected across the flow cells at 5 ul/min for 10 mins in the presence or absence of excess purified IL-13bc-Ig. Binding was quantified as the difference in RU before and after sample injection. Specific IL-13 binding of 481.9 RU was observed only for immobilized IL-13bc-Ig whereas coinjection of IL-13 plus IL-13bc-Ig resulted in no binding to the immobilized IL-13bc-Ig (4 RU). No IL-13 binding was observed for either immobilized IgG or IL-11R-Ig (5.4 and 3.7 RU respectively).

Sample	IL-13bc-Ig (10,383 RU)	IgG control (13,399 RU)	IL-11R-Ig (17,182 RU)
100 ng/ml human IL-13	481.9 RU bound	5.4 RU bound	3.7 RU bound
100 ng/ml human IL-13 + soluble IL-13bc-Ig	4.0 RU bound	not tested	not tested

Example 4

Binding of IL-13 Expressed in COS Cells to

Labeled IL-13BC-Ig Fusion Protein:

COS in situ Detection of IL-13 with IL-13bc-Fc

Expression vectors for IL-13, IL-4, IL-11 or empty vector were transfected into COS-1 cells in duplicated plates via the DEAE-dextran method. Two days after transfection cells were washed twice in phosphate buffered saline (PBS) and fixed in the culture dish for 10' at 4° C with methanol. Following fixation cells were washed twice with PBS then rinsed once with binding buffer (PBS, 1% (w/v) bovine serum albumin,).1% (w/v) sodium azide) and incubated for two hours at 4° C in binding buffer with IL-13bc-Fc at 1.0ug/ml or with relevant anti-cytokine antisera. Cells were washed twice with PBS and incubated at 4o C with shaking in alkaline phosphatase labeled Rabbit F(ab)2' anti-human IgG diluted 1:500 in binding buffer (for Fc fusion detection) or Rabbit F(ab)2' anti-rat IgG (for anti-cytokine detection) . Cells were again washed twice in PBS. Alkaline phosphatase activity was visualized using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate.

Specific binding was visualized under the microscope. Only cells transfected with IL-13 showed specific binding to IL13bc-Ig. (See photograph of transfected cells, Figure 1).

Example 5

Other Systems for Determination Biological Activity of IL-13bc Protein

Other systems can be used to determine whether a specific IL-13bc protein exhibits a "biological activity" of IL-13bc as defined herein. The following are examples of such systems.

Assays for IL-13 Binding

The ability of a IL-13bc protein to bind IL-13 or a fragment thereof can be determine by any suitable assays which can detect such binding. Some suitable examples follow.

Binding of IL-13 to the extracellular region of the IL-13bc protein will specifically cause a rapid induction of phosphotyrosine on the receptor protein. Assays for ligand binding activity as measured by induction of phosphorylation are described below.

Alternatively, a IL-13bc protein (such as, for example, a soluble form of the extracellular domain) is produced and used to detect IL-13 binding. For example, a DNA construct is prepared in which the extracellular domain (truncated prior, preferably immediately prior, to the predicted transmembrane domain) is ligated in frame to a cDNA encoding the hinge C_H2 and C_H3 domains of a human immunoglobulin (Ig) $\gamma 1$. This construct is generated in an appropriate expression vector for COS cells, such as pEDΔC or pMT2. The plasmid is transiently transfected into COS cells. The secreted IL-13bc-Ig fusion protein is collected in the conditioned medium and purified by protein A chromatography.

The purified IL-13bc-Ig fusion protein is used to demonstrate IL-13 binding in a number of applications. IL-13 can be coated onto the surface of an enzyme-linked immunosorbent assay (ELISA) plate, and then additional binding sites blocked with bovine serum albumin or casein using standard ELISA buffers. The IL-13bc-Ig fusion protein is then bound to the solid-phase IL-13, and binding is detected with a secondary goat anti-human Ig conjugated to horseradish peroxidase. The activity of specifically bound enzyme can be measured with a colorimetric substrate, such as tetramethyl benzidine and absorbance readings.

IL-13 may also be expressed on the surface of cells, for example by providing a transmembrane domain or glucosyl phosphatidyl inositol (GPI) linkage. Cells expressing the membrane bound IL-13 can be identified using the IL-13bc-Ig fusion protein. The soluble IL-13bc-Ig fusion is bound to the surface of these cells and detected with goat anti-human Ig conjugated to a fluorochrome, such as fluorescein isothiocyanate and flow cytometry.

Interaction Trap

A yeast genetic selection method, the "interaction trap" [Gyuris et al, Cell 75:791-803, 1993], can be used to determine whether a IL-13bc protein has a biological activity of IL-13bc as defined herein. In this system, the expression of reporter genes from both LexAop-Leu2 and LexAop-LacZ relies on the interaction between the bait protein,

for example in this case a species which interacts with human IL-13bc, and the prey, for example in this case the human IL-13bc protein. Thus, one can measure the strength of the interaction by the level of Leu2 or LacZ expression. The most simple method is to measure the activity of the LacZ encoded protein, β-galactosidase. This activity can be judged by the degree of blueness on the X-Gal containing medium or filter. For the quantitative measurement of β-galactosidase activity, standard assays can be found in "Methods in Yeast Genetics" Cold Spring Harbor, New York, 1990 (by Rose, M.D., Winston, F., and Hieter, P.).

In such methods, if one wishes to determine whether the IL-13bc protein interacts with a particular species (such as, for example, a cytosolic protein which binds to the intracellular domain of the IL-13bc *in vivo*), that species can be used as the "bait" in the interaction trap with the IL-13bc protein to be tested serving as the "prey", or *vice versa*.

Example 6

Inhibition of Fibrosis Using Soluble IL-13R

The development of fibrous tissue is part of the normal process of healing after injury. Nevertheless, in some circumstances there is a destructive accumulation of excess collagen that interferes with the normal function of the affected tissue. Indeed collagen synthesis and tissue scaring are the major pathological manifestations of a number of chronic and debilitating illnesses, including several autoimmune, allergic, and infectious diseases [1-7]. While there is a great deal of mechanistic information regarding the process of scar tissue formation [8,9], there are still large gaps in our understanding of the role of inflammatory cells and cytokines in initiating the fibrotic process.

As used herein "fibrosis" includes any condition which involves the formation of fibrous tissue (whether such formation is desireable or undesireable). Such conditions include, without limitation, fibrositis, formation of fibromas (fibromatosis), fibrogenesis (including pulmonary fibrogenesis), fibroelastosis (including endocardial fibroelastosis), formation of fibromyomas, fibrous ankylosis, formation of fibroids, formation of fibroadenomas, formation of fibromyxomas, and fibrocystotitis (including cystic fibrosis).

The IL-13 receptor complex is composed of at least three distinct components, including the IL-4 receptor, the low-affinity binding IL-13Rα1 chain, and the high affinity

binding chain, IL-13Rα2 [35,42-44]. Recently, a soluble IL-13Ra2-Fc fusion protein was prepared and has been used successfully to neutralize IL-13 both in vitro [35] and in vivo [30,39-41]. Since the fusion protein binds IL-13 with high affinity, but fails to neutralize IL-4, the protein provided an excellent tool to determine the specific roles of IL-13. In the present study, we used the IL-13 antagonist in wild type and IL-4-deficient mice in order to dissect the contributions of IL-13 and IL-4 to the development of hepatic fibrosis in murine schistosomiasis. In these studies, granuloma formation was examined in detail, focusing on eosinophil and mast cell recruitment and, more importantly, the development of egg-induced fibrosis was quantified using biochemical, histological, and molecular techniques. We also examined the contributions of IL-4 and IL-13 to the regulation of Th1/Th2-type cytokine responses both *in vitro*, in mesenteric lymph node cultures and, *in vivo*, in the granulomatous livers. While the results from this study show that IL-13 and IL-4 exhibit some redundant activities in schistosomiasis pathogenesis, distinct functions for both cytokines were also clearly elucidated. Probably the most important and novel finding was the observation that IL-13, not IL-4, was the major Th2-type cytokine driving type I and type III collagen mRNA production and hepatic fibrosis in infected mice. Thus, our findings establish that an IL-13 inhibitor/antagonist, such as sIL-13Rα2-Fc, can be of therapeutic benefit in preventing fibrosis, such as, for example, that associated with chronic infectious disease.

RESULTS

Comparative effect of IL-4, IL-13 or double IL-4/IL-13 deficiencies in schistosomiasis pathogenesis: sIL-13Rα2-Fc treatment significantly reduces hepatic fibrosis in S. mansoni-infected mice

To compare the regulatory roles of IL-4 and IL-13 in the pathogenesis of schistosomiasis, we infected C57BL/6 WT and IL-4-deficient mice percutaneously with 25 *S. mansoni* cercariae. Separate groups of animals were treated with either sIL-13Rα2-Fc or with control-Fc, as described in the Materials and Methods. The treatments began on week 5, at the start of egg laying, and all animals were sacrificed 8 wk postinfection and examined for several parasitologic and immunologic parameters. As shown in Table III,

{TABLE III INSERTED HERE}

all four groups of mice harbored similar worm burdens, and tissue eggs produced per worm pair did not vary among the groups. At 8 wk postinfection, the time of the peak tissue response [45], WT mice showed no significant change in granuloma size as a result of IL-13 blockade (Fig. 2A). Interestingly, control-Fc-treated IL-4-deficient mice also failed to show a reduced granulomatous response, and in fact, granulomas were significantly larger in these mice. In striking contrast to these observations, the IL-4-deficient mice displayed a markedly reduced granulomatous response when IL-13 was inhibited (Fig. 2A, far right). Indeed, the double IL-4-deficient/sIL-13Rα2-Fc-treated mice displayed on average a 40 to 50% reduction in granuloma volume when compared with either control or sIL-13Rα2-Fc-treated WT animals, and more than a 75% reduction when compared with control-Fc-treated IL-4-deficient mice.

The cellular composition of the lesions was also evaluated in Giemsa-stained liver sections and as shown in Table III, IL-4-deficient mice displayed a marked reduction in granuloma-associated mast cells. In contrast, there was no change in mast cell numbers by IL-13 inhibition alone, and IL-13 blockade had no additional effect on the already highly reduced numbers of mast cells in IL-4-deficient mice. Somewhat similar, yet distinct findings were observed when granuloma-associated eosinophils were evaluated (Fig. 2B). Here, the numbers of eosinophils were increased from 46 to 64% in WT mice by IL-13 blockade and significantly decreased (28%) as consequence of IL-4 deficiency. Despite the apparent contrasting roles for IL-13 and IL-4 in the tissue eosinophilia, an even more striking combined inhibitory effect was observed when the IL-4-deficient mice were treated with the IL-13 inhibitor. In these mice, the average number of granuloma eosinophils was below 10%. Finally, there was no change in the degree of parenchymal or egg-associated liver necrosis in the WT versus IL-4-deficient animals, while both sIL-13Rα2-Fc-treated WT and IL-4-deficient groups showed marked reductions in overall parenchymal necrosis.

Perhaps most importantly, the sIL-13Rα2-Fc treatment alone significantly reduced the collagen content of liver granulomas in WT mice, as assessed in tissue sections stained with picrosirius red (Table III and Fig. 3). In contrast, infected IL-4-deficient mice showed no detectable change in granuloma collagen deposition by microscopic analysis. Interestingly, there appeared to be no combined or synergistic role for IL-13 and IL-4 in this parameter since there was no significant difference between sIL-13Rα2-Fc-treated-

WT and -IL-4 deficient mice (Table III). Fig. 3 shows that while similar worm numbers, tissue egg burdens, and granuloma sizes were found in control and sIL-13Rα2-Fc treated WT mice, IL-13 blockade had a substantial inhibitory effect on collagen deposition within the liver. Finally, the extent of hepatic fibrosis was also measured by the assessment of hydroxyproline levels (Fig. 2C), which is more quantitative than the histological techniques described above. The soluble IL-13 antagonist alone markedly decreased liver hydroxyproline levels, while the IL-4-deficiency resulted in a less significant reduction. The dual IL-4/IL-13 deficiency failed to reduce hydroxyproline to levels below that already observed in the sIL-13Rα2-Fc treated WT mice (Fig. 2C), although there was a slight trend in a second study (not significant). Together, these data demonstrate that IL-13 is the dominant Th2-associated cytokine responsible for the development of hepatic fibrosis in murine schistosomiasis.

Th2-type cytokine production is reduced in IL-4-deficient mice but unaffected by IL-13 inhibition.

While it is well-known that IL-4 is the primary cytokine driving $CD4^+$ Th2 cell development [21,22], the role of IL-13 in the generation and maintenance of Th2-type responses has been controversial and may be influenced by both host genetics and the infectious disease model under study [30,34,38]. Therefore, to determine whether the sIL-13Rα2-Fc-induced changes in liver pathology were generated by alterations in the Th1/Th2 cytokine balance, we isolated mesenteric lymph nodes and spleens from infected mice, prepared single cell suspensions, and restimulated the cultures *in vitro* with parasite antigens. Additional cell cultures were exposed to parasite antigens in the presence of anti-CD4 mAbs to determine whether cytokine production was dependent upon a $CD4^+$ T cell response. Culture supernatants were analyzed by ELISA for IL-4, IL-13, IL-5, IL-10, and IFN-γ. As might be predicted [15], mesenteric (Fig. 5) and splenic cultures (data not shown) prepared from WT mice displayed a highly polarized Th2-type cytokine response. They produced high levels of IL-4, IL-5, IL-10, and IL-13 in response to SEA stimulation and little or no IFN-γ. IL-4-deficient mice in contrast showed a more mixed Th1/Th2-type profile. Indeed, a significant SEA-specific IFN-γ response was detected in IL-4-deficient mice, which is consistent with previous studies [23,24]. IL-13, IL-10, and to a lesser extent IL-5, were also detected in these animals, although the levels of these cytokines were

markedly decreased when compared with WT mice. Importantly, the maintenance of the low but significant IL-4-independent IL-13 response likely explains the marked granulomatous response that is maintained in the absence IL-4 (Fig. 2). Surprisingly, despite its marked inhibitory effect on hepatic fibrosis, sIL-13Rα2-Fc had no significant effect on Th1 or Th2-type cytokine responses in either WT or IL-4-deficient mice. It should also be noted that in all cases, cytokine production was highly dependent on a $CD4^+$ T cell response, since little or no cytokine expression was detected in any of the anti-CD4 mAb-treated SEA-stimulated cultures.

Changes in Th1/Th2-type cytokine mRNA expression in the granulomatous livers of IL-4-deficient and sIL-13Ra2-Fc-treated mice.

To determine whether a similar pattern of cytokine expression was observed *in vivo* at the site of granuloma formation, we isolated liver mRNA from the various groups of mice at 8 wk postinfection and performed quantitative RT-PCR. As shown in Figure 5, infected WT mice displayed a strong Th2-type cytokine mRNA profile, showing marked increases in IL-4, IL-13, IL-5, and IL-10 mRNA. The WT mice also showed modest increases in the expression of IFN-γ mRNA, which was consistent with previous observations [19]. In contrast to these findings, IL-13 and IL-5 mRNA levels were much lower in IL-4-deficient mice, while IL-10 and TNF-α mRNA significantly increased and IFN-γ mRNA expression did not change. Again, similar to the in vitro results obtained from mesenteric lymph node and splenocyte cultures, IL-13 blockade had no significant effect on the pattern of cytokine mRNA expression in either WT or IL-4-deficient mice. There was however, a modest increase in IL-10 mRNA levels in IL-4-deficient mice treated with the sIL-13Rα2-Fc, although this is unlikely to explain the decreases in fibrosis, since highly divergent levels of IL-10 were detected in sIL-13Rα2-Fc-treated WT versus IL-4-deficient mice, yet a similar decrease in fibrosis was observed. TGF-β1 and TGF-β2 mRNA expression was also examined in the granulomatous tissues, however no significant differences were observed in either infected IL-4-deficient mice or in animals treated with sIL-13Rα2-Fc (data not shown).

Collagen I and collagen III mRNA levels are reduced in the livers of sIL-13Rα2-Fc-treated mice but unaffected by IL-4-deficiency.

The *in vitro* and *in vivo* cytokine studies described above suggested that the anti-fibrotic effect of sIL-13Rα2-Fc was unlikely to be explained by changes in Th1 or Th2-type cytokine expression. Therefore, in subsequent experiments, we investigated the patterns of collagen I (Col I) and collagen III (Col III) mRNA expression to determine whether the sIL-13Rα2-Fc-induced reduction in fibrosis was accompanied by direct changes in the expression of these two important collagen producing genes [19]. As shown in Figure 6, IL-13 blockade significantly reduced Col I and Col III mRNA expression in both WT and IL-4-deficient mice. There was no change in the infection-induced levels Col I or Col III mRNAs in IL-4-deficient mice and when compared with sIL-13Rα2-Fc-treated WT mice, there was no further reduction in similarly treated IL-4-deficient mice.

IL-13 stimulates collagen production in mouse 3T3 fibroblasts.

Having shown that IL-13 blockade *in vivo* significantly reduced Col I and Col III mRNA expression in the liver of infected WT and IL-4-deficient mice, we wanted to determine whether IL-13 would directly stimulate collagen synthesis in fibroblasts. To answer this question, we examined the induction of type I collagens in murine 3T3 fibroblasts by Western blotting. As shown in Fig. 7, IL-13 induced collagen synthesis 48 h after stimulation. Minimal type I collagen was detected in unstimulated cells (Fig 7, lane 1) or at earlier time points in the cytokine-activated cultures (data not shown). IL-4 also induced collagen I synthesis (lane 2) and high levels of secreted collagen were easily detectable in the supernatants obtained from both cytokine-stimulated cultures (data not shown). The specificity of the reaction was confirmed by using purified collagen type I (lane 5) and bacterial collagenase treatments showed that the antibodies were specific for collagen (data not shown).

DISCUSSION

A $CD4^+$ Th2-type cytokine pattern dominates the immune response in mice infected with *S. mansoni* [12,13]. Previous IL-4 depletion studies and experiments with IL-4-deficient mice however, failed to show an indispensable role for this cytokine in the pathogenesis of schistosomiasis [15,23,24]. Indeed, while a partial reduction in fibrosis was observed in some studies [15], egg-induced granuloma formation could proceed in the complete absence of IL-4 [23,24]. In contrast to these observations, granuloma formation

and the development of hepatic fibrosis was severely impaired in Stat6-deficient mice [16], which display a major defect in the production of several Th2-associated cytokines [46]. IL-4 and IL-13 both signal through Stat6, therefore the apparent differences in pathology observed between infected IL-4-deficient and Stat6-deficient mice may be explained by IL-13. Nevertheless, the distinct contributions of IL-4 and IL-13 in disease progression can not be discerned from studies in Stat6 or IL-4-deficient mice alone. In this study, we used a potent inhibitor of IL-13 in infected WT and IL-4-deficient mice and demonstrate that IL-13 and IL-4 exhibit redundant, as well as unique roles in the pathogenesis of schistosomiasis.

Several studies have shown that Th2-type cytokine responses can develop *in vivo* in the absence of IL-4 or the IL-4 receptor [26,39], which is consistent with our findings since reduced but significant IL-13, IL-10, and IL-5 expression was detected in the mesenteric lymph nodes (Fig. 4) and livers (Fig. 5) of infected IL-4-deficient mice. Their production was also highly dependent on a $CD4^+$ T cell response (Fig. 4), further indicating that a conventional Th2-type response was established. These findings provide evidence that while maximal IL-13 expression is dependent on IL-4, the continued production of IL-13 might explain the maintenance of a significant granulomatous response in the absence of IL-4 [23-25]. Indeed, while blocking IL-13 alone had no effect on granuloma size in WT mice, inhibiting the residual IL-13 in IL-4-deficient mice resulted in a marked and highly significant reduction in granuloma volume (Fig. 2A). These findings demonstrate that IL-4 and IL-13 are both sufficient to mediate granuloma development, and formally explain the production of granulomas in IL-4-deficient mice versus the nearly complete lack of granulomas in Stat6-deficient mice [16,24]. They also support recent findings in the pulmonary egg granuloma model[30]. Because granulomas serve an important host-protective role by walling off potentially lethal hepatotoxins released by the eggs [47], the host may have evolved redundant mechanisms for granuloma formation in order to ensure a favorable host-parasite relationship.

While these observations clearly demonstrate that IL-4 and IL-13 actively participate in granuloma formation, unique roles for both cytokines in mast cell recruitment, tissue eosinophilia, and most importantly, the generation of hepatic fibrosis were revealed in these studies. Histological examinations of liver sections from infected mice demonstrated that IL-13 is not required for mast cell (Table III) or eosinophil (Fig.

2B) differentiation and recruitment, since granulomas of sIL-13Ra2-Fc-treated WT mice showed no decrease in either cell type. In fact, eosinophil numbers were significantly increased in the lesions of IL-13-inhibited WT mice (Fig. 2B), suggesting that IL-13 may partially antagonize this effect. In contrast, mast cells were almost completely absent from the lesions in IL-4-deficient mice and eosinophils were decreased by over 50%. Interestingly, IL-13 appears to partially support the reduced but significant egg-induced tissue eosinophilia in IL-4-deficient mice since eosinophils were reduced to below 10% in the IL-4-deficient/sIL-13Rα2-Fc-treated animals. Nevertheless, these data indicate that IL-4 is the dominant cytokine responsible for the development of eosinophil and mast cell populations within granulomas.

Probably the most important advance from this study was the finding that hepatic fibrosis could be blocked by sIL-13Rα2-Fc. Indeed, microscopic (Table III), biochemical (Fig.2C), and molecular techniques (Fig. 6) all indicated that IL-13, not IL-4, plays the major role in the development of egg-induced liver fibrosis. Previous studies showed that the Th1/Th2 cytokine balance can significantly effect the extent of tissue fibrosis in *S. mansoni* infected mice [19]. Nevertheless, this study suggests that the effects of sIL-13Rα2-Fc were not mediated through a skewing of the Th cell cytokine response. Blocking IL-13 had no significant effect on the production of IFN-γ, IL-4, IL-5, IL-10, or IL-13 by mesenteric lymph node (Fig. 4) or spleen cells *in vitro* and there was also no change in cytokine mRNA expression *in vivo*, at the site of lesion formation (Fig. 5). In contrast to these observations, IL-4-deficient mice displayed an increased IFN-γ response in the draining lymph nodes (Fig. 4) and decreased IL-5 and IL-13 expression in both the lymph nodes (Fig. 4) and liver (Fig. 5). Thus, the slight reduction in fibrosis detected in IL-4-deficient mice by hydroxyproline analysis (Fig. 2C) may be attributable to decreased IL-13 production. The fact that IL-4 production was unaffected by IL-13 blockade, yet fibrosis was maximally reduced in these animals emphasizes the important role played by IL-13. Indeed, sIL-13Rα2-Fc-treated IL-4-deficient mice showed little additional decrease in hydroxyproline levels (Fig. 2C) and no difference in Collagen I or III mRNA expression (Fig. 6) over that observed in similarly-treated WT mice. There was also no change in Collagen I or III mRNA expression in control-Fc-treated IL-4-deficient mice when compared with WT animals, further de-emphasizing the contribution of IL-4. Moreover, *in vitro* studies with 3T3 cells demonstrated for the first time the ability of IL-13 to

stimulate collagen production in fibroblasts (Fig. 7), thus the effects of IL-13 on fibrosis may be more direct and not dependent upon modulations in the Th1/Th2 cytokine response. In support of this conclusion, recent studies demonstrated that IL-13 receptors are expressed on fibroblasts [32] and that IL-13 increases adhesion molecule and inflammatory cytokine expression in human lung fibroblasts [48]. Finally, although IL-13 (Fig. 7) and IL-4[49] are both capable of promoting collagen production in fibroblasts, the fact that cultured lymph node cells produced nearly 100-fold more IL-13 than IL-4 (Fig. 4), only serves to emphasize the potentially important contribution of IL-13 in this process. Indeed, studies in the pulmonary granuloma model revealed that IL-4 mRNA expression is more tightly regulated at the site of lesion formation, while the induction of IL-13 mRNA is much more sustained over time [30]. Nevertheless, we have not examined the kinetics of IL-4 and IL-13 mRNA expression in infected animals, so we can not say whether a similar pattern holds in the granulomatous livers.

IL-13 was also recently shown to be important for resistance against intestinal nematodes [27,37-39]. Studies in IL-4 [39] and IL-13-deficient mice [37,38] suggested that IL-13, in contrast to IL-4, plays a requisite role in expulsion of both *N. brasiliensis* and *T. muris*. Nevertheless, the specific mechanism of worm expulsion remains unknown, although IL-4 and IL-13-induced changes in epithelial cells and gut physiology have been suggested as possible targets [50,51]. IL-13 also plays a central role in murine asthma models. In these studies, IL-13 was found to be necessary and sufficient for the expression of allergic asthma [40,41]. Subepithelial fibrosis and airway smooth muscle hypertrophy are common features of chronic severe asthma [5] and chronic pulmonary fibrosis is associated with the production of type III and type I collagen in the early and late stages of the disease, respectively. Thus the link between IL-13 and fibrosis revealed in our study elucidates the etiology of several important human diseases and provides more effective modes of treatment of fibrotic diseases in general.

Our previous studies showed that an egg specific IL-12-induced Th1 memory response could effectively reduce hepatic fibrosis in subsequently-infected mice[19]. The reduction in pathology was accompanied by a switch in the normal Th2 response to one dominated by Th1-type cytokines. Findings from the current study suggest that the anti-pathology effects of this IL-12 -based vaccination protocol may be explained by the inhibition of IL-13. Interestingly, a second study using a different protocol showed that

repeated rIL-12 injections given at 6 to 8 weeks, during the Th2-dominated phase of granuloma development, was almost completely ineffective at blocking granuloma formation and fibrosis [52]. Related studies have suggested that IL-12 is less capable of modulating established Th2-type responses [53], which likely explains the failure to modulate pathology in the latter study [52]. In contrast to these findings, sIL-13Rα2-Fc was extremely effective at reducing hepatic fibrosis, even though administered only during the later stages of infection. These findings indicate that IL-13 antagonism is a much more effective therapeutic approach to reduce fibrosis in situations where pathogenic Th2-type immune responses have already been established. In summary, our findings provide evidence that IL-13 inhibitors, such as the sIL-13Rα2-Fc, are of general therapeutic benefit in preventing fibrosis associated with chronic infectious disease and demonstrate the important and non-redundant role of IL-13 in the pathogenesis of schistosomiasis.

METHODS

Animals, Parasites and Ag preparations

6-8 week old female C57BL/6 and IL-4-deficient mice (C57BL/6 background, 10^{th} backcross) were obtained from Taconic Farms, Inc. (Germantown, NY). All mice were housed in a NIH American Association for the Accreditation of Laboratory Animal Care-approved animal facility in sterile filter-top cages and maintained on sterile water. Cercariae of a Puerto Rican strain of *Schistosoma mansoni* (NMRI) were obtained from infected *Biomphalaria glabrata* snails (Biomedical Research Institute, Rockville, MD). Soluble egg antigen (SEA) was purified from homogenized eggs, as previously described [15].

Reagents

The soluble IL-13 receptorα2-Fc fusion protein (sIL-13Rα2-Fc) was prepared as previously described [35] and provided by Genetics Institute, Cambridge MA. Endotoxin contamination was <2 EU/mg, as determined with the Cape Cod Associates LAL assay (Limulus Amebocyte Lysate, Woods Hole, MA). The in vitro ID50, as determined by the ability to neutralize 3ng/ml of murine IL-13 in the B9 proliferation assay, was approximately 10 ng/ml. Human IgG (control-Fc), which was used as a control for sIL-13Rα2-Fc, was affinity purified by recombinant Protein A-Sepharose chromatography, as

described for sIL-13Rα2-Fc [35]. As described previously, the control-Fc had no detectable effect on pathology or cytokine expression in infected mice [30].

Infection and treatments

Mice were infected by percutaneous challenge of tail skin for 40 min in water containing between 20 and 25 cercariae. Animals were treated with either a human control-Fc or with the sIL-13Rα2-Fc by i.p. injection in 0.5 ml PBS, every other day after the onset of egg production (week 5). The optimal concentration for in vivo use (200 μg/mouse/day) was chosen based on kinetic assays and on dose response experiments in sensitized/i.v. egg-injected mice [30]. Sera were collected from mice on the day of sacrifice. All animals were sacrificed by i.p. administration of sodium pentobarbital (18 mg/mouse, Sigma, St. Louis, MO) on week 8 and perfused with citrated saline to assess worm burdens [15]. No mortality was observed among any of the treated groups.

Histopathology and fibrosis measurement

For measurement of granulomas, approximately half of the liver was fixed with Bouin-Hollande fixative and processed as previously described [15]. The size of hepatic granulomas was determined in histological sections stained by Wright's Giemsa stain (Histopath of America, Clinton, MD). The diameters of each granuloma containing a single viable egg were measured with an ocular micrometer and the volume of each granuloma calculated assuming a spherical shape. The mean of the longest diameter and the diameter perpendicular to that was used. The percentage of eosinophils, mast cells and other cell types were evaluated in the same sections. Parenchymal necrosis was scored on a scale of 0-4, with 0 being the least and 4 being the most extensive necrosis. The frequency of mast cells was also assigned on a similar scale, using a range from 0-4. The number of schistosome eggs in the liver and gut and the collagen content of the liver, determined as hydroxyproline, were measured as described previously [15]. Fibrosis was also scored histologically using sections stained with picrosirius red. The picrosirius reagent stains collagen specifically and when sections are viewed under polarizing light, the bright areas where collagen is deposited are illuminated. All granulomas within each section were scored for picrosirius (red) "density" based on a scale 1-4, and a second measurement of "area involved" was also determined using the same scale. The total

fibrosis score was determined by multiplying the density and area for each granuloma (ie. a score of 16 would be the maximum). An average of 30 granulomas per mouse was included in all analyses. To control for consistency, the same individual scored all histological features and had no knowledge of the experimental design.

Isolation and purification of RNA

Two portions of the liver from each animal were combined and placed in 1 ml of RNA-STAT 60 (Tel-Test), frozen on dry ice and kept at -70°C until use. Tissues were homogenized using a tissue polytron (Omni International Inc., Waterbury, CT) and total RNA was extracted following the recommendations of the manufacturer. The RNA was resuspended in DEPC-treated water and quantitated spectrophotometrically.

RT-PCR detection of cytokine mRNA

A RT-PCR procedure was used to determine relative quantities of mRNA for IL-4, IL-5, IL-10, IL-13, IFN-γ, collagen I, collagen III, TGFβ1, TGFβ2, and HPRT (hypoxanthine-guanine phosphoribosyl transferase). The cDNA was obtained after reverse transcription of 1 μg of RNA as described [14]. The primers and probes for all genes were previously published [14,19,54]. The PCR cycles used for each cytokine were as follows: IL-4 (33), IL-5 (31), IFN-γ (29), collagen I (26), collagen III (22), TGFβ1 (34), TGFβ2 (34), and HPRT (23).

Analysis and quantification of PCR products

The amplified DNA was analyzed by electrophoresis, Southern blotting and hybridization with non-radioactive cytokine-specific probes as previously described [14]. The PCR products were detected using a ECL detection system (Amersham). The chemiluminescent signals were quantified using a flat-bed scanner (Microtek model 600 ZS, Torrance, CA). The amount of PCR product was determined by comparing the ratio of cytokine-specific signal density to that of HPRT-specific signal density for individual samples. Arbitrary densitometric units for individual samples were subsequently multiplied by a factor of 100.

In vitro cultures

Mesenteric lymph node (MLN) cells and spleens were extracted from the mice and single cell suspensions were prepared. Red blood cells were lysed by osmotic treatment with ACK lysing buffer (Biofluids, Inc., Rockville, MD). Cells were placed in RPMI 1640 medium supplemented with 10% FCS, 2mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPES, 1mM sodium pyruvate, 0.1 mM nonessential amino acids, and 50 μM 2-ME at 37°C in 5% CO_2. Cells were plated in 24-well plates (3 x 10^6/ml, 1ml) and stimulated with SEA (20 μg/ml) and supernatants were collected after 72 h to measure the levels of IL-4, IL-5, IL-10, IL-13 and IFN-g. Additional SEA-stimulated cultures were also treated with 50 μg/ml of anti-CD4 mAb (GK1.5). Cultures treated with anti-CD4 mAb alone showed no change in cytokine expression when compared with that observed in medium control cultures (data not shown). IL-5, IL-10, and IFN-g were measured using specific sandwich ELISA [15]. IL-13 levels were measured using murine IL-13 ELISA kits (R&D Systems, Minneapolis, MN). Cytokine levels were calculated from curves prepared with recombinant cytokines. IL-4 was measured using the IL-4 sensitive cell line CT.4S. Proliferation of these cells was quantified by (^{3}H)TdR incorporation, and the amount of cytokine was determined by comparison with known amounts of recombinant IL-4.

Western blot detection of collagen I

3T3 fibroblasts were cultured in RPMI 1640 medium supplemented with 10% FCS, 2mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPES, 1mM sodium pyruvate, 0.1 mM nonessential amino acids, and 50 μM 2-ME at 37°C in 5% CO_2. Confluent cells were plated in 24-well plates (500,000 cells/ml) and stimulated with IL-4 (1000 U/ml) or rIL-13 (R&D Systems, Minneapolis, MN) (20 ng/ml) for 6, 24 and 48 hs. Culture supernatants were collected to analyze secreted collagen I. Cells were washed once with phosphate buffered saline and lysed with SDS-PAGE sample buffer. Cell lysates and culture supernatants were submitted to electrophoretic separation in 6% tris-glycine gels (Novel Experimental Technology, San Diego, CA) using reducing conditions, and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Blots were probed with rabbit IgG anti-mouse type I collagen (Biodesign International, Kennenbunk, ME) and peroxidase labelled anti-rabbit IgG (Amersham Pharmacia Biotech,

Inc., Piscataway,NJ) was used as a second Ab. The bands were visualized using a western blot chemiluminescence reagent (NEN Life Science Products, Boston, MA). To confirm identity of the collagen bands, cell lysates were treated with 0.5 mg/ml of collagenase (Boehringer Mannheim, Indianapolis, IN) in PBS, supplemented with 1 mM $CaCl_2$ and 1% FCS, for 1 h at 37°C. A purified rat collagen I preparation was also used as a control.

Statistics

Schistosome worm and egg numbers, changes in cytokine mRNA, and values for secreted cytokine proteins were compared using Student's two-tailed t test. Hepatic fibrosis was compared by analysis of covariance, using the log of total liver eggs as the covariate and the log of hydroxyproline per egg. $p < 0.05$ was considered significant.

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All patent and literature references cited herein are incorporated by reference as if fully set forth.

What is claimed is:

1. A method of treating tissue fibrosis in a mammalian subject, said method comprising administering a therapeutically effective amount of a pharmaceutical composition comprising a protein and a pharmaceutically acceptable carrier, wherein said protein comprises an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence of SEQ ID NO:2;

(b) the amino acid sequence of SEQ ID NO:2 from amino acids 22 to 334;

(c) the amino acid sequence of SEQ ID NO:2 from amino acids 357 to 383;

(d) the amino acid sequence of SEQ ID NO:4;

(e) the amino acid sequence of SEQ ID NO:4 from amino acids 26 to 341;

(f) the amino acid sequence of SEQ ID NO:4 from amino acids 363 to 380; and

(g) fragments of (a)-(f) having a biological activity of the IL-13 receptor binding chain..

2. The method of claim 1 wherein said tissue fibrosis affects a tissue selected from the group consisting of liver, skin epidermis, skin endodermis, muscle, tendon, cartilage, cardiac tissue, pancreatic tissue, lung tissue, uterine tissue, neural tissue, testis, ovary, adrenal gland, artery, vein, colon, small intestine, biliary tract and gut.

3. The method of claim 2 wherein said tissue is liver.

4. The method of claim 2 wherein said fibrosis is that resulting from infection with schistosoma.

5. The method of claim 1 wherein said fibrosis is that resulting from healing of a wound.

6. A method of inhibiting formation of tissue fibrosis in a mammalian subject, said method comprising administering a therapeutically effective amount of a pharmaceutical composition comprising a protein and a pharmaceutically acceptable carrier, wherein said protein comprises an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence of SEQ ID NO:2;

(b) the amino acid sequence of SEQ ID NO:2 from amino acids 22 to 334;

(c) the amino acid sequence of SEQ ID NO:2 from amino acids 357 to 383;

(d) the amino acid sequence of SEQ ID NO:4;

(e) the amino acid sequence of SEQ ID NO:4 from amino acids 26 to 341;

(f) the amino acid sequence of SEQ ID NO:4 from amino acids 363 to 380; and

(g) fragments of (a)-(f) having a biological activity of the IL-13 receptor binding chain..

7. The method of claim 6 wherein said tissue fibrosis affects a tissue selected from the group consisting of liver, skin epidermis, skin endodermis, muscle, tendon, cartilage, cardiac tissue, pancreatic tissue, lung tissue, uterine tissue, neural tissue, testis, ovary, adrenal gland, artery, vein, colon, small intestine, biliary tract and gut.

8. The method of claim 7 wherein said tissue is liver.

9. The method of claim 7 wherein said fibrosis is that resulting from infection with schistosoma.

10. The method of claim 6 wherein said fibrosis is that resulting from healing of a wound.

11. The method of claim 10 wherein said wound is a surgical incision.

12. The method of claim 5 wherein said wound is a surgical incision.

13. A method of treating tissue firbrosis in a mammalian subject, said method comprising administering a therapeutically effective amount of a composition comprising (a) a molecule selected from the group consisting of an IL-13 antagonist and an IL-4 antagonist, and (b) a pharmaceutically acceptable carrier.

14. The method of claim 13 wherein said antagonist is selected from the group consisting of an IL-13bc protein, a soluble form of IL-13Rα1, an antibody to IL-13 or an IL-13-binding fragment thereof, an antibody to IL-13bc or an IL-13bc-binding fragment thereof, an antibody to IL-13Rα1 or an IL-13Rα1-binding fragment thereof, IL-13R-binding mutants of IL-4, a small molecule capable of inhibiting the interaction of IL-13 with IL-13bc and a small molecule capable of inhibiting the interaction of IL-13 with IL-13Rα1.

15. The method of claim 14 wherein said IL-13bc protein is a protein comprising an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence of SEQ ID NO:2;

(b) the amino acid sequence of SEQ ID NO:2 from amino acids 22 to 334;

(c) the amino acid sequence of SEQ ID NO:2 from amino acids 357 to 383;

(d) the amino acid sequence of SEQ ID NO:4;

(e) the amino acid sequence of SEQ ID NO:4 from amino acids 26 to 341;

(f) the amino acid sequence of SEQ ID NO:4 from amino acids 363 to 380; and

(g) fragments of (a)-(f) having a biological activity of the IL-13 receptor binding chain.

16. The method of claim 13 wherein said tissue fibrosis affects a tissue selected from the group consisting of liver, skin epidermis, skin endodermis, muscle, tendon, cartilage, cardiac tissue, pancreatic tissue, lung tissue, uterine tissue, neural tissue, testis, ovary, adrenal gland, artery, vein, colon, small intestine, biliary tract and gut.

17. The method of claim 16 wherein said tissue is liver.

18. The method of claim 17 wherein said fibrosis is that resulting from infection with schistosoma.

19. The method of claim 13 wherein said fibrosis is that resulting from healing of a wound.

20. The method of claim 19 wherein said wound is a surgical incision.

21. A method of inhibiting formation of tissue firbrosis in a mammalian subject, said method comprising administering a therapeutically effective amount of a composition comprising (a) a molecule selected from the group consisting of an IL-13 antagonist and an IL-4 antagonist, and (b) a pharmaceutically acceptable carrier.

22. The method of claim 21 wherein said antagonist is selected from the group consisting of an IL-13bc protein, a soluble form of IL-13Rα1, an antibody to IL-13 or an IL-13-binding fragment thereof, an antibody to IL-13bc or an IL-13bc-binding fragment thereof, an antibody to IL-13Rα1 or an IL-13Rα1-binding fragment thereof, IL-13R-binding mutants of IL-4, a small molecule capable of inhibiting the interaction of IL-13 with IL-13bc and a small molecule capable of inhibiting the interaction of IL-13 with IL-13Rα1.

23. The method of claim 22 wherein said IL-13bc protein is a protein comprising an amino acid sequence selected from the group consisting of:

(a) the amino acid sequence of SEQ ID NO:2;

(b) the amino acid sequence of SEQ ID NO:2 from amino acids 22 to 334;

(c) the amino acid sequence of SEQ ID NO:2 from amino acids 357 to 383;

(d) the amino acid sequence of SEQ ID NO:4;

(e) the amino acid sequence of SEQ ID NO:4 from amino acids 26 to 341;

(f) the amino acid sequence of SEQ ID NO:4 from amino acids 363 to 380; and

(g) fragments of (a)-(f) having a biological activity of the IL-13 receptor binding chain.

24. The method of claim 21 wherein said tissue fibrosis affects a tissue selected from the group consisting of liver, skin epidermis, skin endodermis, muscle, tendon, cartilage, cardiac tissue, pancreatic tissue, lung tissue, uterine tissue, neural tissue, testis, ovary, adrenal gland, artery, vein, colon, small intestine, biliary tract and gut.

25. The method of claim 24 wherein said tissue is liver.

26. The method of claim 25 wherein said fibrosis is that resulting from infection with schistosoma.

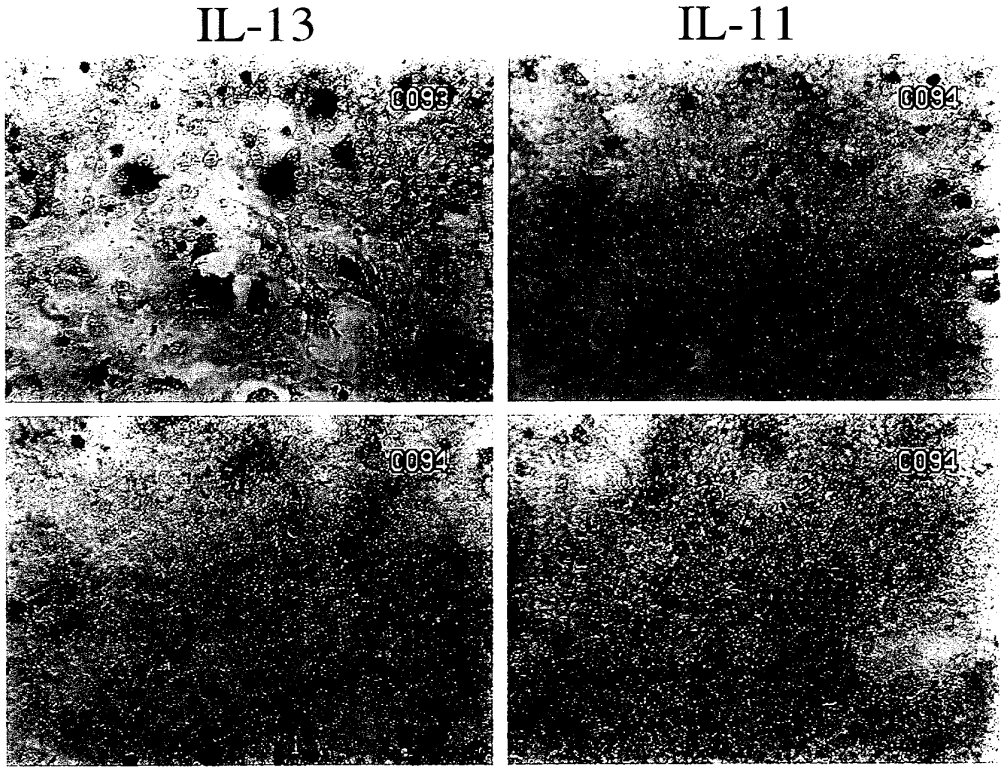
27. The method of claim 21 wherein said fibrosis is that resulting from healing of a wound.

28. The method of claim 27 wherein said wound is a surgical incision.

29. The method of claim 21 wherein said antagonist is selected from the group consisting of a soluble form of IL-4R, an antibody to IL-4 or an IL-4-binding fragment thereof, an antibody to IL-4R or an IL-4R-binding fragment thereof, and a small molecule capable of inhibiting the interaction of IL-4 with IL-4R.

30. The method of claim 13 wherein said antagonist is selected from the group consisting of a soluble form of IL-4R, an antibody to IL-4 or an IL-4-binding fragment thereof, an antibody to IL-4R or an IL-4R-binding fragment thereof, and a small molecule capable of inhibiting the interaction of IL-4 with IL-4R.

Figure 1



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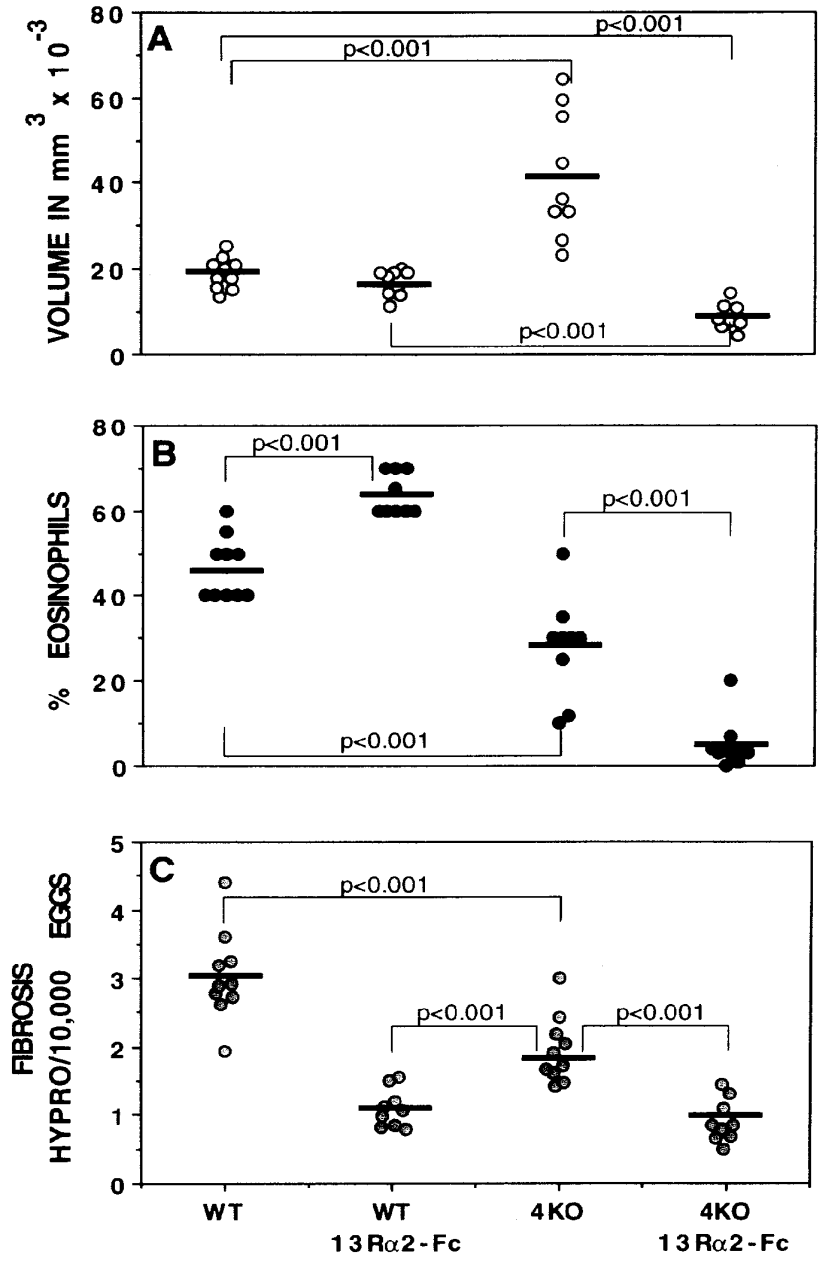
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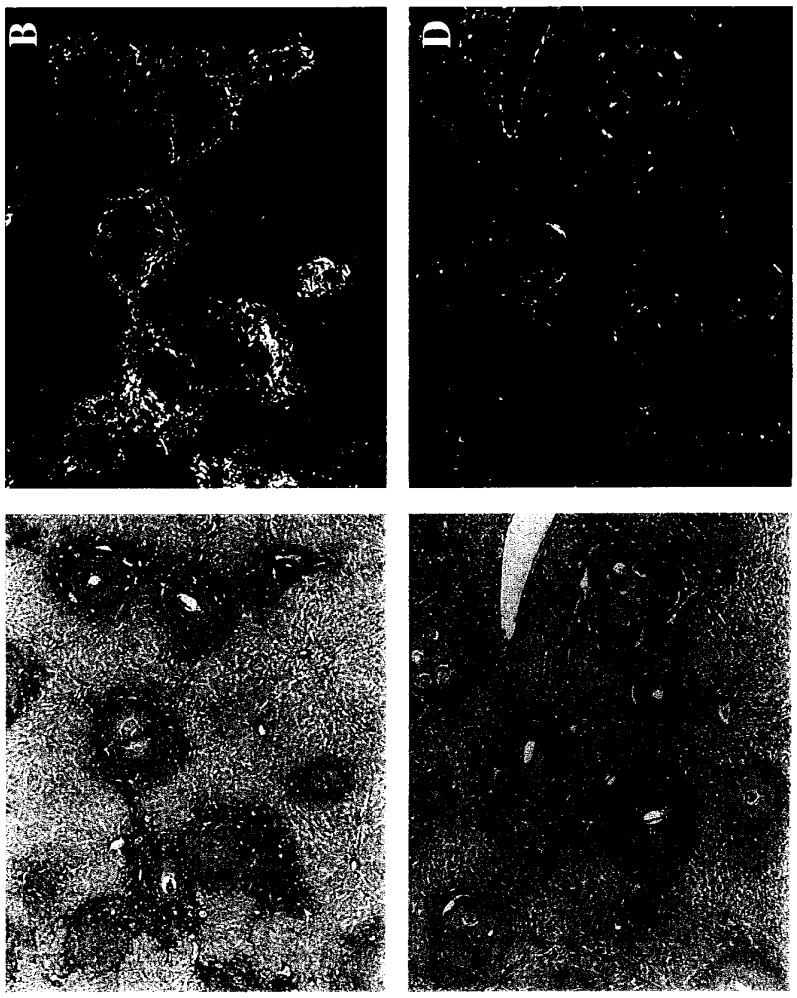
Figure 3



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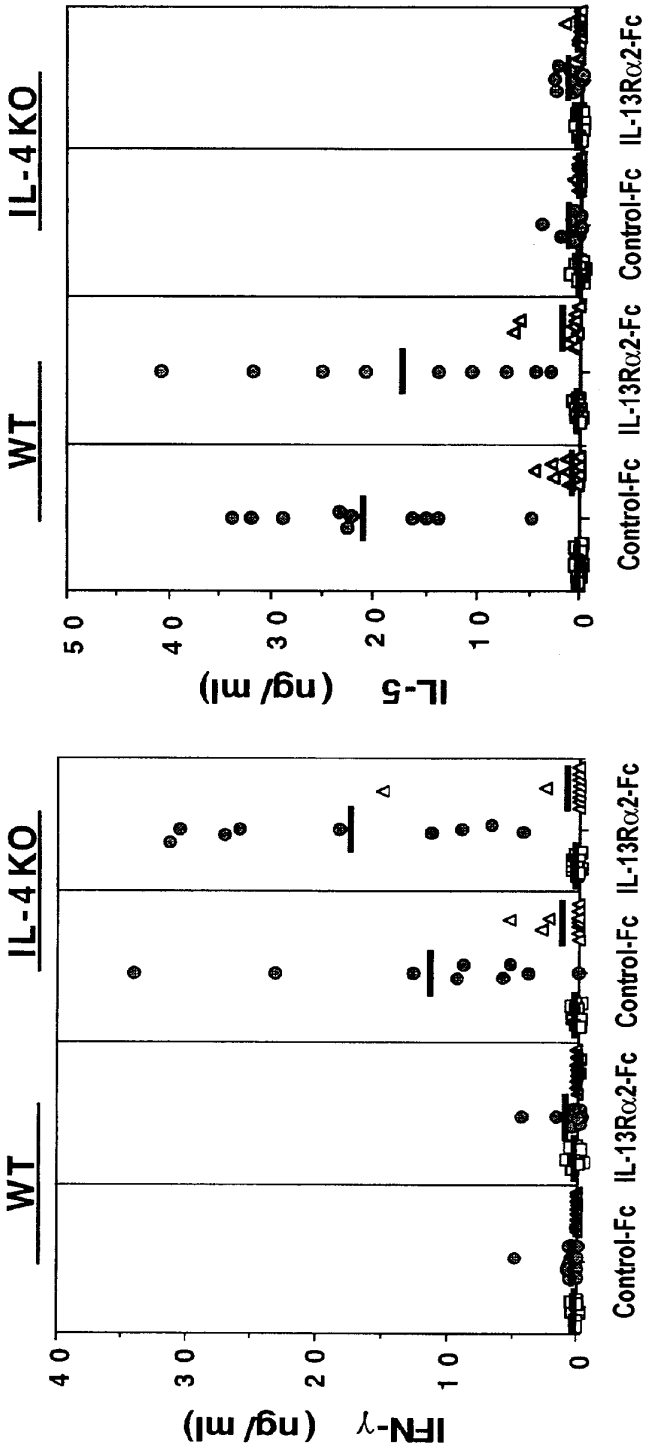
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Figure 4B

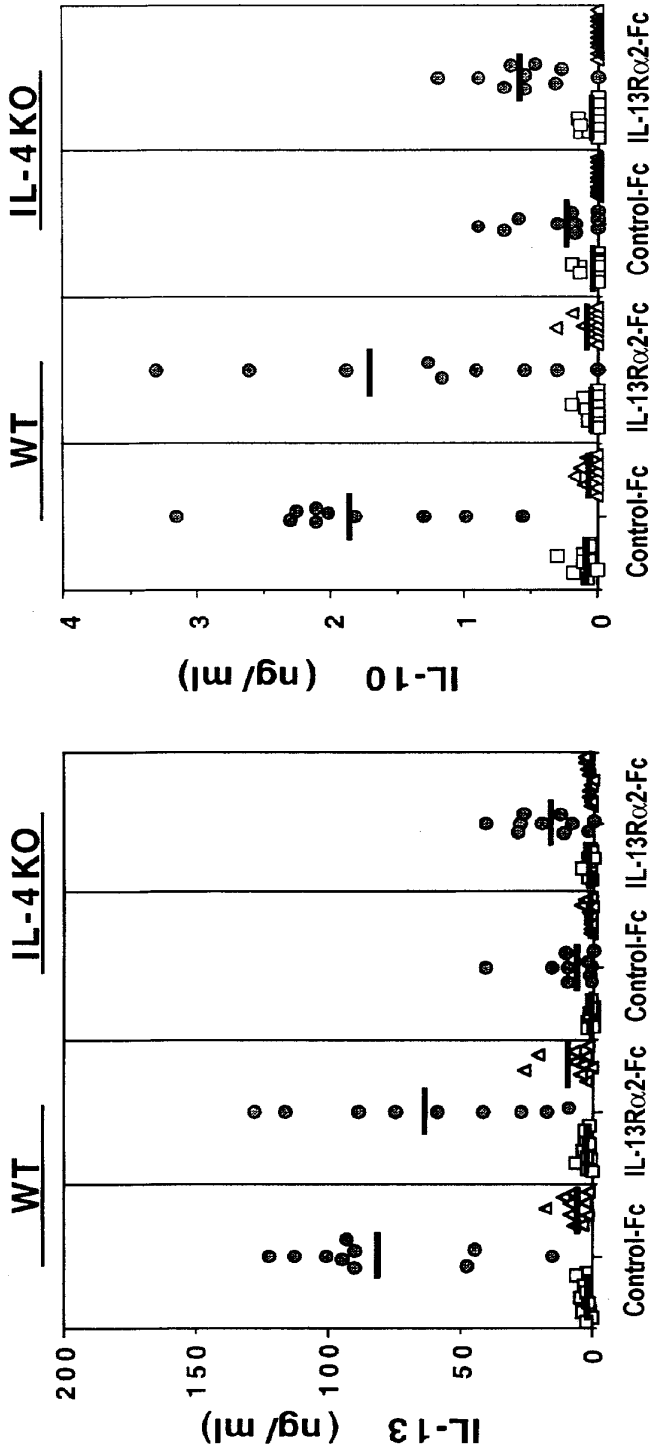
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Figure 4C

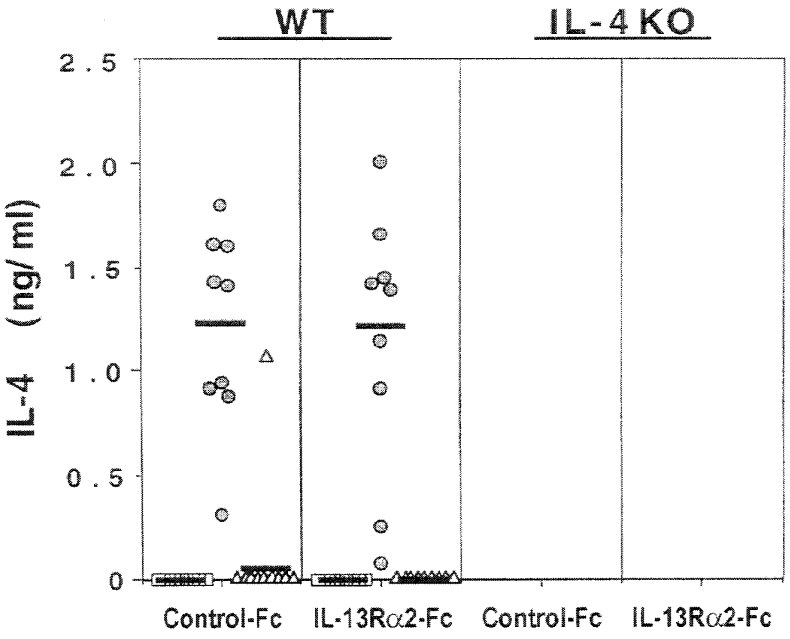
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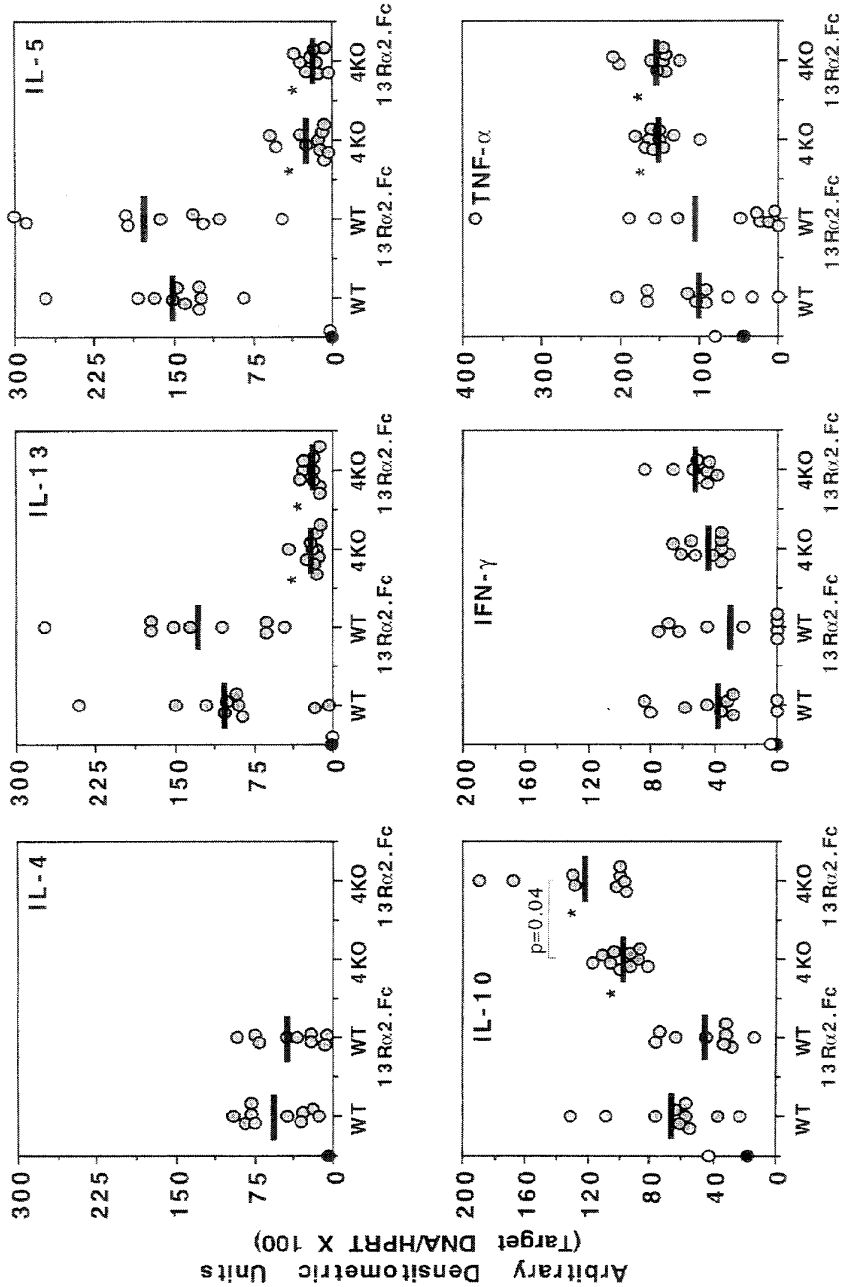
Figure 5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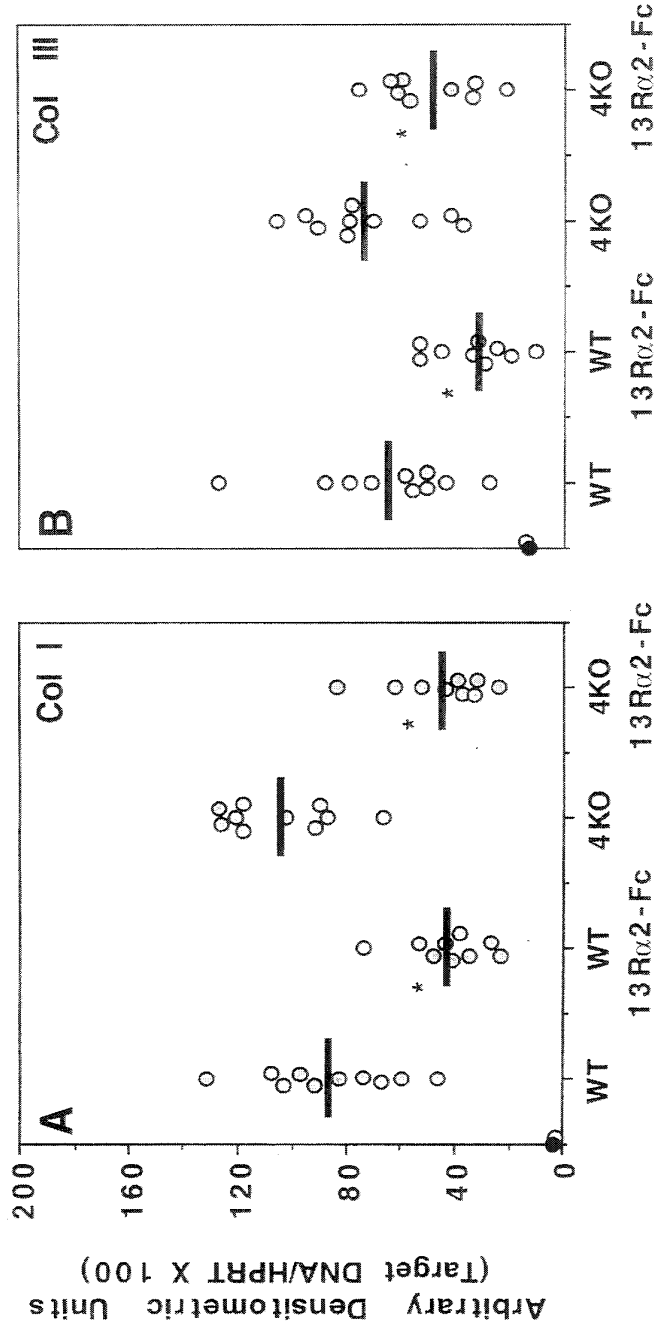
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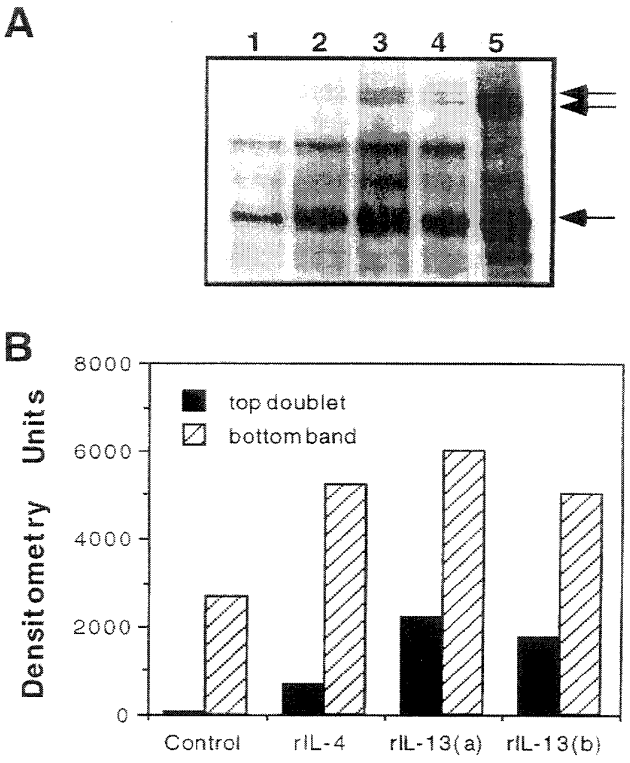
Figure 7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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/17103

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A61K 38/16, 38/20
US CL :514/2, 12, 917; 424/145.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2, 12, 917; 424/145.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

West-US Patent full, STN via medline, embase, caplus, biosis. Search terms: interleukin 13 inhibit? or antagonis?, fibrosis, treatment or prevention

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CAPUT et al. Cloning and Characterization of a Specific Interleukin (IL)-13 Binding Protein Structurally Related to the IL-5 Receptor Alpha Chain. Journal Of Biological Chemistry. 12 July 1996. Vol.271, No.28, pages 16921-16926, see entire document.	1-30
A	HANCOCK et al. Production of Interleukin-13 by Alveolar Macrophages from Normal and Fibrotic Lung. American Journal of Respiratory Cell and Molecular Biology. January 1998. Vol.18, No.1, pages 60-67, especially see second and last paragraphs of page 65.	1-30

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
24 SEPTEMBER 2000	16 OCT 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer FOZIA HAMUD Telephone No. (703) 308-0196

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/20609 A2

(54) Title: NEW USE

(57) Abstract: New uses for interleukin 11 polypeptides and polynucleotides encoding IL-11 polypeptides are disclosed. Such uses include their use in therapy and in identifying agonist compounds which are potentially useful in therapy.

New Use

Field of the Invention

This invention relates to new uses for interleukin 11 polypeptides and polynucleotides encoding such polypeptides, to their use in therapy and in identifying agonist or antagonist compounds which are potentially useful in therapy.

Summary of the Invention

In one aspect, the invention relates to new uses of interleukin 11 (hereinafter IL-11) polypeptides disclosed in Paul SR et al *Proc Natl Acad Sci U S A* (1990) 87(19):7512-6. Such uses include the treatment of stroke and neuropathies hereinafter referred to as "the Diseases", amongst others. In another aspect the invention relates to methods for identifying agonists and antagonists using the IL-11 polypeptides and IL-11 receptor polypeptides, and the use of these agonists or antagonists for treating conditions such as stroke.

Description of the Invention

Stroke is a disease of major clinical significance in the western world, in the United States alone it is the third largest cause of death. As the population of developed countries has aged, the incidence of stroke has increased in accordance. While stroke mortality occurs in only 20% of cases, it is a leading cause of long term disability. Despite huge research efforts by both academic and industrial organisations and the great economic burden induced by stroke, there are at present few treatment options. Thrombolytics, primarily tissue-type plasminogen activator (tPA), are the most common treatment, and the window of opportunity for their use (usually within 3 hrs of stroke) are such that they are effective in fewer than 10% of cases.

Interleukin 11 (IL-11) was first identified as an activity in bone marrow derived stromal cell line. It is synthesised as a 199 amino acid precursor that undergoes proteolytic cleavage to remove the signal peptide and allow the mature, 178 amino acid peptide to be secreted. The mature cytokine forms a four-helix bundle structure that signals through a heteromeric receptor complex of a specific alplha subunit, IL-11R, and a beta subunit that

is shared amongst a number of cytokine receptors, the gp130 subunit. The gp130 family of cytokines, as they are known, includes IL-6, LIF, Oncostatin M and CNTF in addition to IL-11, and are functionally as well as mechanistically linked, at least partly as a result of the common receptor signaling pathway. The binding of cytokine to the receptor elicits a response through the JAK kinase/STAT transcription factor signalling pathway which ultimately leads to transcriptional changes in the nucleus.

IL-11 was initially described as a growth factor, and has been shown to be a potent stimulator of thrombopoiesis. Indeed it is approved for the treatment of chemotherapy induced thrombocytopenia, where its platelet restorative capacity is of benefit. A clue that this may not be the sole function of IL-11 comes from the fact that it is rarely detected in the circulation, but can be detected at sites of chronic inflammation such as seen with Rheumatoid Arthritis (RA) and Inflammatory bowel syndrome (IBS). In vitro, IL-11 has been shown to downregulate macrophage function, in particular, IL-11 has been shown to inhibit the induction of proinflammatory mediators such as TNFalpha, IL-1beta, IL-12 and iNOS which are normally induced in response to challenge. This anti-inflammatory role for IL-11 is supported by the beneficial outcomes noted for IL-11 treatment of both acute inflammation such as lipopolysaccharide (LPS) treatment and chronic conditions such as RA and IBS in various organs in animal models. Indeed IL-11 is in clinical trials for the treatment of IBS, chemotherapy induced mucositis and psoriasis. These properties, either alone or in the aggregate, are hereinafter referred to as "IL-11 activity" or "IL-11 polypeptide activity" or "biological activity of IL-11". Preferably an IL-11 polypeptide of the invention exhibits at least one biological activity of IL-11.

The present invention is based on the finding that IL-11 levels are significantly increased in a rat stroke model. In a first aspect, the present invention relates to the use of a compound selected from:

(a) an IL-11 polypeptide;

(b) a compound which mimics an IL-11 polypeptide;

(c) a compound which activates an IL-11 receptor;

(d) a compound which inhibits activation of an IL-11 receptor; or

(d) a polynucleotide encoding an IL-11 polypeptide,

for the manufacture of a medicament for treating stroke or neuropathies.

In a preferred embodiment the present invention relates to the use of an IL-11 polypeptide or a compound which mimics an IL-11 polypeptide for the manufacture of a medicament for treating stroke or neuropathies.

Such IL-11 polypeptides include those having at least 95% identity to the IL-11 polypeptide of SEQ ID NO:2 or SEQ ID NO:3, and further including sequence variants and short forms of IL-11 that retain IL-11 activity, as hereinabove defined. By "short form" it is meant an IL-11 polypeptide that is truncated at one or both of the N-terminal or C-terminal ends whilst retaining IL-11 activity as herinabove defined. Such polypeptides include those comprising a polypeptide having the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:3 as well as the polypeptides of SEQ ID NO:2 or SEQ ID NO:3.

The polypeptides of the present invention may be in the form of the "mature" protein (for example SEQ ID NO:3) or may be a part of a larger protein such as the IL-11 precursor protein (for example SEQ ID NO:2) or a fusion protein. It is often advantageous to include an additional amino acid sequence which contains secretory or leader sequences, pro-sequences, sequences which aid in purification such as multiple histidine residues, or an additional sequence for stability during recombinant production.

The present invention also includes variants of the aforementioned polypeptides, that is polypeptides that vary from the referents by conservative amino acid substitutions, whereby a residue is substituted by another with like characteristics. Typical such substitutions are among Ala, Val, Leu and Ile; among Ser and Thr; among the acidic residues Asp and Glu; among Asn and Gln; and among the basic residues Lys and Arg; or aromatic residues Phe and Tyr. Particularly preferred are variants in which several, 5-10, 1-5, 1-3, 1-2 or 1 amino acids are substituted, deleted, or added in any combination.

IL-11 polypeptides of the present invention can be prepared in any suitable manner. Such polypeptides include isolated naturally occurring polypeptides, recombinantly produced polypeptides prepared using IL-11 encoding polynucleotides, synthetically produced polypeptides, or polypeptides produced by a combination of these methods. The mature IL-11 polypeptide is available commercially, for example from R&D Systems (R&D Systems Europe Ltd, 4-10 The Quadrant, Barton Ln., Abingdon, Oxon, OX143YS, United Kingdom). Alternatively means for preparing such polypeptides are well understood in the art.

In a further aspect, the present invention relates to IL-11 polynucleotides. Such polynucleotides may be used, for example, for gene therapy. Such gene therapy includes introducing the IL-11 polynucleotide sequence into somatic cells to replace a defective IL-11 gene, or to enhance the production of IL-11 in, for example, acute illness such as stroke. Such polynucleotides include isolated polynucleotides comprising a nucleotide sequence encoding a polypeptide which has at least 95% identity to the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:3, over the entire length of SEQ ID NO:2 or SEQ ID NO:3. In this regard, polypeptides which have at least 97% identity are highly preferred, whilst those with at least 98-99% identity are more highly preferred, and those with at least 99% identity are most highly preferred. Such polynucleotides include a polynucleotide comprising the nucleotide sequence contained in SEQ ID NO:1 encoding the polypeptide of SEQ ID NO:2 or SEQ ID NO:3.

Further polynucleotides of the present invention include isolated polynucleotides comprising a nucleotide sequence which has at least 95% identity to SEQ ID NO:1 over the entire length of SEQ ID NO:1. In this regard, polynucleotides which have at least 97% identity are highly preferred, whilst those with at least 98-99% identiy are more highly preferred, and those with at least 99% identity are most highly preferred. Such polynucleotides include a polynucleotide comprising the polynucleotide of SEQ ID NO:1 as well as the polynucleotide of SEQ ID NO:1

The nucleotide sequence of SEQ ID NO:1 is a human cDNA sequence and comprises a polypeptide encoding sequence (137-736) encoding an IL-11 precursor polypeptide of 199 amino acids, the polypeptide of SEQ ID NO:2. The active IL-11 polypeptide, from amino acid 22 to 199 (the polypeptide of SEQ ID NO:3), is cleaved from the precursor by processing enzymes. The nucleotide sequence encoding the polypeptide of SEQ ID NO:2 or SEQ ID NO:3 may be identical to the polypeptide encoding sequence contained in SEQ ID NO:1 or it may be a sequence other than the one contained in SEQ ID NO:1, which, as a result of the redundancy (degeneracy) of the genetic code, also encodes the polypeptides of SEQ ID NO:2 or SEQ ID NO:3.

Preferred polypeptides and polynucleotides of the present invention are expected to have, *inter alia*, similar biological functions/properties to their homologous polypeptides and polynucleotides. Furthermore, preferred polypeptides and polynucleotides of the present invention have at least one IL-11 activity.

IL-11 encoding polynucleotides of the present invention may be obtained, using standard cloning and screening techniques (Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.(1989)), from a cDNA library derived from, for example, mRNA in cells of bone marrow fibroblast (eg Paul, SR et al *supra*). Polynucleotides of the invention can also be obtained from natural sources such as genomic DNA libraries or can be synthesized using well known and commercially available techniques.

When polynucleotides of the present invention are used for the recombinant production of IL-11 polypeptides of the present invention, the polynucleotide may include the coding sequence for the mature polypeptide, by itself; or the coding sequence for the mature polypeptide in reading frame with other coding sequences, such as those encoding a leader or secretory sequence, a pre-, or pro- or prepro- protein sequence, or other fusion peptide portions. In a preferred embodiment the polynucleotide will include a polynucleotide sequence encoding the natural IL-11 leader sequence, preferably the natural polynucleotide sequence (as in SEQ ID NO:1). The polynucleotide may also contain non-coding 5' and 3'

sequences, such as transcribed, non-translated sequences, splicing and polyadenylation signals, ribosome binding sites and sequences that stabilize mRNA.

Further embodiments of the present invention include polynucleotides encoding polypeptide variants which comprise the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:3 and in which several, for instance from 5 to 10, 1 to 5, 1 to 3, 1 to 2 or 1, amino acid residues are substituted, deleted or added, in any combination.

Recombinant polypeptides of the present invention may be prepared by processes well known in the art from genetically engineered host cells comprising expression systems. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention.

For recombinant IL-11 production, host cells can be genetically engineered to incorporate expression systems or portions thereof for IL-11 polynucleotides of the present invention. Introduction of polynucleotides into host cells can be effected by methods described in many standard laboratory manuals, such as Davis et al., Basic Methods in Molecular Biology (1986) and Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989). Preferred such methods include, for instance, calcium phosphate transfection, DEAE-dextran mediated transfection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape loading, ballistic introduction or infection.

Representative examples of appropriate hosts include bacterial cells, such as *Streptococci*, *Staphylococci*, *E. coli*, *Streptomyces* and *Bacillus subtilis* cells; fungal cells, such as yeast cells and *Aspergillus* cells; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 or sf21cells; animal cells such as CHO, COS, HeLa, C127, 3T3, BHK, HEK 293 and Bowes melanoma cells; and plant cells.

A great variety of expression systems can be used, for instance, chromosomal, episomal and virus-derived systems, e.g., vectors derived from bacterial plasmids, from bacteriophage,

from transposons, from yeast episomes, from insertion elements, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids. The expression systems may contain control regions that regulate as well as engender expression. Generally, any system or vector which is able to maintain, propagate or express a polynucleotide to produce a polypeptide in a host may be used. The appropriate nucleotide sequence may be inserted into an expression system by any of a variety of well-known and routine techniques, such as, for example, those set forth in Sambrook *et al.*, Molecular Cloning, A Laboratory Manual (supra). Appropriate secretion signals may be incorporated into the desired polypeptide to allow secretion of the translated protein into the lumen of the endoplasmic reticulum, the periplasmic space or the extracellular environment. These signals may be endogenous to the polypeptide or they may be heterologous signals.

In a preferred embodiment the IL-11 polypeptide is expressed using a baculovirus system in sf9 or sf21 cells.

Polypeptides of the present invention can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography is employed for purification. Well known techniques for refolding proteins may be employed to regenerate active conformation when the polypeptide is denatured during isolation and or purification.

The polypeptides of the invention or their fragments or analogs thereof, or cells expressing them, can also be used as immunogens to produce antibodies immunospecific for polypeptides of the present invention. The term "immunospecific" means that the

antibodies have substantially greater affinity for the polypeptides of the invention than their affinity for other related polypeptides in the prior art.

Antibodies generated against polypeptides of the present invention may be obtained by administering the polypeptides or epitope-bearing fragments, analogs or cells to an animal, preferably a non-human animal, using routine protocols. For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler, G. and Milstein, C., Nature (1975) 256:495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor *et al.*, Immunology Today (1983) 4:72) and the EBV-hybridoma technique (Cole *et al.*, Monoclonal Antibodies and Cancer Therapy, 77-96, Alan R. Liss, Inc., 1985).

Techniques for the production of single chain antibodies, such as those described in U.S. Patent No. 4,946,778, can also be adapted to produce single chain antibodies to polypeptides of this invention. Also, transgenic mice, or other organisms, including other mammals, may be used to express humanized antibodies.

The above-described antibodies may be employed to isolate or to identify clones expressing the polypeptide or to purify the polypeptides by affinity chromatography.

Antibodies against polypeptides of the present invention may also be employed to treat the Diseases, amongst others. For example antibodies which mimic the effect of IL-11 may be useful in this regard.

In a further aspect, the present invention relates to genetically engineered soluble fusion proteins comprising an IL-11 polypeptide of the present invention, or a fragment thereof, and various portions of the constant regions of heavy or light chains of immunoglobulins of various subclasses (IgG, IgM, IgA, IgE). Preferred as an immunoglobulin is the constant part of the heavy chain of human IgG, particularly IgG1, where fusion takes place at the hinge region. In a particular embodiment, the Fc part can be removed simply

by incorporation of a cleavage sequence which can be cleaved with blood clotting factor Xa. Furthermore, this invention relates to processes for the preparation of these fusion proteins by genetic engineering, and to the use thereof for drug screening, diagnosis and therapy. A further aspect of the invention also relates to polynucleotides encoding such fusion proteins. Examples of fusion protein technology can be found in International Patent Application Nos. WO94/29458 and WO94/22914.

In a further aspect of the invention there is provided screening methods useful to identify compounds which activate (agonists) or inhibit activation of (antagonists) the IL-11 receptor, which compounds may then be used in the manufacture of a medicament for use in the treatment of stroke or neuropathies. Such agonists would include, for example, compounds which mimic the IL-11 polypeptide or which otherwise act as an agonist at the IL-11 receptor. Such IL-11 receptor polypeptides may be prepared by recombinant means (see for example Cherel et al (1995) Blood 86(7):2534-40 which discloses two isoforms of the IL-11 receptor, formed by alternative splicing). The aforesaid screens, may be formatted to use either or both isoforms of the IL-11 receptor (Cherel et al *supra*), optionally in combination the gp130 subunit protein with which it is known to associate in the membrane. In general, agonists or antagonists so identified may be employed for therapeutic and prophylactic purposes for such Diseases as hereinbefore mentioned. Compounds may be identified from a variety of sources, for example, cells, cell-free preparations, chemical libraries, and natural product mixtures. Such agonists or antagonists, so-identified may be natural or modified substrates, ligands, receptors, enzymes, etc., as the case may be, of the polypeptide; or may be structural or functional mimetics thereof (see Coligan *et al.*, Current Protocols in Immunology 1(2):Chapter 5 (1991)).

The screening method may simply measure the binding of a candidate compound to the IL-11 polypeptide or IL-11 receptor or to cells or membranes bearing these polypeptides, or a fusion protein thereof by means of a label directly or indirectly associated with the candidate compound. Alternatively, the screening method may involve competition with a labeled competitor. In a preferred embodiment these screening methods test whether

the candidate compound results in a signal generated by activation of the IL-11 receptor polypeptide, using detection systems appropriate to the cells bearing the polypeptide. Specifically such signalling, wherein the JAK kinase/STAT transcription factor signalling pathway is activated, can be detected using methods known in the art. In a further embodiment the screening methods test whether the candidate compound blocks a signal generated by activation of the IL-11 receptor polypeptide using the aforesaid detection systems.

The polynucleotides, polypeptides and antibodies to the polypeptide of the present invention may also be used to configure screening methods for detecting the effect of added compounds on the production of mRNA and polypeptide in cells. For example, an ELISA assay may be constructed for measuring secreted or cell associated levels of polypeptide using monoclonal and polyclonal antibodies by standard methods known in the art. This can be used to discover agents which may enhance or reduce the production of IL-11 polypeptide from suitably manipulated cells or tissues.

Examples of potential polypeptide agonists and antagonists include antibodies or oligonucleotides or proteins which are closely related to the IL-11 ligands of the IL-11 receptor polypeptide, eg. a fragment of the IL-11 polypeptide.

Thus, in another aspect, the present invention relates to a screening kit for identifying agonists or antagonists for the IL-11 receptor polypeptides of the present invention; comprising

(a) an IL-11 or IL-11 receptor polypeptide, optionally including the gp130 subunit;

(b) a recombinant cell expressing an IL-11 receptor polypeptide, optionally with the gp130 subunit;

(c) a cell membrane comprising an IL-11 receptor polypeptide, optionally with the gp130 subunit; or

(d) antibody to an IL-11 or IL-11 receptor polypeptide of the present invention;

which polypeptide is preferably an IL-11 polypeptide of SEQ ID NO:2 or SEQ ID NO:3.

It will be appreciated that in any such kit, (a), (b), (c) or (d) may comprise a substantial component.

It will be readily appreciated by the skilled artisan that a polypeptide of the present invention may also be used in a method for the structure-based design of an IL-11 agonist or antagonist by:

(a) determining in the first instance the three-dimensional structure of the IL-11 polypeptide,

(b) deducing the three-dimensional structure for the likely reactive or binding site(s) of an agonist or antagonist;

(c) synthesing candidate compounds that are predicted to bind to or react with the deduced binding or reactive site; and

(d) testing whether the candidate compounds are indeed agonists or antagonists.

Alternatively the structure based design may be carried out as described in (a) to (d) above wherein the polypeptide is an IL-11 receptor polypeptide optionally in association with the gp130 subunit.

It will be further appreciated that this will normally be an iterative process.

In a further aspect, the present invention provides methods of treating abnormal conditions such as, for instance, stroke or neuropathies, related to an under-expression or over-expression of IL-11 polypeptide activity. For treating abnormal conditions related to an under-expression of IL-11 and its activity, several approaches are also available. One approach comprises administering to a subject a therapeutically effective amount of a compound which activates an IL-11 or IL-11 receptor polypeptide of the present invention, i.e., an agonist as described above, in combination with a pharmaceutically acceptable carrier, to thereby alleviate the abnormal condition. Alternatively, gene therapy may be employed to effect the endogenous production of IL-11 by the relevant cells in the subject. For example, a polynucleotide of the invention may be engineered for expression in a replication defective retroviral vector, as discussed above. The retroviral expression construct may then be isolated and introduced into a packaging cell transduced with a retroviral plasmid vector containing RNA encoding a polypeptide of the present invention

such that the packaging cell now produces infectious viral particles containing the gene of interest. These producer cells may be administered to a subject for engineering cells *in vivo* and expression of the polypeptide *in vivo*. For an overview of gene therapy, see Chapter 20, Gene Therapy and other Molecular Genetic-based Therapeutic Approaches, (and references cited therein) in Human Molecular Genetics, T Strachan and A P Read, BIOS Scientific Publishers Ltd (1996). Another approach is to administer a therapeutic amount of an IL-11 polypeptide, or active fragment thereof, of the present invention in combination with a suitable pharmaceutical carrier.

If the activity of the IL-11 polypeptide is in excess, several approaches are available. One approach comprises administering to a subject in need thereof an inhibitor compound (antagonist) as hereinabove described, optionally in combination with a pharmaceutically acceptable carrier, in an amount effective to inhibit the function of the IL-11 polypeptide, such as, for example, by preventing the binding of the IL-11 polypeptide to it's receptor and thereby alleviating the abnormal condition. In another approach, soluble forms of the IL-11 polypeptides still capable of binding the IL-11 receptor, but unable to activate the receptor, can be used. Typical examples of such competitors include inactive fragments of the IL-11 polypeptide.

In still another approach, expression of the gene encoding the endogenous IL-11 polypeptide can be inhibited using expression blocking techniques. Known such techniques involve the use of antisense sequences, either internally generated or externally administered (see, for example, O'Connor, J Neurochem (1991) 56:560 in Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988)). Alternatively, oligonucleotides which form triple helices ("triplexes") with the gene can be supplied (see, for example, Lee *et al.,* Nucleic Acids Res (1979) 6:3073; Cooney *et al.,* Science (1988) 241:456; Dervan *et al.*, Science (1991) 251:1360). These oligomers can be administered *per se* or the relevant oligomers can be expressed *in vivo*. Synthetic antisense or triplex oligonucleotides may comprise modified bases or modified backbones. Examples of the latter include methylphosphonate, phosphorothioate or peptide nucleic acid backbones. Such backbones are incorporated in

the antisense or triplex oligonucleotide in order to provide protection from degradation by nucleases and are well known in the art. Antisense and triplex molecules synthesised with these or other modified backbones also form part of the present invention.

In addition, expression of the human IL-11 polypeptide may be prevented by using ribozymes specific to the human IL-11 mRNA sequence. Ribozymes are catalytically active RNAs that can be natural or synthetic (see for example Usman, N, et al., Curr. Opin. Struct. Biol (1996) 6(4), 527-33.) Synthetic ribozymes can be designed to specifically cleave human IL-11 mRNAs at selected positions thereby preventing translation of the human IL-11 mRNAs into functional polypeptide. Ribozymes may be synthesised with a natural ribose phosphate backbone and natural bases, as normally found in RNA molecules. Alternatively the ribosymes may be synthesised with non-natural backbones to provide protection from ribonuclease degradation, for example, 2'-O-methyl RNA, and may contain modified bases.

Alternatively, such expression blocking techniques can be employed to reduce the levels of expression of the IL-11 receptor, rather than the IL-11 polypeptide itself.

In a further aspect, the present invention provides for pharmaceutical compositions comprising a therapeutically effective amount of an IL-11 polypeptide, or active fragment thereof, such as the soluble form of a polypeptide of the present invention, agonist or antagonist peptide or small molecule compound, in combination with a pharmaceutically acceptable carrier or excipient. Such carriers include, but are not limited to, saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The invention further relates to pharmaceutical packs and kits comprising one or more containers filled with one or more of the ingredients of the aforementioned compositions of the invention. Polypeptides and other compounds of the present invention may be employed alone or in conjunction with other compounds, such as therapeutic compounds.

The composition will be adapted to the route of administration, for instance by a systemic or an oral route. Preferred forms of systemic administration include injection, typically by

intravenous injection. Other injection routes, such as subcutaneous, intramuscular, or intraperitoneal, can be used. Alternative means for systemic administration include transmucosal and transdermal administration using penetrants such as bile salts or fusidic acids or other detergents. In addition, if a polypeptide or other compounds of the present invention can be formulated in an enteric or an encapsulated formulation, oral administration may also be possible. Administration of these compounds may also be topical and/or localized, in the form of salves, pastes, gels, and the like.

The dosage range required depends on the choice of peptide or other compounds of the present invention, the route of administration, the nature of the formulation, the nature of the subject's condition, and the judgment of the attending practitioner. Suitable dosages, however, are in the range of 0.1-100 μg/kg of subject. Wide variations in the needed dosage, however, are to be expected in view of the variety of compounds available and the differing efficiencies of various routes of administration. For example, oral administration would be expected to require higher dosages than administration by intravenous injection. Variations in these dosage levels can be adjusted using standard empirical routines for optimization, as is well understood in the art.

Polypeptides used in treatment can also be generated endogenously in the subject, in treatment modalities often referred to as "gene therapy" as described above. Thus, for example, cells from a subject may be engineered with a polynucleotide, such as a DNA or RNA, to encode a polypeptide *ex vivo*, and for example, by the use of a retroviral plasmid vector. The cells are then introduced into the subject.

The following definitions are provided to facilitate understanding of certain terms used frequently hereinbefore.

"Antibodies" as used herein includes polyclonal and monoclonal antibodies, chimeric, single chain, and humanized antibodies, as well as Fab fragments, including the products of an Fab or other immunoglobulin expression library.

"Isolated" means altered "by the hand of man" from the natural state. If an "isolated" composition or substance occurs in nature, it has been changed or removed from its original environment, or both. For example, a polynucleotide or a polypeptide naturally present in a living animal is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein.

"Polynucleotide" generally refers to any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. "Polynucleotides" include, without limitation, single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, "polynucleotide" refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The term "polynucleotide" also includes DNAs or RNAs containing one or more modified bases and DNAs or RNAs with backbones modified for stability or for other reasons. "Modified" bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications may be made to DNA and RNA; thus, "polynucleotide" embraces chemically, enzymatically or metabolically modified forms of polynucleotides as typically found in nature, as well as the chemical forms of DNA and RNA characteristic of viruses and cells. "Polynucleotide" also embraces relatively short polynucleotides, often referred to as oligonucleotides.

"Polypeptide" refers to any peptide or protein comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds, i.e., peptide isosteres. "Polypeptide" refers to both short chains, commonly referred to as peptides, oligopeptides or oligomers, and to longer chains, generally referred to as proteins. Polypeptides may contain amino acids other than the 20 gene-encoded amino acids. "Polypeptides" include amino acid sequences modified either by natural processes, such as post-translational processing, or by chemical modification techniques which are well

known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature. Modifications may occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present to the same or varying degrees at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications. Polypeptides may be branched as a result of ubiquitination, and they may be cyclic, with or without branching. Cyclic, branched and branched cyclic polypeptides may result from post-translation natural processes or may be made by synthetic methods. Modifications include acetylation, acylation, ADP-ribosylation, amidation, biotinylation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination (see, for instance, Proteins - Structure and Molecular Properties, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York, 1993; Wold, F., Post-translational Protein Modifications: Perspectives and Prospects, pgs. 1-12 in Post-translational Covalent Modification of Proteins, B. C. Johnson, Ed., Academic Press, New York, 1983; Seifter *et al.*, "Analysis for protein modifications and nonprotein cofactors", Meth Enzymol (1990) 182:626-646 and Rattan *et al.*, "Protein Synthesis: Post-translational Modifications and Aging", Ann NY Acad Sci (1992) 663:48-62).

"Variant" or "active fragment" refers to a polynucleotide or polypeptide that differs from a reference polynucleotide or polypeptide, but retains essential properties. A typical variant of a polynucleotide differs in nucleotide sequence from another, reference polynucleotide. Changes in the nucleotide sequence of the variant may or may not alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide.

Nucleotide changes may result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence, as discussed below. A typical variant of a polypeptide differs in amino acid sequence from another, reference polypeptide. Generally, differences are limited so that the sequences of the reference polypeptide and the variant are closely similar overall and, in many regions, identical. A variant, or active fragment, and reference polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions in any combination. A substituted or inserted amino acid residue may or may not be one encoded by the genetic code. A variant of a polynucleotide or polypeptide may be a naturally occurring such as an allelic variant, or it may be a variant that is not known to occur naturally. Non-naturally occurring variants of polynucleotides and polypeptides may be made by mutagenesis techniques or by direct synthesis.

"Identity," as known in the art, is a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as the case may be, as determined by the match between strings of such sequences. "Identity" and "similarity" can be readily calculated by known methods, including but not limited to those described in (*Computational Molecular Biology*, Lesk, A.M., ed., Oxford University Press, New York, 1988; *Biocomputing: Informatics and Genome Projects*, Smith, D.W., ed., Academic Press, New York, 1993; *Computer Analysis of Sequence Data*, Part I, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; *Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press, 1987; and *Sequence Analysis Primer*, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; and Carillo, H., and Lipman, D., SIAM *J. Applied Math., 48:* 1073 (1988). Preferred methods to determine identity are designed to give the largest match between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs. Preferred computer program methods to determine identity and similarity between two sequences include, but are not limited to, the GCG program package (Devereux, J., et al., *Nucleic Acids Research 12(1):* 387 (1984)), BLASTP, BLASTN, and FASTA (Atschul, S.F. et

al., *J. Molec. Biol. 215:* 403-410 (1990). The BLAST X program is publicly available from NCBI and other sources (*BLAST Manual*, Altschul, S., *et al.*, NCBI NLM NIH Bethesda, MD 20894; Altschul, S., *et al.*, *J. Mol. Biol. 215*: 403-410 (1990). The well known Smith Waterman algorithm may also be used to determine identity.

Preferred parameters for polypeptide sequence comparison include the following:
1) Algorithm: Needleman and Wunsch, J. Mol Biol. 48: 443-453 (1970)
Comparison matrix: BLOSSUM62 from Hentikoff and Hentikoff, Proc. Natl. Acad. Sci. USA. 89:10915-10919 (1992)
Gap Penalty: 12
Gap Length Penalty: 4

A program useful with these parameters is publicly available as the "gap" program from Genetics Computer Group, Madison WI. The aforementioned parameters are the default parameters for peptide comparisons (along with no penalty for end gaps).

Preferred parameters for polynucleotide comparison include the following:
1) Algorithm: Needleman and Wunsch, J. Mol Biol. 48: 443-453 (1970)
Comparison matrix: matches = +10, mismatch = 0
Gap Penalty: 50
Gap Length Penalty: 3
Available as: The "gap" program from Genetics Computer Group, Madison WI. These are the default parameters for nucleic acid comparisons.

By way of example, a polynucleotide sequence of the present invention may be identical to the reference sequence of SEQ ID NO:1, that is be 100% identical, or it may include up to a certain integer number of nucleotide alterations as compared to the reference sequence. Such alterations are selected from the group consisting of at least one nucleotide deletion, substitution, including transition and transversion, or insertion, and wherein said alterations may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either

individually among the nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence. The number of nucleotide alterations is determined by multiplying the total number of nucleotides in SEQ ID NO:1 by the numerical percent of the respective percent identity(divided by 100) and subtracting that product from said total number of nucleotides in SEQ ID NO:1, or:

$$n_n \leq x_n - (x_n \bullet y),$$

wherein n_n is the number of nucleotide alterations, x_n is the total number of nucleotides in SEQ ID NO:1, and y is, for instance, 0.70 for 70%, 0.80 for 80%, 0.85 for 85%, 0.90 for 90%, 0.95 for 95%,etc., and wherein any non-integer product of x_n and y is rounded down to the nearest integer prior to subtracting it from x_n. Alterations of a polynucleotide sequence encoding the polypeptide of SEQ ID NO:2 may create nonsense, missense or frameshift mutations in this coding sequence and thereby alter the polypeptide encoded by the polynucleotide following such alterations.

Similarly, a polypeptide sequence of the present invention may be identical to the reference sequence of SEQ ID NO:2, that is be 100% identical, or it may include up to a certain integer number of amino acid alterations as compared to the reference sequence such that the % identity is less than 100%. Such alterations are selected from the group consisting of at least one amino acid deletion, substitution, including conservative and non-conservative substitution, or insertion, and wherein said alterations may occur at the amino- or carboxy-terminal positions of the reference polypeptide sequence or anywhere between those terminal positions, interspersed either individually among the amino acids in the reference sequence or in one or more contiguous groups within the reference sequence. The number of amino acid alterations for a given % identity is determined by multiplying the total number of amino acids in SEQ ID NO:2 by the numerical percent of the respective percent identity(divided by 100) and then subtracting that product from said total number of amino acids in SEQ ID NO:2, or:

$$n_a \leq x_a - (x_a \bullet y),$$

wherein n_a is the number of amino acid alterations, x_a is the total number of amino acids in SEQ ID NO:2, and y is, for instance 0.70 for 70%, 0.80 for 80%, 0.85 for 85% etc., and

wherein any non-integer product of x_a and **y** is rounded down to the nearest integer prior to subtracting it from x_a.

"Fusion protein" refers to a protein encoded by two, often unrelated, fused genes or fragments thereof. In one example, EP-A-0 464 discloses fusion proteins comprising various portions of constant region of immunoglobulin molecules together with another human protein or part thereof. In many cases, employing an immunoglobulin Fc region as a part of a fusion protein is advantageous for use in therapy and diagnosis resulting in, for example, improved pharmacokinetic properties [see, e.g., EP-A 0232 262]. On the other hand, for some uses it would be desirable to be able to delete the Fc part after the fusion protein has been expressed, detected and purified.

Examples

Example 1 - Representational Difference Analysis (RDA)

Representational Difference Analysis (RDA) was performed to identify gene products that were up-regulated following pMCAO (permanent middle cerebral artery occlusion) in the rat. RDA was performed essentially as described previously (Hubank and Schatz, (1994) Nucleic Acids Res. 22(25):5640-8) on polyA RNA extracted from the cerebral cortex 24 hrs after rats had either undergone sham surgery or permanent middle cerebral artery occlusion (pMCAO). The subtracted library was cloned into pcDNA3.1 (Invitrogen) and subjected to automated sequence analysis using vector primers.

Two of the clones isolated (contigs 578 and 594) showed significant homology to rat IL-11 (GenBank Accession number: AF347935) and alignment with rat IL-11 showed 90% identity (e.g. bestfit alignment of contig 578 and AF347935, see Figure 2). These clones thus represent the rat orthologue of IL-11.

contig 578 (SEQ ID NO:4):

TGGTCTTTAAAGACGATGTSaGATGaGTGGCTTTCAGWTCMTGAGTTACAGCCGAGTCTT
TARCARCAGCAGRCGCCGCDCGRCCCAGTCCAAGGTCAGGWgCAGCCCTCCTAGGATGGC
ATAADCTGCCNGKATGCTTCCCCAGRCSGAGGCAGGAGGKCCCAGAGRGAYCGGAAtGGW
WGTAMMRGGTCTTGGRGCMAGAGgCATACatCAAGAGCTGTAAGCGACGAAGTAGCCGTT
CCAGTCGGGCTTGCAGGGCACCCAGCTCTGGCTCCAGAGTCTTTAGGGAAGGACCAGCTG
CCCGGCGCAACCcACTGTACATGTCGGAAGTAGGACATTAAGTCTACTCGAAGCCTTGTC
AGCACTCCAGGAAGCTGCAAAGATC

contig 594 (SEQ ID NO:5):

NATCCCAGTGTTCCAGCGCTCACGGCCTAYGTAGGTAGGGAGTCCCANATTGCGGCCTTC
CATCAGCKTGGGAATTTGTCTCTCTGGTGAGAAAGAAGCTGTGAGGGGAGCCCYATGCCC
TTCTCCCTCTTTGGTNGCCTNANANAGAGAANGNGACTGTNANTTTAAATATCCCCCTTG
TTTCCCTCNNANTCCTCATACCATCTTGTGCNATCTNWGTNNCCGGTGTGCTNTGCCANN
AANGGACCCYGGTTAACNANGGACAAGNCTTTTTTCNNAAYTCCCNCTANTRTC

PCR primers were generated to the rat IL-11 sequence (SEQ ID NO:6: CTTCCGACATGTACAGTGG and SEQ ID NO:7: ATACATCAAGAGCTGTAAGCG) and these were used to amplify IL-11 from SMART RACE cDNA templates generated from mRNA extracted from the cortex of rat that had either undergone pMCAO or sham

surgery. PCR amplification was monitored in real time using SybrGreen dye and a ABI 7700 Sequence detection system (Applied Biosystems). Fold increase in expression following pMCAO is expressed in Figure 1 by dividing the signal seen in the pMCAO samples from the sham control. By means of negative controls, beta actin and GAPDH (GAPDH: ACC ACA GTC CAT GCC ATC AC, TCC ACC ACC CTG TTG CTG TA. SEQ ID NO:13,14 ; β-Actin: AAC CGT GAA AAG ATG ACC, CTG ATC CAC ATC TGC TGG, SEQ ID NO:15,16) were amplified from the same cDNAs under identical conditions. As can be seen in Figure 1, while GAPDH and beta actin changed 2 and 4 fold respectively, IL-11 was confirmed to be expressed at approximately 700 fold higher levels in the pMCAO over the sham cDNA.

Example 2 - Timecourse of IL-11 induction following permanent Middle Cerebral Artery Occlusion (pMCAO)

To assess the timecourse of the accumulation of IL-11 mRNA following pMCAO, TaqMan quantitative RT-PCR was performed. cDNA templates were generated from RNA harvested from naïve, sham-operated and pMCAO treated rats at 3, 6, 12 and 24 hrs following surgical intervention, and these served as templates for quantitative RT-PCR as described previously (Harrison, D.C., et al Brain Res. Mol. Brain Res., 75 (2000) 143-149.). Briefly, total RNA was extracted using TRIZOL reagent from the left and right cortices of pMCAO, sham-operated and naïve rats (four rats in each group). First strand cDNA synthesis was carried out in triplicate by oligo(dT) priming from 1μg of each RNA pool. PCR conditions were as follows: 50°C for 2 minutes, 95°C for 10 minutes followed by forty cycles of 95°C for 15 seconds, 60°C for 1 minute. RT-PCR primers and probes were designed to rat IL-11 sequence (SEQ ID NO:8,Forward primer ACTTCCGACATGTACAGTGGGTT; SEQ ID NO:9, Reverse primer GCAGGGCACCCAGCTCT; SEQ ID NO:10, Taqman probe CGGGCAGCTGGTCCTTCCCTAAAG) and the amplification of specific cDNA products was monitored using an ABI 7700 Sequence detection system. All values were normalised to naïve control animals and then expressed as fold difference versus naïve.

The data again showed a robust increase in IL-11 mRNA following pMCAO on the lesioned side of the brain (left side), with detectable increases in message levels 6hrs following MCAO (100-fold) which continued to rise throughout the timecourse up to 1470 fold by 24hrs. A small but significant induction was also noted on the right side of brain (100 fold at 12hrs), suggesting that cells even at some distance from the lesion were also responding to the stimulus. Significantly, IL-11 mRNA levels were unaffected by the sham surgery, indicating that the IL-11 accumulation was directly in response to the MCAO procedure.

Example 3 - Isolation of cDNA clones for Human, mouse and rat IL-11

cDNA clones for human and mouse IL-11 were isolated using standard means of RT-PCR from human bone marrow stromal cell RNA and mouse 7 day embryo respectively.

SEQ ID NO:11, Human IL-11

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SEQ ID NO:12, Mouse IL-11

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Example 4 - Treatment with IL-11 reduces lesion volume in the pMCAO model

The effect of recombinant mouse IL-11 (rmIL-11) on lesion volume was assessed twenty four hours following permanent middle cerebral artery occlusion (pMCAO) in the rat. Male Sprague-Dawley rats (300-350g) underwent pMCAO using the intraluminal thread model as described previously (Irving et al., Mol Brain Research (2000) 77 : 65-75). rmIL-11 (300μg/kg, i.v.) or 0.1% BSA was administered one hour following the onset of cerebral ischaemia (n=10/group). Twenty-four hours later the animals were killed by transcardiac perfusion of saline followed by 4% Neutral Buffered Formalin and the brains processed for lesion volume assessment. Statistical analysis was conducted using a Student's T-Test.

rmIL-11 significantly reduced total lesion volume ($259\pm9mm^3$, T-Test, $p= 0.029$) and cortical lesion volume ($157\pm6mm^3$, T-test, $p=0.036$) compared to vehicle (0.1% BSA) treated animals (Total volume = $289\pm8mm^3$; Cortical volume = $173\pm4mm^3$). There was no effect of rm-IL11 on striatal lesion volume ($41\pm2mm^3$) compared to vehicle treated animals ($41\pm1mm^3$).

rm-IL provided significant neuroprotection 24h following pMCAO in the normotensive rat.

SEQUENCE INFORMATION

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SEQ ID NO:3

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SEQ ID NO:4

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SEQ ID NO:5

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SEQ ID NO:15
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SEQ ID NO:16
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Brief Description of Figure 1

Figure 1 shows the fold-increase in expression levels of IL-11, GAPDH and beta actin in pMCAO operated rats compared with sham operated rats (fold increase in expression is calculated by dividing by the signal seen in the pMCAO samples from the sham control). Error bars reflect the standard error between duplicate PCR reactions.

Brief Description of Figure 2

Alignment of the nucleotide sequences of conitg 578 (SEQ ID NO 4) identified from the RDA subtraction versus rat IL-11. The Bestfit algorithm was used to compare sequence identity between contig 578 and the published IL-11 (Accession number AF347935), and the data expressed as a sequence alignment. Identity was indicated by a line (|) and similarity indicated by a dot (:).

Brief Description of Figure 3

Time-course of gene expression changes following pMCAO as monitored by TaqMan® RT-PCR. RNA extracted from both left (operated) and right (un-operated) cortices of MCAO (4 individuals), sham-treated (4 individuals) or naive rats (2 individuals) at 3, 6, 12 and 24 hrs was used to generate cDNA which served as template for PCR amplification of IL-11. Amplification was performed in triplicate and monitored using a fluorescently labelled gene-specific probes and ABI TaqMan® technology. Fold induction versus naive rats for MCAO left hemisphere (black circles), right hemisphere (black square), sham left hemisphere (white square) and sham right hemisphere (white square) are plotted on the x-axis versus time in hours on the y-axis. Error bars reflect standard error between triplicate PCR reactions.

Brief Description of Figure 4

Reduction in pMCAO induced lesion volume following IL-11 treatment. Lesion volume (mm3) is plotted against individual brain regions and total brain for both 0.1% BSA injected (grey bars) and rmIL-11 (300ug/kg) injected (white bars) rats. The protection afforded by IL-11 treatment is indicated by the reduction in lesion volume seen with rmIL-11 as compared to the BSA control. * indicates a statistically significant reduction in lesion volume compared to vehicle as deduced using the Student's T-Test.

Claims

1. The use of a compound selected from:

(a) an IL-11 polypeptide;

(b) a compound which mimics an IL-11 polypeptide;

(c) a compound which activates an IL-11 receptor;

(d) a compound which inhibits activation of an IL-11 receptor; or

(d) a polynucleotide encoding an IL-11 polypeptide,

for the manufacture of a medicament for treating stroke or neuropathies.

2. The use according to claim 1 wherein the medicament is for use in the treatment of stroke.

3. The use according to claims 1 or 2 wherein the medicament comprises an isolated polypeptide which comprises a polypeptide having at least 95% identity to the IL-11 polypeptide of SEQ ID NO:2 or SEQ ID NO:3.

4. The use according to claim 3 wherein the isolated polypeptide is the IL-11 polypeptide of SEQ ID NO:3.

5. The use according to claims 1 or 2 wherein the medicament comprises a compound which mimics an IL-11 polypeptide.

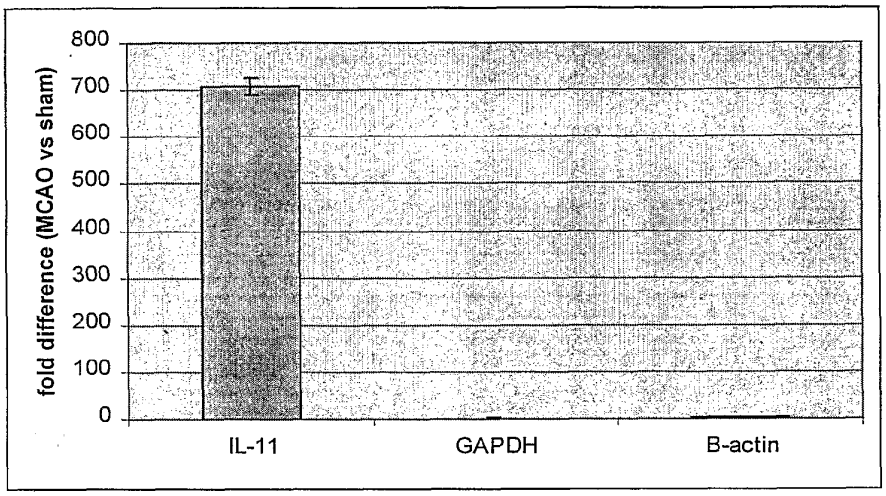
6. The use according to claims 1 or 2 wherein the medicament comprises a compound which is an agonist of an IL-11 receptor polypeptide.

7. The use according to claims 1 or 2 wherein the medicament comprises a polynucleotide encoding a polypeptide having at least 95% identity with the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:3.

8. The use according to claim 7 wherein the polynucleotide comprises a polynucleotide having at least 95% identity with the polynucleotide of SEQ ID NO:1.

9. The use according to claims 7 or 8 wherein the polynucleotide has the polynucleotide sequence of SEQ ID NO:1.

Figure 1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600
500
400
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100
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IL-11
GAPDH
B-actin

Figure 2 BESTFIT Query description: **rat IL-11 versus contig 578**

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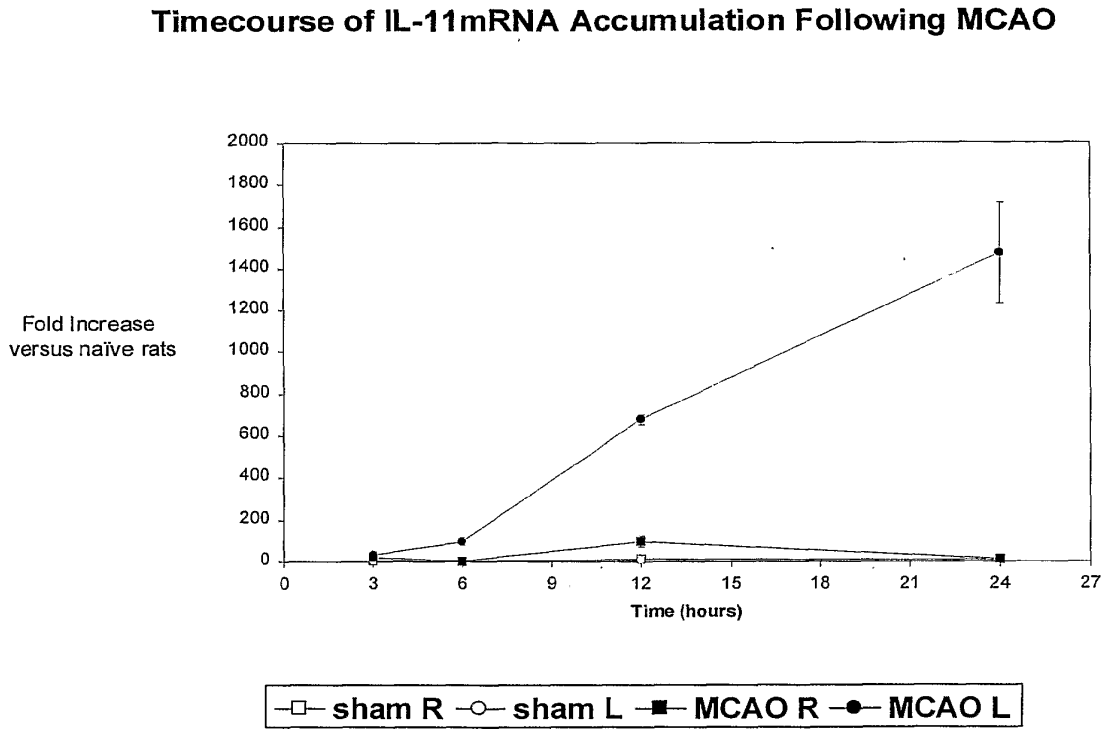
Figure 3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800
600
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200
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Time (hours)
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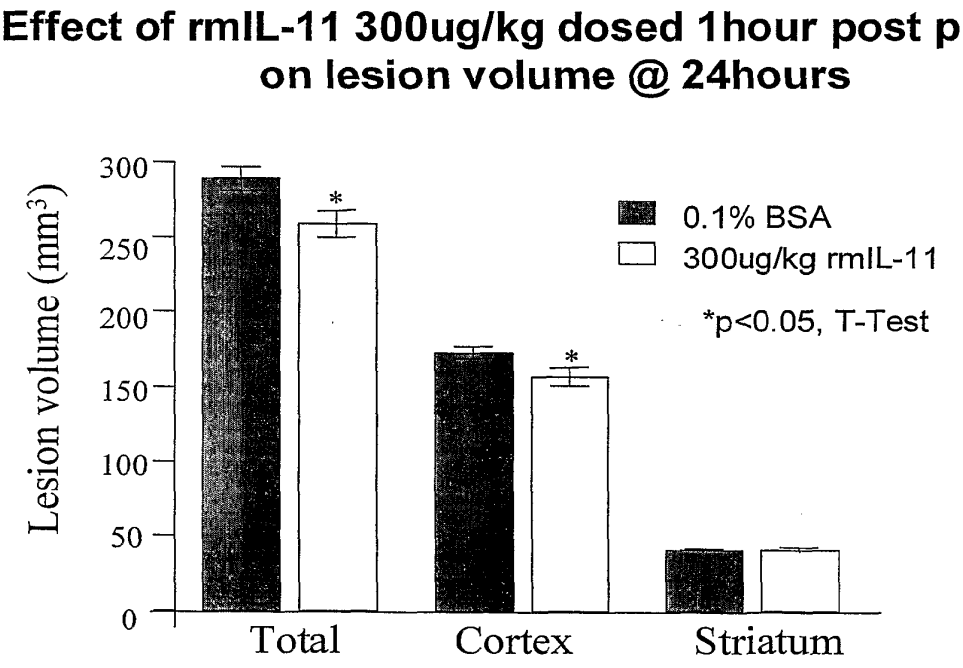
Figure 4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200
150
100
50
0
*
*
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Total
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Ex. 2001 - Page583

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(54) **Title:** METHOD FOR TREATING CARDIAC REMODELING FOLLOWING MYOCARDIAL INJURY

(57) **Abstract:** The invention concerns methods for treating cardiac remodeling in a subject who has undergone myocardial injury, said method comprising the administration of natriuretic peptide to said subject. Preferably the natriuretic peptide is brain natriuretic peptide. The invention also concerns methods for treating structural heart disorders arising from myocardial injury, said method comprising the administration of a natriuretic peptide to a patient in need thereof.

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METHOD FOR TREATING CARDIAC REMODELING FOLLOWING MYOCARDIAL INJURY

This application claims priority to U.S. provisional application Serial No. 60/537,221. The 60/537,221 provisional application is herein incorporated by reference in its entirety.

Field of the Invention

The present invention concerns methods of treatment using one or more natriuretic peptides or derivatives thereof. More specifically, the invention concerns methods of treating or preventing cardiac dysfunction in a subject after said subject has undergone myocardial injury.

BACKGROUND

Myocardial infarction is a major cause of significant disability and death in the United States and in many other countries around the world, and accounts for approximately 2/3 of all heart failure. Hunt et al, AMERICAN COLLEGE OF CARDIOLOGY/AMERICAN Heart Association. ACC/AHA guidelines for the evaluation and management of chronic heart failure in the adult: executive summary. A report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Committee to revise the 1995 Guidelines for the Evaluation and Management of Heart Failure). Journal of the American College of Cardiology 2001; 38: 2101-2113. Several disease-initiating events (e. g. myocardial infarction, untreated hypertension, congenital mutations of contractile proteins) can

result in a common heart disease phenotype that consists of dilation of the cardiac chambers, resulting in reduction in contractile function (i.e., a decrease in the fraction of total blood ejected from each chamber during systole) that leads to the clinical syndrome of heart failure. This phenotype generally involves a compensatory aspect that results from myocardial infarction when the normal compensatory hypertrophy of surviving, non-infarcted myocardium is insufficient. Often this compensatory mechanism is a result of the profibrotic response associated with cardiac injury.

Available therapies for heart dysfunction are insufficient, and new methods of treatment are needed. The heart responds to infarction by hypertrophy of surviving cardiac muscle in an attempt to maintain normal contraction. However, when the hypertrophy is insufficient to compensate, cardiac remodeling and reduced cardiac function result, leading to heart failure and death. Despite important advances in medical therapies for preventing cardiac dysfunction and heart failure after myocardial infarction, these problems remain a significant unsolved public health problem.

No pharmacological therapy for post MI cardiac remodeling is curative or satisfactory, and many patients die or, in selected cases, undergo heart transplantation. Presently available pharmacological therapies for reducing cardiac dysfunction and reducing mortality in patients with heart failure fall into three main categories: angiotensin-converting enzyme (ACE) inhibitors, beta adrenergic receptor (βAR) antagonists, and aldosterone antagonists. Despite reducing mortality, patients treated with these medicines remain at significantly increased risle for death compared to age- matched control patients without heart failure. ACE inhibitors, ßAR antagonists and (at least one type of) aldosterone receptor antagonist can significantly reduce the

incidence and extent of cardiac dysfunction and heart failure after myocardial infarction.

ACE inhibitors are associated with cough in 10% of patients and can result in renal failure in the setting of bilateral renal artery stenosis or other severe kidney disease. βAR antagonists are associated with impotence and depression, and are contraindicated in patients with asthma; furthermore, patients may develop worsened heart failure, hypotension, bradycardia, heart block, and fatigue with initiation of βAR antagonists. Aldosterone receptor antagonism causes significant hyperkalemia and painful gynecomastia in 10% of male patients. Agents without a demonstrated mortality benefit are also associated with problems; most notable is the consistent finding that many cardiac stimulants improve symptoms, but actually increase mortality, likely by triggering lethal cardiac arrhythmias. In summary, presently available pharmacological therapies are ineffective and are limited by significant unwanted side effects, and so development of new therapies with improved efficacy and less severe side effects is an important public health goal.

SUMMARY OF THE INVENTION

The present invention is directed to the use of natriuretic peptides for the prevention and/or treatment of cardiac remodeling in a subject that has undergone myocardial injury. In a preferred embodiment, the natriuretic peptide(s) comprise brain natriuretic peptide (BNP), also known as nesiritide. In another embodiment, the invention is directed to the treatment of cardiac dysfunction, said treatment comprising the administration of a therapeutically effective amount of natriuretic peptide to a subject that has undergone myocardial injury.

In another related embodiment, the invention is directed to a method of alleviating or reversing the effect of TGFβ mediated cell activation in cardiac tissue on the expression of one or more genes associated with fibrosis, comprising contacting one or more cells or tissues in which the expression of said genes is altered as a result of TGFβ mediated activation, with BNP. In another related embodiment, the targeted gene(s) associated with fibrosis are selected from the group consisting essentially of Collagen1, Collagent 3, Fibronectin, CTGF, PAI-1, and TIMP3.

In another embodiment, the invention is directed to a method of inhibiting the production of Collagen 1, Collagen 3 or Fibronectin proteins by the administration of a therapeutically effective amount of BNP to a subject in need thereof.

In another related embodiment, the invention is directed to a method of inhibiting TGFβ mediated myofibroblast conversion by administration of a therapeutically effective amount of BNP to a mammalian subject in need thereof.

In another related embodiment, the invention is directed to a method of alleviating or reversing the effect of TGFβ mediated cell activation in cardiac tissue on the expression of one or more genes associated with cell proliferation, comprising contacting one or more cells or tissues in which the expression of said genes is altered as a result of TGFβ mediated activation, with BNP. In another related embodiment, the targeted gene(s) associated with cell proliferation are selected from the group consisting essentially of PDGFA, IGF1, FGF18, and IGFBP10.

In another related embodiment, the invention is directed to a method of alleviating or reversing the effect of TGFβ mediated cell activation in cardiac tissue on the expression of one or more genes associated with inflammation, comprising contacting one or more cells or tissues in which the expression of said genes is altered as a result of TGFβ mediated activation, with BNP. In another related embodiment,

the targeted gene(s) associated with inflammation are selected from the group comprise COX1, IL6, TNFα-inducted protein 6, TNF superfamily, member 4.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Gene expression changes induced by TGFβ and BNP in human cardiac fibroblasts at 24 and 48 h. Histograms show the number of gene expression changes that were up-regulated and down-regulated by TGFβ and BNP treatment. Hybridizations using fluorescently-labeled cDNA probes compare untreated (control) to TGFβ-treated cells and control to BNP-treated cells. See Experimental for details related to the gene expression values. Histogram bars: 24 h (white) and 48 h (black).

Figure 2. Effects of BNP on TGFβ-induced gene expression in human cardiac fibroblasts. Hybridizations using fluorescently-labeled cDNA probes compare TGFβ-treated to TGFβ BNP-treated cells at 24 and 48 h. Strong and weak effects represent 1.8- and 1.5- fold gene expression levels, respectively. See Experimental for details related to statistical significance. Histogram bars: no effect (white), weak effect (grey), and strong effect (black).

Figure 3. Gene expression patterns in TGFβ-treated human cardiac fibroblasts. Data was generated using the hierarchical clustering algorithm contained in Spotfire™ software. Each row represents one of 524 genes, and each column represents the results from duplicate hybridizations: (A) control vs. TGFβ, 24 h; (B) control vs. TGFβ, 48 h; (C) TGFβ vs. TGFβ + BNP 24 h; (D) TGFβ vs. TGFβ + BNP 48 h; (E) control vs. BNP 24 h; and (F) control vs. BNP 48 h. Normalized data values depicted in shades of red and green represent elevated and repressed expression, respectively. See Table 2 in Experimental section for gene identities and expression values.

Figure 4. Gene expression clusters in human cardiac fibroblasts: (A) fibrosis and ECM, (B) cell proliferation, and (C) inflammation. See Fig. 4 legend for descriptions of the hybridizations and gene expression color codes.

Figure 5. Effects of BNP on TGFβ-induced Collagen 1 (A and B) and Fibronectin (C and D) mRNA and protein levels in cultured human cardiac fibroblasts. Histograms show control cells (white), cells treated with BNP (gray), cells treated with TGFβblack), and cells co-treated with BNP and TGFβ(hatched). (A and C) Real-time RT-PCR expression levels were normalized to 18S rRNA and plotted relative to the level in the 6 h control cells. Error bars reflect duplicate biological replicates; real-time RT-PCR reactions were performed in triplicate. (B and D) Western blot analyses are presented as mean ± SD from three separate experiments; *p<0.01 vs. control; **p<0.01 vs. TGFβ.

Figure 6. Effects of BNP on TGFβ–induced fibrotic and inflammatory genes. Real-time RT-PCR expression levels were normalized to 18S rRNA and plotted relative to the level in the 6 h control cells. See Fig. 5 for key to histogram bar labels and error bars.

Fig 7. Effect of PKG and MEK inhibitors on BNP-dependent inhibition of TGFβ signaling in human cardiac fibroblasts. (A) Western analysis of ERK phosphorylation. Cells were treated with BNP (0.5 μmol/L) in the presence or absence of KT5823 (1 μmol/L) or U0126 (10 μmol/L) for 15 min. (B) Western blot and (C) real-time RT-PCR analysis to detect Collagen 1 expression. Cells were treated with 5 ng/ml TGFβ and/or BNP (100 nmol/L, three times daily) in the presence or absence of KT5823 (1 μmol/L), U0126 (0.1-10 μmol/L) or PD98059 (10 μmol/L) for 48 h. Control (C); KT5823 (KT); U0126 (U); TGFβ (TGF).

Figure 8. Summary of BNP effects on gene expression in TGFβ–stimulated human cardiac fibroblasts.

Figure 9. Effects of BNP on TGFβ-stimulated fibroblast proliferation. Histograms show fold induction of BrdU labeled cells treated with TGFβ alone, BNP alone or co-treated with BNP and TGFβ. Cells were co-treated with BNP and TGFβ for 24 h, then labeled with BrdU and cultured for an additional 24 h. Pooled data represent the mean ± SD from three individual experiments: *$p < 0.01$ vs. the control; **$p<0.05$ vs. TGFβ.

Figure 10. Changes in plasma aldosterone level. The increased plasma aldosterone level by L-NAME/AngII was reduced by BNP ($p<0.05$, n=7)

Figure 11. Changes in heart/body weight ratio. BNP abolished L-NAME/AngII-induced increase in heart/body weight ratio ($p<0.01$, n=12)

Figure 12. Real time RT-PCR results. Expression of mRNA of collagen I (A), collagen III (B) and fibronectin (C) in the heart. BNP abolished the fibrotic genes that enhanced by L-NAME plus Angiotensin II ($p<0.01$ in all cases).

Figure 13. Cardiac function parameters including heart rate (A), stroke volume (B), ejection fraction (C), cardiac output (D), stroke work (E), maximum dP/dt (F), minimum dP/fy (G), and arterial elastance (H). L-NAME/AngII induced deterioration of cardiac function. Administration of BNP significantly improved cardiac function as judged by increases in stroke volume, ejection fraction, cardiac output, stroke work and decrease in arterial elastance ($p<0.001$, n=8). BNP also increased maximum dP/dt ($p<0.05$) and minimum dP/dt. BNP had no effect on heart rate.

DETAILED DESCRIPTION

A. Definitions

As used herein, any reference to "reversing the effect of TGF-β-mediated cell activation on the expression of a gene associated with fibrosis" means partial or complete reversal the effect of TGF-β-mediated cell activation of that gene, relative to a normal sample of the same cell or tissue type. It is emphasized that total reversal (i.e. total return to the normal expression level) is not required, although is advantageous, under this definition.

The term "cardiac remodeling" generally refers to the compensatory or pathological response following myocardial injury. Cardiac remodeling is viewed as a key determinant of the clinical outcome in heart disorders. It is characterized by a structural rearrangement of the cardiac chamber wall that involves cardiomyocyte hypertrophy, fibroblast proliferation, and increased deposition of extracellular matrix (ECM) proteins. Cardiac fibrosis is a major aspect of the pathology typically seen in the failing heart. The proliferation of interstitial fibroblasts and increased deposition of extracellular matrix components results in myocardial stiffness and diastolic dysfunction, which ultimately leads to heart failure. A number of neurohumoral or growth factors have been implicated in the development of cardiac fibrosis. These include angiotensin II (AII), endothelin-1 (ET-1), cardiotrophin-1 (CT-1), norepinephrine (NE), aldosterone, FGF2, PDGF, and transforming growth factor-β (TGFβ). TGFβ expression is also stimulated by AII and ET-1 in cardiac myocytes and fibroblasts, further supporting its involvement in cardiac fibrosis.

The term "cardiac dysfunction" refers to the pathological decline in cardiac performance following myocardial injury. Cardiac dysfunction may be manifested through one or more parameters or indicia including changes to stroke volume, ejection fraction,

end diastolic fraction, stroke work, arterial elastance, or an increase in heart weight to body weight ratio.

The terms "differentially expressed gene," "differential gene expression" and their synonyms, which are used interchangeably, refer to a gene whose expression is activated to a higher or lower level in a test sample relative to its expression in a normal or control sample. For the purpose of this invention, "differential gene expression" is considered to be present when there is at least an about 2.5-fold, preferably at least about 4-fold, more preferably at least about 6-fold, most preferably at least about 10-fold difference between the expression of a given gene in normal and test samples.

"Myocardial injury" means injury to the heart. It may arise from myocardial infarction, cardiac ischemia, cardiotoxic compounds and the like. Myocardial injury may be either an acute or nonacute injury in terms of clinical pathology. In any case it involves damage to cardiac tissue and typically results in a structural or compensatory response.

As used herein, "natriuretic peptides" means a composition that includes one or more of an Atrial natriuretic peptide (ANP), a Brain natriuretic peptide (BNP), or a C-type natriuretic peptide (CNP). It is contemplated that analogues and variants of these peptides be included in the definition. Examples of such include anaritide (ANP analogue of different length) or combinations of natriuretic peptide including but not limited to ANP/BNP, ANP/CNP, an BNP/CNP variants. Preferably, natriuretic peptide means BNP (nesiritide).

The terms "treating" or "alleviating" refer to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) the targeted pathologic condition or disorder. Those in need of treatment

include those already with the disorder as well as those prone to have the disorder or those in whom the disorder is to be prevented. In the treatment of a fibroproliferative disease, a therapeutic agent may directly decrease the pathology of the disease, or render the disease more susceptible to treatment by other therapeutic agents.

The term " subject" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, cats, cattle, horses, sheep, pigs, goats, rabbits, etc. Preferably, the subject is human.

Administration "in combination with" one or more further therapeutic agents includes simultaneous (concurrent) and consecutive administration in any order.

A "therapeutically effective amount", in reference to the treatment of cardiac or renal fibrosis, e.g. when inhibitors of the present invention are used, refers to an amount capable of invoking one or more of the following effects: (1) inhibition (i.e., reduction, slowing down or complete stopping) of the development or progression of fibrosis and/or sclerosis; (2) inhibition (i.e., reduction, slowing down or complete stopping) of consequences of or complications resulting from such fibrosis and/or sclerosis; and (3) relief, to some extent, of one or more symptoms associated with the fibrosis and/or sclerosis, or symptoms of consequences of or complications resulting from such fibrosis and/or sclerosis.

B. Modes of Carrying out the Invention

Natriuretic peptides comprise a family of vasoactive hormones that play important roles in the regulation of cardiovascular and renal homeostasis. Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are predominantly produced in the heart and exert vasorelaxant, natriuretic, and anti-growth activities.

Binding of ANP and BNP to type-A natriuretic peptide receptor (NPRA) leads to the generation of cyclic guanosine monophosphate (cGMP), which mediates most biological effects of the peptides. Mice lacking NPRA exhibit cardiac hypertrophy, fibrosis, hypertension and increased expression of fibrotic genes including *TGFβ1*, *TGFβ3* and *Collagen 1*. Furthermore, targeted disruption of the BNP gene in mice results in cardiac fibrosis and enhanced fibrotic response to ventricular pressure overload, suggesting that BNP is involved in cardiac remodeling.

TGFβ mediates fibrosis by modulating fibroblast proliferation and ECM production, particularly of collagen and fibronectin. TGFβ also promotes the phenotypic transformation of fibroblasts into myofibroblasts characterized by expression of *α-smooth muscle actin*. Studies have demonstrated that increased myocardial TGFβ expression is associated with cardiac hypertrophy and fibrosis. Moreover, functional blockade of TGFβ prevents myocardial fibrosis and diastolic dysfunction in pressure overloaded rats, indicating that TGFβ has a crucial role in the process of myocardial remodeling, particularly in cardiac fibrosis. However, the implication of natriuretic peptide(s) in this process has not been previously explored.

The present invention is directed to the treatment or prevention of cardiac remodeling following myocardial injury. In a preferred embodiment, the myocardial injury comprises an acute myocardial infarction. Preferably the administration of natriuretic peptide occurs as soon as possible after the injury event.

In another embodiment, the invention involves the treatment of cardiac dysfunction in a subject in need thereof comprising the administration of a natriuretic peptide to a subject in need thereof wherein said administration occurs after said subject has undergone myocardial injury.

The manner of administration and formulation of the natriuretic peptide(s) useful in the invention will depend on the nature of the condition, the severity of the condition, the particular subject to be treated, and the judgment of the practitioner; formulation will depend on mode of administration. The peptides of the invention are conveniently administered by oral administration by compounding them with suitable pharmaceutical excipients so as to provide tablets, capsules, syrups, and the like. Suitable formulations for oral administration may also include minor components such as buffers, flavoring agents and the like. Typically, the amount of active ingredient in the formulations will be in the range of about 5%-95% of the total formulation, but wide variation is permitted depending on the carrier. Suitable carriers include sucrose, pectin, magnesium stearate, lactose, peanut oil, olive oil, water, and the like.

The peptides useful in the invention may also be administered through suppositories or other transmucosal vehicles. Typically, such formulations will include excipients that facilitate the passage of the compound through the mucosa such as pharmaceutically acceptable detergents.

The peptides may also be administered by injection, including intravenous, intramuscular, subcutaneous, intrarticular or intraperitoneal injection. Preferably the natriuretic peptide(s) are administered intravenously. Typical formulations for such use are liquid formulations in isotonic vehicles such as Hank's solution or Ringer's solution.

Alternative formulations include aerosol inhalants, nasal sprays, liposomal formulations, slow-release formulations, and the like, as are known in the art.

Any suitable formulation may be used. A compendium of art-known formulations is found in Remington's Pharmaceutical Sciences, latest edition, Mack Publishing Company, Easton, PA. Reference to this manual is routine in the art.

The dosages of the peptide(s) of the invention will depend on a number of factors which will vary from patient to patient. The dose regimen will vary, depending on the conditions being treated and the judgment of the practitioner. Further information regarding related formulations and dosages for brain natriuretic peptide can be found in the package insert or the latest version of Physicians Desk Reference (PDR) for nesiritide or the Natrecor® product.

It should be noted that the peptides useful for the invention can be administered as individual active ingredients, or as mixtures of several different compounds. In addition, the peptide(s) can be used as single therapeutic agents or in combination with other therapeutic agents. Drugs that could be usefully combined with these compounds include natural or synthetic corticosteroids, particularly prednisone and its derivatives, monoclonal antibodies targeting cells of the immune system or genes associated with the development or progression of fibrotic diseases, and small molecule inhibitors of cell division, protein synthesis, or mRNA transcription or translation, or inhibitors of immune cell differentiation or activation.

As implicated above, although the peptide(s) of the invention may be used in humans, they are also available for veterinary use in treating non-human mammalian subjects.

Further details of the invention will be apparent from the Experimental section as provided below.

EXPERIMENTAL

In vitro

Cell Culture

Two lots of primary human cardiac fibroblasts, derived from an 18-year old Caucasian male (lot 1) and a 56-year old Caucasian male (lot 2), were provided by Cambrex Bio Science (Walkersville, MD). Cells stained positive for α-smooth muscle actin and vimentin antibodies corroborating their identity as cardiac fibroblasts and myofibroblasts. Both lots were used for the real-time RT-PCR studies; lot 1 was used for the microarray analysis. Cells at passage 3-5 were cultured in FGM containing 15% FBS. At confluence, cells were split and cultured in 6-well plates for 24 h. Cells were changed to serum-free medium and treated with human BNP (American Peptide Company, Sunnyvale, CA) in the presence or absence of 5 ng/ml of TGFβ (R&D systems, Minneapolis, MN) for 6, 24 and 48 h. BNP and/or TGFβ–treated cells were also incubated in the presence of cGMP-dependent protein kinase (PKG) inhibitor KT5823 (1 μmol/L, Calbiochem, San Diego, CA), MAP kinase kinase (MEK) inhibitor U0126 (0.1-10 μmol/L, Sigma, St. Louis, MO) or PD98059 (10 μmol/L, Sigma) for 48 h. BNP (100 nmol/L) was added into the medium three times a day, such that the total calculated concentrations of exogenous BNP were 200 nmol/L, 600 nmol/L, and 900 nmol/L at 6, 24, and 48 h, respectively. This dosing protocol was necessary to maintain the levels of BNP in culture, since two distinct clearance pathways are responsible for the rapid degradation of natriuretic peptides. Without this treatment regime, it was found that BNP was significantly degraded in the cardiac fibroblasts; 50% of added BNP was metabolized within 24 h as measured by immunoreactive assays and cGMP stimulation cell bioassays.

Intracellular cGMP assay

Cells were cultured in 6-well plates for 24 h, then changed to serum-free medium, and pre-incubated with 0.1 mmol/L of 3-isobutyl-1-methylxanthine (IBMX) for 1 h before treating with 10^{-9} - 10^{-6} mol/L of BNP for 10 min. The medium was aspirated and 0.5 ml of cold PBS was added into each well. Cells were scraped and mixed with 2 volumes of cold ethanol by vortex. After a 5 min room temperature incubation, the precipitate was removed by centrifugation at 1500 x g for 10 min. The supernatant was dried by vacuum centrifugation, and levels of cGMP were measured using the cyclic GMP EIA kit (Cayman Chemical, Ann Arbor, MI).

BrdU incorporation

Cells were placed in 96-well plates and cultured for 24 h before changing to serum-free medium. Cells were treated with BNP (100 nmol/L, three times a day) in the presence or absence of 5 ng/ml of TGF-β for 24 h. Subsequently, 10 μmol/L of 5-bromo-2'-deoxyuridine (BrdU) was added to the cells, and they were cultured for an additional 24 h. BrdU incorporation was detected using the Cell Proliferation ELISA kit (Roche, Indianapolis, IN). Data was analyzed by ANOVA using the Newman-Keuls test to assess significance.

cDNA Microarray

Gene expression profiles were determined from cDNA microarrays containing 8,600 elements derived from clones isolated from normalized cDNA libraries or purchased from ResGen (Invitrogen Life Technologies, Carlsbad, CA). DNA for spotting was generated by PCR amplification using 5'amino-modified primers (BD Biosciences Clontech, Palo Alto, CA) derived from flanking vector sequences. Amplified DNA

was purified in a 96-well format using Qiagen's Qiaquick columns (Valencia, CA) according to the manufacturer's recommendations. Samples were eluted in Milli-Q purified water, dried to completion and resuspended in 7 µl of 3X SSC. A fluorescent assay using PicoGreen (Molecular Probes, Eugene, OR) was randomly performed on 12% of the PCR products to determine the average yield after purification; yields were ~1.5 µg of DNA which corresponds to a concentration of 214 µg/ml. Purified DNA was arrayed from 384-well microtiter plates onto lysine-coated glass slides using an OmniGrid II microarrayer (GeneMachines, San Carlos, CA). After printing, DNA was cross-linked to the glass with 65 mjoules UV irradiation and reactive amines were blocked by treatment with succinic anhydride.

mRNA Isolation, Labeling, and Hybridizations

Total RNA was extracted from cells using Qiagen's RNeasy kit; two wells from a 6 well plate were pooled to yield a total of 4 x 10^5 cells per treatment. RNA was amplified using a modified Eberwine protocol[51] that incorporated a polyA tail into the amplified RNA. Fluorescently-labeled cDNA probes were generated by reverse transcription of 4 µg of RNA with SuperScript II (Invitrogen Life Technologies, Carlsbad, CA) using anchored dT primers in the presence of Cy3 or Cy5 dUTP (Amersham, Piscataway, NJ). Labeled cDNA probe pairs were precipitated with ethanol and purified using Qiaquick columns. Twenty µg each of poly(A) DNA, yeast tRNA, and human Cot1 DNA (Applied Genetics, Melbourne, FL) was added to the eluant. The samples were dried to completion and resuspended in 12.5 µl 3XSSC, 0.1%SDS. Probes were heated to 95°C for 5 minutes, applied to the arrays under a 22 mm^2 cover slip and allowed to hybridize for at least 16 h at 65°C. The arrays were

washed at 55°C for 10 minutes in 2XSSC, 0.1% SDS, followed by two washes at room temperature in 1XSSC (10 min) and 0.2XSSC (15 min). Hybridization of each fluorophore was quantified using an Axon GenePix 4000A scanner.

Microarray Data Analysis

Differential expression values were expressed as the ratio of the median of background-subtracted fluorescent intensity of the experimental RNA to the median of background-subtracted fluorescent intensity of the control RNA. For ratios greater than or equal to 1.0, the ratio was expressed as a positive value. For ratios less than 1.0, the ratio was expressed as the negative reciprocal (i.e., a ratio of 0.5 = -2.0). Median ratios were normalized to 1.0 using two pools of 3000 randomly chosen cDNAs in each pool. Six replicates of each of the two pools were printed in 4 evenly distributed blocks of the array. Expression data was rejected if neither channel produced a signal of at least 2.0-fold over background. Differential expression ratios were determined as the mean of the two values from dye-swapped duplicates.

A statistically significant differential expression threshold value was empirically determined according to the method of Yang *et al.*[53] Seven independent self-self- hybridizations were performed in which the same RNA sample was labeled with Cy3 dUTP and Cy5 dUTP and hybridized to arrays containing 8,448 elements. Only elements that gave a signal greater than 2.0-fold over background in at least one of the dyes were considered in the analysis. Expression ratios were converted to $\log_{(2)}$ and normalized to a mean = 0. Combining data from all hybridizations, the 3 standard deviation limit was equivalent to a 1.48 fold change (+/- 0.563 $\log_{(2)}$). Of the 45,633 elements analyzed, 0.85% fell outside this threshold. Therefore, at this standard deviation limit, genes with fold changes greater than 1.48 can be considered

differentially expressed at a 99% confidence level for any given hybridization. The percentage of elements that reproducibly fell outside the 3 standard deviation limit between any two duplicates of the seven self-self hybridizations was determined by comparing all 21 pair-wise combinations. An average of 18.9 elements +/- 15.6 per hybridization duplicated at a fold change of 1.5, corresponding to a false positive rate of 0.29%. At a fold change of 1.8, an average of 0.71 elements +/-0.97 duplicated, corresponding to a false positive rate of 0.01%. A 1.8-fold threshold value was used to identify differentially expressed genes, except in Fig. 3, a 1.5-fold threshold value was used to designate "weak effects".

Real-time RT-PCR

Real-time RT-PCR[18] was performed in a two-step manner. cDNA synthesis and real-time detection were carried out in a PTC-100™ Thermal Cycler (MJ Research Inc, Waltham, MA) and an ABI Prism™ 7700 Sequence Detection System (Applied Biosystems, Foster City, CA), respectively. Random hexamers (Qiagen, Valencia, CA) were used to generate cDNA from 200ng RNA as described in Applied Biosystems User Bulletin #2. TaqMan™ PCR Core Reagent Kit or TaqMan™ Universal PCR Master Mix (Applied Biosystems) were used in subsequent PCR reactions according to the manufacturer's protocols. Relative quantitation of gene expression was performed using the relative standard curve method. All real-time RT-PCR reactions were performed in triplicate.

Sequence specific primers and probes were designed using Primer Express Version 2 software (Applied Biosystems). Sequences of primers and probes can be found in Table 1 below. Expression levels were normalized to 18S rRNA. The

selection of 18S rRNA as an endogenous control was based on an evaluation of the ΔC_T levels (Applied Biosystems document # 4308134C) of 6 "housekeeping" genes: *Cyclophilin A, 18S, GAPDH, β-actin, β-Glucuronidase,* and *Hypoxanthine Guanine Phosphoribosyl Transferase.* The ΔC_T levels of *18S* did not differ significantly between treatment conditions; thus, they were expressed at constant levels between samples.

Table 1. Real-time PCR primers and probes.

Western blot analysis

Gene	Forward	Probe	Reverse
18S	5'-GCGGCTAGAGGTGAAATTCTTG-3'	5'-6FAM-ACCGGTCCAAGAGGACCAG-TAMRA-3'	5'-CATTCTTGGCAAATGCTTTCG-3'
Collagen1	5'-CGAATTCGGCTTGGACGTT-3'	5'-6FAM-TCTCCTTCCTGTAAACTCCCTCCATCCG-TAMRA-3'	5'-TTCAGTTTGGGTTGCTTGTCTGT-3'
Fibronectin	5'-AGATCTACCTGTACACCTTGAATGACA-3'	5'-6FAM-TGTCGTCATCGACTTTCTCCA-TAMRA-3'	5'-CATGATACCAGCAAGGAATTGG-3'
TIMP3	5'-TGTGTCATGTGTACGCTGTAATATGTG3'	5'-6FAM-CACATCCCCCCATTTTCCTGAATCAA-TAMRA-3'	5'-GCCTAGAAGTATTTTCCTCTCCATTG-3'
PAI-1	5'-GCCTGACTTCACGAGTCTTTCA-3	5'-6FAM-ACCAAGACCCTCTCCACGTCCCG-TAMRA-3'	5'-GTTCACCTCGATCTTCACTTTCTG-3'
CTGF	5'-TGTGTGACGAGCCCAAGGA-3'	5'-6FAM-CTCCCCTCCCGCCTTACCGA-TAMRA-3'	5'-TAGTTGGGTCTGCCCAAAG-3'
IL11	5'- AGAACACGGAATTAAATGTGTCATACA-3'	5'-6FAM-AGACAAATCCCCCTCAAGTGGA-TAMRA-3'	5'-CCCAGTTACCCAAGCATCCA-3'
COX2	5'-GCTCAAACATGATGTTTCCATTC-3'	5'-6FAM-TTGCCCACCACTTCACCCATCAG-TAMRA-3'	5'-CCCCTCGCTTATGATCTGTCTT-3'
IL6	5'- ATGTACCATCGCCACCTCAGAT-3'	5'-6FAM-TGGTCAGAAACCTGTCCACTCCCA-TAMRA-3'	5'-TAACCCTCATACTTTAGTTCTCCATAGA-3'
α-smoothmuscle actin	5'- GCCCAGAGACCCTGTTCCA-3'	5'-6FAM-CCCAGCAGACTCCATCCCGA-TAMRA-3'	5'-TGATCCTGTTGTAGGTGGTTTCA-3'

Cells were cultured in 6-well plates and treated with BNP (100 nM, three times daily) in the presence or absence of 5 ng/ml TGFβ for 48 h. Lysis was induced with 0.2 ml of buffer containing 20 mM Tris-HCL, pH 7.9, 137 mM NaCl, 1% Triton X-100, 5 mM EDTA, 10 mM NaF, 1mM β-glycerophosphate, and protease inhibitor cocktail. The protein concentration of each lysate was measured using coomassie protein reagent from PIERCE. Twenty μg of protein from each sample was loaded and electrophoresed on 4-12% gradient polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes (Invitrogen, San Diego, CA). The membranes were incubated with rabbit anti-human Collagen 1 antibody (Cortex Biochem, San

Leandro, CA), HRP-conjugated anti-human Fibronectin antibody, or goat anti-Actin antibody (Santa Cruz Biotehnology, Santa Cruz, CA) in TBST buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20, and 5% nonfat dried milk at 4°C for ~16 h. For ERK phosphorylation, cells were treated with 0.5 μmol/L BNP in the presence of 1 μmol/L KT5823 or 10 μmol/L U0126 for 15 min; the membranes were incubated with rabbit anti-human phospho-ERK 1/2 antibody or rabbit anti-human ERK 1/2 antibody (Cell Signaling, Beverly, MA). For secondary antibody detection, membranes were incubated with HRP-conjugated anti-rabbit antibody or anti-goat antibody at room temperature for 1 h and washed 3 times with TBST buffer. The blots were soaked in ECL Plus reagent for 5 min and exposed to KODAK x-ray film. Signals were identified and quantified using a Typhoon Scanner and Densitometer from Amersham Biosciences (Piscataway, NJ). Data was analyzed by ANOVA using the Newman-Keuls test to assess significance.

Results

cGMP Production in Cardiac Fibroblasts

To determine if NPRA was expressed in the cultured fibroblast cells, cGMP accumulation assays were utilized. BNP dose-dependently induced intracellular cyclic GMP production in cardiac fibroblasts with an EC50 of 50 nmol/L. These results are consistent with the report of Cao and Gardner showing NPRA expression in cardiac fibroblasts.

Effects of BNP on TGFβ–Induced Fibroblast Proliferation

To examine the effects of TGFβ and BNP on cell proliferation, BrdU incorporation was measured in cardiac fibroblasts treated with TGFβ in the presence or absence of

BNP. TGFβ modestly increased (~50%) cardiac fibroblast proliferation, and BNP inhibited TGFβ–induced proliferation by ~65% (Figure 9).

Effects of BNP on TGFβ-Induced Gene Expression

In order to determine the effects of BNP on gene expression profiles induced by TGFβ in cardiac fibroblasts, a microarray analysis was performed. Fluorescently-labeled cDNA probes were prepared from pooled mRNAs generated from duplicate wells of cells from four groups: unstimulated (control), TGFβ-treated, BNP-treated, and co-treated with TGFβ and BNP for 24 and 48 h (as described above). Arrays were probed in duplicate for a total of 12 hybridizations (6 at each time point): control compared to TGFβ-treated, TGFβ-treated compared to TGFβ + BNP-treated, and control compared to BNP-treated.

It was observed that BNP had no significant effects on gene expression in unstimulated human cardiac fibroblasts (Fig 1). In contrast, TGFβ induced 394 and 501 gene expression changes at 24 and 48 h, respectively. These differentially expressed genes represent ~7-8% of the target genes on the array. Interestingly, BNP had dramatic effects on the gene expression changes induced by TGFβ (Fig 2). Approximately, 88% and 85% of TGFβ–regulated gene expression events were opposed by BNP at 24 and 48 h, respectively. These results demonstrate that BNP has strikingly different effects on gene expression in TGFβ stimulated fibroblasts compared to unstimulated cells.

Gene Expression Clustering

To identify different gene expression patterns following TGFβ stimulation, we performed a hierarchical cluster analysis. A visualization of this analysis is shown in

Fig. 3. A complete listing of differentially expressed genes is provided in Table 2. The clustered expression patterns showed temporal effects of TGFβ responsive genes (compare A to B). In addition, the dramatic effects of BNP in opposing TGFβ induced up- and down-regulated gene changes were revealed in the clusters (compare A and B to C and D). The insignificant effects of BNP on gene expression in unstimulated cardiac fibroblast cells were evident in groups E and F.

Genes were grouped according to functional categories by using a combination of gene expression clustering and functional annotations. A cluster of genes involved in fibrosis and ECM production was up-regulated in cells stimulated with TGFβ; these genes were down-regulated when treated with BNP (Fig. 4a). This cluster includes extracellular matrix components: *Collagen 1a2 (COL1A2), Collagen 15A (COL15A), Collagen 7A1 (COL7A1), Microfibril-associated glycoprotein-2 (MAGP2), Matrilin 3 (MATN3), Fibrillin 1 (FBN1),* and *Cartilage oligomeric matrix protein (COMP).* Also included in the cluster are known markers of fibrosis such as *TIMP3, CTGF, IL11, and SERPINE1 (PAI-1).* Furthermore, the cluster revealed that BNP opposed TGFβ-induction of myofibroblast markers including *α-smooth muscle actin 2 (ACTA2)* and *non-muscle myosin heavy chain (MYH9).*

Many genes involved in cell proliferation were also regulated by TGFβ and were opposed by BNP (Fig. 4B). For example, TGFβ induced the expression of positive regulators of cell proliferation, including *PDGFA, IGFBP10, IGF1, and Parathyroid hormone-like hormone (PTHLH).* It was also found that TGFβ down-regulated both positive and negative regulators of proliferation, such as, *CDC25B* and *Cullin 5 (CUL5),* respectively. All of these TGFβ–regulated gene events were opposed by BNP.

BNP affected TGFβ–induced genes involved in inflammation (Fig. 4C). For example, BNP reversed TGFβ-induction of *PTGS2 (COX2), TNF α-induced protein 6 (TNFAIP6),* and *TNF superfamily, member 4 (TNFSF4)* (Fig 4C and data not shown). *TNFAIP6* and *TNFSF4* were not included in Fig 4C, since some of the data points at 48 h did not meet acceptable criteria (see Experimental); at 24 h both genes were elevated ~3–fold by TGFβ and opposed by BNP. TGFβ also down-regulated many pro-inflammatory genes including *IL1B, CCR2 (MCP1-R)*, CXCL1 (GRO1), CXCL3 (GRO3), and CCL13 (MCP4), which were reversed by BNP. The significance of these inflammatory changes is discussed below.

Table 2. Expression data for differentially expressed genes in TGFβ-treated human cardiac fibroblasts. Median differential expression values are shown for each hybridization: control vs. TGFβ 24 h (column 2); control vs. TGFβ 48 h (column 3); TGFβ vs. TGFβ + BNP 24 h (column 4); TGFβ vs. TGFβ + BNP 48 h (column 5); control vs. BNP 24 h (column 6); and control vs. BNP 48 h (column 7).

Clone ID	TGF 24 h	TGF BNP 24 h	BNP 24 h	TGF 48 h	TGF BNP 48 h	BNP 48 h	Symbol	Name	Accession
P00777_A03	2.5	-2.8	1.1	1.5	-1.6	1.1		EST	
P00777_A04	8.9	-5.7	1.2	3.3	-2.4	1		EST	
P00777_A12	2.1	-2.4	-1	1.8	-1.9	-1.1		EST	
P01061_E01	2.7	-3	1	2.6	-2.8	-1		EST	
P01061_B10	-2.7	2.3	1.1	-4	2.4	-1.2		EST	
P01077_A08	-1.8	3.1	1.3	-2.2	1.9	1.2		No Sequence	
P01111_A08	-1.3	1.4	1.3	-1.8	1.7	1.1		EST	
P01113_E11	-1.7	1.8	1.1	-1.8	1.6	-1		EST	
P01111_F07	-4.5	5.5	1.3	-5.3	4.2	1.1		EST	
P01111_A07	2	-2.7	1.3	1.4	-1.5	-1.1		EST	
P01110_G03	-1.2	1.5	1.3	-3.9	2.1	1.1		No Sequence	
P01108_G07	4.2	-4.4	-1.1	3.9	-4.5	-1		EST	
P01099_G03	-1.9	1.9	1.1	-2.2	1.9	1.2		EST	
P01113_B03	6.4	-5.1	1	4.3	-3.7	-1		EST	
P01080_A11	4	-3	1	4.2	-4.1	-1		EST	
P01076_E01	-1.7	1.8	1.1	-1.8	1.8	-1.1		EST	
P01075_H09	-3.1	3.6	1.4	-2.9	3.2	1.4		No Sequence	
P01139_D10	3	-2.6	1.1	2.1	-2.1	1		EST	
P01132_B01	-2.1	2	1	-1.4	1.3	1		EST	
P01123_H03	2.2	-2.2	1.2	1.9	-1.9	1.1		EST	
P01117_D08	-1.7	1.5	1.1	-4.9	2.4	-1		EST	
P01115_F08	-2.2	1.6	-1	-2.3	1.7	-1		EST	
P01081_F02	2.4	-1.8	1.2	2.4	-2.1	1.1		No Sequence	
P01087_A12	2.4	-2	1	2.6	-2.6	-1		EST	
P01077_A02	2.2	-2	1	1.4	-1.3	-1		No Sequence	

P01136_G11	-2	2.5	1.3	-3	2.5	1		EST	
P01130_B03	-3.3	3.5	1.1	-4.2	5.3	1.1		EST	
P01124_A05	-1.2	-1	1.1	-1.8	1.5	1		EST	
P01124_A10	2.1	-2	-1	2.7	-2.5	-1.1		EST	
P01124_B04	-1.9	2	1.3	-1.6	1.7	1.1		EST	
P01120_G06	-2.3	2.2	-1.1	-2.4	2.2	-1.1		EST	
P01117_B11	1.8	-2.4	1	2.4	-2	1		EST	
P01116_A02	-3.1	2.7	1.1	-3.7	2.2	-1.4		EST	
P01088_C10	2.1	-2	-1	1.6	-2.1	-1.1		EST	
P01093_C04	2.6	-2.3	1	1.8	-1.9	-1		EST	
P01095_H01	-1.8	1.8	1	-1.4	1.2	1		EST	
P01099_D03	1.9	-1.8	1.1	1.1	-1.2	1.1		EST	
P01100_A07	1	-1	1.1	-3	1.7	-1.1		EST	
P01100_D09	-1.6	1.6	-1	-2.1	1.8	-1.1		EST	
P01101_C11	-2.4	1.7	-1	-1.4	1.6	1		No Sequence	
P01101_E11	-1.4	1.5	1.1	-2	1.8	-1		EST	
P01103_H04	-3.2	2.9	1.1	-5.6	4.3	-1		EST	
P01104_A09	-1.9	1.6	1.1	-1.8	1.5	1		No Sequence	
P01104_E03	-2.5	2.3	-1	-2.8	2	-1.1		EST	
P01104_G04	2.5	-2	-1	1.1	-1.3	-1.1		EST	
P01104_G12	-3.7	2.7	-1.1	-4.9	3.2	-1		EST	
P01105_A05	2.3	-2.3	1.3	1.3	-1.3	1		EST	
P01105_D09	1.8	-1.1	1.1	1.8	-2.1	-1		EST	
P01109_A01	-1.4	1.4	1.2	-2.2	1.7	1.1	A2M	alpha-2-macroglobulin	NM_000014
P01109_G11	1.4	-1	1.1	2	-1.6	1	ABCG1	ATP-binding cassette, sub-family G (WHITE), member 1	NM_004915
P01092_E08	2.3	-2	1.2	1.5	-1.3	1.1	ACLY	ATP citrate lyase	NM_001096
P01088_C02	-1.9	1.8	1.2	-2.1	2	1	ACO1	aconitase 1, soluble	NM_002197
P00777_G09	2.6	-2.2	-1.5	1	1	-1.3	ACTA1	actin, alpha 1, skeletal muscle	NM_001100
P01094_F04	2.6	-2.5	-1.4	-1	-1	-1.4	ACTA2	actin, alpha 2, smooth muscle, aorta	NM_001613
P01091_G04	1.9	-1.6	1.1	1.2	-1.3	-1	ACTR3	ARP3 actin-related protein 3 homolog (yeast)	NM_005721
P01096_D02	-1.3	1.5	1.1	-2.3	2.2	1	ADAMTS1	a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 1	NM_006988
P01097_D04	1.7	-1.9	-1	2.1	-1.8	-1.1	ADAMTS6	a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 6	NM_014273
P01092_D03	-6.5	6	-1.1	-6.3	6.5	-1	ADFP	adipose differentiation-related protein	NM_001122
P01070_D09	-5	4.1	1.3	-9.7	3.8	1.3	ADH1B	alcohol dehydrogenase IB (class I), beta polypeptide	NM_000668
P01134_D11	-1.7	2	1.3	-3.6	1.6	1.2	ADH1C	alcohol dehydrogenase 1C (class I), gamma polypeptide	NM_000669
P01070_D05	-1.3	-1.4	1.2	-2.2	1.7	1.1	ADH5	alcohol dehydrogenase 5 (class III), chi polypeptide	NM_000671
P01094_D10	-2.3	2.5	1.1	-2.2	1.8	-1	ADORA2B	adenosine A2b receptor	NM_000676
P01124_F09	-1.5	1.6	1.1	-1.8	1.9	1	AHR	aryl hydrocarbon receptor	NM_001621
P01101_B03	-2.4	1	1	-3	2.8	1.1	AKAP2	A kinase (PRKA) anchor protein 2	NM_007203
P01120_C03	-1.9	2	1.2	-1.2	1.5	1.2	AKR1B1	aldo-keto reductase family 1, member B1 (aldose reductase)	NM_001628
P01134_B08	-2.7	2.6	1.1	-1.4	1.9	1.2	AKR1B10	aldo-keto reductase family 1, member B10 (aldose reductase)	NM_020299

P01069_C01	-2.8	3.1	1.2	-2.2	2.6	1.1	AKR1C1	aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase)	NM_001353
P01081_A11	-2.3	3.3	1.6	-2.2	1.9	1.3	AKR1C2	aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type III)	NM_001354
P01143_D10	-2.8	3.2	1.3	-2.1	2.7	1.1	AKR1C2	aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type III)	NM_001354
P01106_C11	-2.3	2.8	1.2	-2	2.5	1.1	AKR1C3	aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II)	NM_003739
P01094_D12	-2.8	3.6	1.2	-2.5	1.7	1.2	ALDH1A3	aldehyde dehydrogenase 1 family, member A3	NM_000693
P01094_E01	-1.4	1.8	1.1	-2.1	1.6	1.1	ALDH3A2	aldehyde dehydrogenase 3 family, member A2	NM_000382
P01140_G11	-1.9	2.7	1.4	-2.5	1.8	1.1	ALDH3A2	aldehyde dehydrogenase 3 family, member A2	NM_000382
P01118_A12	-1.9	1.6	1.1	-2.6	2.2	1	ALEX1	ALEX1 protein	NM_016608
P01096_E12	-2.4	2	1	-2.1	2.2	1	ANG	angiogenin, ribonuclease, RNase A family, 5	NM_001145
P01145_E08	-2	2.3	1.2	-2.9	2.6	-1	ANGPT1	angiopoietin 1	NM_001146
P01091_G02	-1.2	1.5	1.2	-2.7	2	1.1	ANGPT2	angiopoietin 2	NM_001147
P01094_D06	-2.1	1.9	-1	-1.9	1.3	-1.1	ANK3	ankyrin 3, node of Ranvier (ankyrin G)	NM_001149
P01128_A07	-1.5	1.8	1.2	-2.2	2.3	1.2	AOX1	aldehyde oxidase 1	NM_001159
P01116_H05	-1.1	1.4	1.2	-2	1.8	1	APELIN	apelin; peptide ligand for APJ receptor	NM_017413
P01103_F06	2.4	-2.4	-1.1	1.4	-1.5	-1.1	APG3	autophagy Apg3p/Aut1p-like	NM_022488
P01123_A07	3.2	-3	-1	1.5	-1.8	-1	APOA1	apolipoprotein A-I	NM_000039
P01105_G06	-2.2	1.8	-1.1	-4.5	5.7	1.1	APOC1	apolipoprotein C-I	NM_001645
P01124_G03	-1.3	1.4	1	-2.4	2	1.1	APOE	apolipoprotein E	NM_000041
P01105_B02	-1.6	1.8	-1	-2.9	1.9	1.2	ARHGAP6	Rho GTPase activating protein 6	NM_001174
P01064_G03	-1.1	1.3	1.1	-2	1.6	1.2	ARHGEF16	Rho guanine exchange factor (GEF) 16	NM_014448
P01110_E10	-2	2.1	1.2	-2.3	1.9	1	ARHGEF3	Rho guanine nucleotide exchange factor (GEF) 3	NM_019555
P01142_C03	-1.6	1.8	1.5	-1.9	1.7	1.2	ARHI	ras homolog gene family, member I	NM_004675
P01138_A09	1.9	-2.2	-1.1	1.8	-1.9	-1.1	ARL4	ADP-ribosylation factor-like 4	NM_005738
P01064_G12	-1.7	1.8	1.1	-1.8	1.6	-1	ARNT2	aryl-hydrocarbon receptor nuclear translocator 2	NM_014862
P01088_H09	-1.5	1.7	1.2	-1.8	1.6	1.1	ASAH1	N-acylsphingosine amidohydrolase (acid ceramidase) 1	NM_004315
P01105_F06	2.9	-2.8	1.1	2.1	-2.4	-1.2	ASNS	asparagine synthetase	NM_001673
P01070_E06	1.8	-1.5	-1.3	1.6	-1.4	1	ATF3	activating transcription factor 3	NM_001674
P01122_G07	-1.2	1.7	1.2	-1.8	1.5	1.3	AXIN2	axin 2 (conductin, axil)	NM_004655
P01115_D06	-1.4	1.6	1	-2	1.5	-1.1	B3GALT2	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 2	NM_003783
P01128_A08	-1.6	1.7	1	-2.4	1.7	-1	B3GALT3	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase,	NM_003781

								polypeptide 3	
P01095_F06	2.4	-2.2	1.1	1.3	-1.5	-1	BAI3	brain-specific angiogenesis inhibitor 3	NM_001704
P01094_C02	-1.8	2	1.2	-2.4	2.7	-1	BF	B-factor, properdin	NM_001710
P01134_E02	-1.7	1.8	1	-2.2	1.6	-1	BFSP1	beaded filament structural protein 1, filensin	NM_001195
P01081_D08	-1.2	1.7	1.2	-3.5	1.8	1.2	BIRC1	baculoviral IAP repeat-containing 1	NM_004536
P01094_B06	-2.6	2.9	1.1	-4	2.5	-1	BMP4	bone morphogenetic protein 4	NM_001202
P01145_A02	-3.2	2.3	1	-3.6	3.2	-1.1	BNIP2	BCL2/adenovirus E1B 19kDa interacting protein 2	NM_004330
P01075_F05	-1.5	1.5	1.2	-1.8	2	1.2	BRE	brain and reproductive organ-expressed (TNFRSF1A modulator)	NM_004899
P01124_B10	-1.3	1.5	1.3	-2.2	1.6	1.2	BST1	bone marrow stromal cell antigen 1	NM_004334
P01094_B08	-1.8	1.6	-1.1	-1.2	1.3	-1.1	BTD	biotinidase	NM_000060
P01093_E08	-2	1.5	-1.1	-1.9	2.7	1.1	C1R	complement component 1, r subcomponent	NM_001733
P01077_E12	-1.4	1.6	1.1	-1.8	1.9	-1.1	C1S	complement component 1, s subcomponent	NM_001734
P01097_G03	1.9	-1.7	-1	1	-1.5	-1.1	C20orf14	chromosome 20 open reading frame 14	NM_012469
P01140_A07	2.3	-3.2	-1	3	-2.6	-1	C20orf97	chromosome 20 open reading frame 97	NM_021158
P01069_E02	-1.7	1.6	1.1	-3.3	3.2	1.1	C6	complement component 6	NM_000065
P01077_E10	-3.1	2.9	1.1	-8.2	4.7	-1	C7	complement component 7	NM_000587
P01099_C10	-1.8	2.1	1.2	-2.7	3.5	1.1	CA12	carbonic anhydrase XII	NM_001218
P01117_G05	-3	2.4	-1.1	-2.2	2.3	1.1	CAMK2B	calcium/calmodulin-dependent protein kinase (CaM kinase) II beta	NM_001220
P01114_A05	-2.7	3.9	1.2	-3.5	2.7	1	CAMK2D	calcium/calmodulin-dependent protein kinase (CaM kinase) II delta	NM_001221
P01080_B05	-2.3	3	1.1	-2.3	2.1	1.1	CAMK2D	calcium/calmodulin-dependent protein kinase (CaM kinase) II delta	NM_001221
P01063_E07	-1.6	2	1.2	-1.8	1.6	1.1	CASP1	caspase 1, apoptosis-related cysteine protease (interleukin 1, beta, convertase)	NM_001223
P01093_G08	-2.4	2.3	-1.2	-2.1	2.4	1	CAV1	caveolin 1, caveolae protein, 22kDa	NM_001753
P01093_E04	1.8	-1.7	-1.1	1.6	-1.9	-1.1	CBS	cystathionine-beta-synthase	NM_000071
P01064_D02	-1.5	1.6	-1.3	-2.2	2.8	-1.1	CCL13	chemokine (C-C motif) ligand 13	NM_005408
P01072_E08	-1.3	1.4	-1.2	-2.2	3.2	-1.1	CCL7	chemokine (C-C motif) ligand 7	NM_006273
P01127_H03	1.1	1.2	-1.3	-2	2.9	-1	CCL8	chemokine (C-C motif) ligand 8	NM_005623
P01070_A04	-1.4	1.9	1.2	-3.2	2.4	1.1	CCR2	chemokine (C-C motif) receptor 2	NM_000647
P01138_B02	-1.2	1.3	1.3	-3.6	1.5	1	CCRL1	chemokine (C-C motif) receptor-like 1	NM_016557
P01069_H09	-1.9	1.9	1.3	-3.6	1.8	1.2	CD36	CD36 antigen (collagen type I receptor, thrombospondin receptor)	NM_000072
P01072_E03	-2.8	2.7	1.2	-2.9	2.8	1.2	CDC25B	cell division cycle 25B	NM_004358
P01093_H07	2	-4.3	1.2	2.1	-2	-1	CDH2	cadherin 2, type 1, N-cadherin (neuronal)	NM_001792
P01129_E07	1.7	-1.4	1.1	2	-1.9	-1.1	CDH4	cadherin 4, type 1, R-cadherin (retinal)	NM_001794

P01130_H07	2.1	-2.4	-1.1	1.9	-1.8	-1	CDH5	cadherin 5, type 2, VE-cadherin (vascular epithelium)	NM_001795
P01116_H02	-3.3	2.1	1.1	-2	2.4	1.1	CDK5RAP2	CDK5 regulatory subunit associated protein 2	NM_018249
P01102_B02	-2.1	2.5	1	-3.4	3.2	-1.1	CDSN	corneodesmosin	NM_001264
P01140_G02	-1.4	1.3	1.1	-2.9	2.4	1	CEACAM5	carcinoembryonic antigen-related cell adhesion molecule 5	NM_004363
P01094_A06	-1.6	1.3	1.3	-4.2	2.9	1	CEACAM5	carcinoembryonic antigen-related cell adhesion molecule 5	NM_004363
P01062_G02	-1.3	1.5	1.3	-2.9	2.1	1.1	CEACAM6	carcinoembryonic antigen-related cell adhesion molecule 6 (non-specific cross reacting antigen)	NM_002483
P01099_B05	-1.8	1.8	1.1	-2.9	3	-1.1	CEACAM7	carcinoembryonic antigen-related cell adhesion molecule 7	NM_006890
P01090_E04	-1.3	1.6	1.3	-1.9	1.8	-1	CEBPD	CCAAT/enhancer binding protein (C/EBP), delta	NM_005195
P01070_A01	-2.6	3.1	-1	-9.2	9.2	1.1	CHI3L1	chitinase 3-like 1 (cartilage glycoprotein-39)	NM_001276
P01125_G02	-2.9	2	1	-5	6.2	1	CHI3L2	chitinase 3-like 2	NM_004000
P01134_F10	8	-6.3	1.2	19.5	-8	1.1	CILP	cartilage intermediate layer protein, nucleotide pyrophosphohydrolase	NM_003613
P01089_A12	-1.9	2.1	1	-2.1	2.1	-1	CITED2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	NM_006079
P01076_A07	2.1	-1.8	-1	1.4	-1.2	1.1	CKAP4	cytoskeleton-associated protein 4	NM_006825
P01104_C09	2.2	-2.4	1.1	4.3	-2.8	1	CKLF	chemokine-like factor	NM_016326
P01103_G05	-1.4	1.6	1.3	-2.5	1.5	1.3	CLDN1	claudin 1	NM_021101
P01105_D03	-3	2.7	1.3	-2.6	2	-1	CLECSF2	C-type (calcium dependent, carbohydrate-recognition domain) lectin, superfamily member 2 (activation-induced)	NM_005127
P01064_F09	2.2	-1.5	1.2	1.2	-1.2	1.1	CNN1	calponin 1, basic, smooth muscle	NM_001299
P01090_A03	-1.1	1.3	1.2	-2.2	1.6	1.1	CNTNAP1	contactin associated protein 1	NM_003632
P01069_F02	1.2	1.2	1.2	3.2	-3	1	COL15A1	collagen, type XV, alpha 1	NM_001855
P01077_E08	1.8	-1.5	1	1.9	-1.9	-1	COL1A2	collagen, type I, alpha 2	NM_000089
P01093_F03	1.7	-2.3	1	1.9	-2.1	-1	COL4A2	collagen, type IV, alpha 2	NM_001846
P01105_C12	1.8	-1.5	1.4	3.1	-2.3	1.1	COL7A1	collagen, type VII, alpha 1 (epidermolysis bullosa, dystrophic, dominant and recessive)	NM_000094
P01120_G04	2.7	-2.1	1.2	3.8	-3.6	1.1	COL8A2	collagen, type VIII, alpha 2	M60832
P01084_A12	-4.9	4.7	1.1	-9.9	6	1	COLEC12	collectin sub-family member 12	NM_030781
P01082_H06	1.3	-1.3	1.2	3.3	-2.4	1.2	COMP	cartilage oligomeric matrix protein (pseudoachondroplasia, epiphyseal dysplasia 1, multiple)	NM_000095
P01129_C12	1.4	-1.5	1.3	2.6	-1.6	1.3	COMP	cartilage oligomeric matrix protein (pseudoachondroplasia, epiphyseal dysplasia 1, multiple)	NM_000095
P01076_C09	-2.2	2.7	1.2	-2.1	1.6	1.1	COPB	coatomer protein complex, subunit beta	NM_016451

P01085_D11	-4	4.2	1.1	-7.7	4.1	1.1	CPA4	carboxypeptidase A4	NM_016352
P01104_A07	-1.9	2	1.2	-2.5	2.2	1	CPD	carboxypeptidase D	NM_001304
P01077_G01	1.9	-1.8	1.1	1.7	-1.9	1.1	CRABP2	cellular retinoic acid binding protein 2	NM_001878
P01095_E03	-1.8	1.8	1.2	-2.1	2	-1.1	CREG	cellular repressor of E1A-stimulated genes	NM_003851
P01124_E01	-2.2	2.1	1	-2.5	2.2	-1	CREM	cAMP responsive element modulator	NM_001881
P01120_B01	1.8	-1.6	1.2	3.9	-3.4	1.1	CRLF1	cytokine receptor-like factor 1	NM_004750
P01120_D10	-1.5	1.9	1.3	-3.5	2.4	1.1	CROT	carnitine O-octanoyltransferase	NM_021151
P01124_F10	-1.2	1.3	1.2	-1.8	1.7	1.1	CRYAA	crystallin, alpha A	NM_000394
P00777_A08	-2	1.6	1.1	-2.6	2.5	-1.1	CRYAB	crystallin, alpha B	NM_001885
P01077_E04	-2.1	1.8	1.1	-2.5	2.6	-1.1	CRYAB	crystallin, alpha B	NM_001885
P01125_B11	-1.8	1.2	1.1	-1.8	1.8	1	CSF1	colony stimulating factor 1 (macrophage)	NM_000757
P01108_G05	3.8	-3	1.1	2.3	-2.4	1	CSPG2	chondroitin sulfate proteoglycan 2 (versican)	NM_004385
P01075_F12	-1.5	1.6	1.1	-2	2	1.1	CSRP2	cysteine and glycine-rich protein 2	NM_001321
P01145_A03	-2.1	2.4	1	-3.7	3.4	-1.1	CST4	cystatin S	NM_001899
P00777_D03	2.5	-2	1.1	1.1	-1.4	-1.2	CTGF	connective tissue growth factor	NM_001901
P01077_D08	2.6	-3.5	-1.2	1.8	-2.7	-1.2	CTGF	connective tissue growth factor	NM_001901
P01069_D11	2	-2.1	1.2	1.9	-1.4	1.2	CTH	cystathionase (cystathionine gamma-lyase)	NM_001902
P01099_B01	-1.7	2	1.1	-2	1.6	-1	CTNNAL1	catenin (cadherin-associated protein), alpha-like 1	NM_003798
P01093_G10	-1.4	1.4	1.1	-1.8	2	1	CTSC	cathepsin C	NM_001814
P01077_G03	-1	1.3	1.2	-1.8	1.7	1.2	CTSH	cathepsin H	NM_004390
P01069_H12	-1.5	1.5	1.1	-2.3	2.6	-1	CTSK	cathepsin K (pycnodysostosis)	NM_000396
P01093_G09	-2.5	2.1	1.1	-2	2.3	-1	CTSL	cathepsin L	NM_001912
P01112_D02	-1.6	1.8	1.2	-2.9	2.1	-1	CUGBP2	CUG triplet repeat, RNA binding protein 2	NM_006561
P01131_G04	-1.3	1.6	1.3	-2.2	1.7	1.3	CUGBP2	CUG triplet repeat, RNA binding protein 2	NM_006561
P01090_H01	-2	1.8	1.3	-1.5	1.9	1.3	CUL5	cullin 5	NM_003478
P01085_C05	-3.8	3.4	1.1	-5.5	5	-1	CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	NM_001511
P01093_A02	-3.7	3.1	1	-5.8	5.4	-1	CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	NM_001511
P01125_H11	-2.4	2	1.1	-2.3	2.1	1	CXCL3	chemokine (C-X-C motif) ligand 3	NM_002090
P01136_B01	-4.5	4.4	1.1	-8.4	10	1.1	CXCL6	chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	NM_002993
P01069_D07	-2.4	2.3	1.3	-2	1.7	1	CYB5	cytochrome b-5	NM_001914
P00777_A11	2	-2.5	-1	1.8	-2	-1	CYR61	cysteine-rich, angiogenic inducer, 61	NM_001554
P00777_C11	1.8	-2.5	-1	1.8	-1.9	-1.1	CYR61	cysteine-rich, angiogenic inducer, 61	NM_001554
P00777_C12	2	-2.6	-1.1	1.9	-1.9	-1.1	CYR61	cysteine-rich, angiogenic inducer, 61	NM_001554
P01108_B04	2.3	-2.4	1.1	1.9	-1.9	-1.1	CYR61	cysteine-rich, angiogenic inducer, 61	NM_001554
P01130_H03	2	-2.4	-1.1	1.9	-1.8	-1.1	CYR61	cysteine-rich, angiogenic inducer, 61	NM_001554
P01100_C06	2.2	-2.4	-1.1	1.8	-1.9	-1	DACT1	dapper homolog 1, antagonist of beta-catenin (xenopus)	NM_016651

P01069_C07	1.7	-1.4	1.1	2.3	-2.1	1.1	DAF	decay accelerating factor for complement (CD55, Cromer blood group system)	NM_000574
P01129_B04	-2.8	2.5	1.2	-4.3	3.5	1	DAPK1	death-associated protein kinase 1	NM_004938
P01092_G02	-2.7	2.6	1.1	-3.8	2.8	1.2	DAPK1	death-associated protein kinase 1	NM_004938
P01065_A02	-1.8	1.9	-1	-1.8	1.7	-1.1	DDX38	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 38	NM_014003
P01105_A10	-3.7	2.9	-1	-7	5.4	-1	DKK1	dickkopf homolog 1 (Xenopus laevis)	NM_012242
P01113_E05	2.8	-2.4	-1.1	1.8	-2.1	-1.1	DLC1	deleted in liver cancer 1	NM_006094
P01093_C11	-1.8	1.9	-1	-4.5	3.3	-1	DPP4	dipeptidylpeptidase 4 (CD26, adenosine deaminase complexing protein 2)	NM_001935
P01073_G11	-1.8	1.7	1	-1.6	1.5	1	DPYSL2	dihydropyrimidinase-like 2	NM_001386
P01090_F08	1.4	-1.5	-1.1	2	-1.9	-1.1	DSCR1	Down syndrome critical region gene 1	NM_004414
P01122_D11	1.7	-1.2	1.3	1.9	-2.1	1.3	EBAF	endometrial bleeding associated factor (left-right determination, factor A; transforming growth factor beta superfamily)	NM_003240
P01123_B11	-1.8	1.7	1	-2.3	1.9	1	ECM2	extracellular matrix protein 2, female organ and adipocyte specific	NM_001393
P01124_E11	-1.6	1.9	1.2	-2.1	1.9	1.2	EDG1	endothelial differentiation, sphingolipid G-protein-coupled receptor, 1	NM_001400
P01103_G08	-1.8	1.8	1.1	-2.4	2.4	1	EDG2	endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2	NM_001401
P01093_C01	-2.1	1.5	-1.1	-2.9	1.9	-1.3	EDN1	endothelin 1	NM_001955
P01105_H10	-1.9	1.9	1	-2.2	2.3	-1	EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1	NM_004105
P01064_A03	-1.4	1.9	1.2	-2	2	1.1	EFNB3	ephrin-B3	NM_001406
P01093_B07	-1.8	1.7	1.3	-1.5	1.3	1.1	EGR2	early growth response 2 (Krox-20 homolog, Drosophila)	NM_000399
P01121_C03	-2	2	1.2	-1.2	1.5	1.2	EHD3	EH-domain containing 3	NM_014600
P01065_E02	1.9	-1.6	1.2	3.4	-3.3	1.1	ELN	elastin (supravalvular aortic stenosis, Williams-Beuren syndrome)	NM_000501
P01096_H11	-3.4	3.7	1.1	-3.5	3.2	-1	EPAS1	endothelial PAS domain protein 1	NM_001430
P01102_E11	-2	2.1	1.2	-2.5	2.1	-1	EPB41L2	erythrocyte membrane protein band 4.1-like 2	NM_001431
P01104_A05	-2.3	3.3	1.1	-2.2	2.3	1.1	EPI64	EBP50-PDZ interactor of 64 kD	NM_031937
P01130_H01	-2	2	1.1	-2.5	3.9	-1	EPOR	erythropoietin receptor	NM_000121
P01077_A07	-1.6	2.7	1.4	-2.3	2.1	1.2	ETV5	ets variant gene 5 (ets-related molecule)	NM_004454
P01097_C06	-5.9	4.9	-1	-15.8	14	-1.1	EVI2B	ecotropic viral integration site 2B	NM_006495
P01077_A01	1.8	-1.8	1.1	1.3	-1.4	1	EXT1	exostoses (multiple) 1	NM_000127
P01069_F04	-1.7	1.6	1.2	-2.1	1.7	1.1	F2R	coagulation factor II (thrombin) receptor	NM_001992
P01128_B02	1.8	-1.9	1.1	-1	-1.1	-1	F3	coagulation factor III (thromboplastin, tissue factor)	NM_001993
P01132_G03	1.9	-1.7	1.2	1.8	-1.6	1.1	FACL3	fatty-acid-Coenzyme A ligase, long-chain 3	NM_004457
P01096_A03	1.8	-2	1	1.8	-2	1	FACL3	fatty-acid-Coenzyme A ligase, long-chain 3	NM_004457

P01083_D07	2.2	-1.6	1.2	1.4	-1.3	1	FADS1	fatty acid desaturase 1	NM_013402
P01093_B02	-2	1.6	1.1	-3.4	3.4	1	FBLN1	fibulin 1	NM_001996
P01123_A08	3.4	-3	1.2	1.6	-1.9	-1	FBLN5	fibulin 5	NM_006329
P01068_H09	1.4	-1.4	1	2.2	-2	-1	FBN1	fibrillin 1 (Marfan syndrome)	NM_000138
P01084_E10	1.9	-1.7	1.3	-1.1	-1.1	1.1	FGF18	fibroblast growth factor 18	NM_003862
P01093_B03	-4.2	4.9	1.2	-5.9	5.6	1	FGF7	fibroblast growth factor 7 (keratinocyte growth factor)	NM_002009
P01092_C04	-3.2	2.9	1.1	-3.1	2.3	-1	FGL2	fibrinogen-like 2	NM_006682
P01126_F06	-5	5.2	-1.1	-6.5	4.9	-1.1	FMO2	flavin containing monooxygenase 2	NM_001460
P01078_G11	-1.9	2.1	1.2	-3.1	2.2	1.1	FMO3	flavin containing monooxygenase 3	NM_006894
P01088_F09	2	-1.8	1.2	1.4	-1.5	1.1	FOXD1	forkhead box D1	NM_004472
P01120_B03	-1.8	1.9	1.3	-1.2	1.5	1.2	FRA	Fos-related antigen	NM_024816
P01138_B06	-1.8	1.5	1	-1.4	1.8	-1	FTHL17	ferritin, heavy polypeptide-like 17	NM_031894
P01068_G11	2.7	-2.1	1.3	2.4	-2.5	1.1	FUT4	fucosyltransferase 4 (alpha (1,3) fucosyltransferase, myeloid-specific)	NM_002033
P01077_A05	1.8	-1.5	1	1.5	-1.2	1	FYN	FYN oncogene related to SRC, FGR, YES	NM_002037
P01124_G01	-1.9	1.9	1.1	-1.4	1.2	1	FZD7	frizzled homolog 7 (Drosophila)	NM_003507
P01083_B09	3.2	-4	-1	4.2	-3.5	-1	GABARAPL2	GABA(A) receptor-associated protein-like 2	NM_007285
P01106_B05	-1.8	1.5	1	-1.1	2.4	1.2	GALT	galactose-1-phosphate uridylyltransferase	NM_000155
P01092_G07	2.5	-2.3	-1.2	1.8	-2.2	-1.1	GARS	glycyl-tRNA synthetase	NM_002047
P01085_D09	-2.9	4.1	1.4	-5.3	2.6	1.3	GAS1	growth arrest-specific 1	NM_002048
P01063_E09	-2	1.7	1.1	-2	1.8	1.2	GBP2	guanylate binding protein 2, interferon-inducible	NM_004120
P01123_D12	-1.9	1.4	-1.2	-2.7	2.7	-1.1	GBP2	guanylate binding protein 2, interferon-inducible	NM_004120
P01135_C03	-1.8	1.9	1.2	-2.7	2.4	1.1	GCNT1	glucosaminyl (N-acetyl) transferase 1, core 2 (beta-1,6-N-acetylglucosaminyltransferase)	NM_001490
P01127_B01	-2.8	2.2	-1.1	-2.9	3.7	-1	GDF5	growth differentiation factor 5 (cartilage-derived morphogenetic protein-1)	NM_000557
P01065_A06	-1.7	1.7	1.1	-1.8	1.6	1	GGA3	golgi associated, gamma adaptin ear containing, ARF binding protein 3	NM_014001
P01076_H05	-2	2.4	1.2	-2.7	1.9	1.1	GM2A	GM2 ganglioside activator protein	NM_000405
P01062_E04	-2.3	1.9	-1	-2.1	1.9	-1	GNPI	glucosamine-6-phosphate isomerase	NM_005471
P01138_C10	-2.1	2.2	-1.1	-2	1.9	-1.1	GNPI	glucosamine-6-phosphate isomerase	NM_005471
P01074_D06	3.5	-4	-1	5.7	-3.6	1.1	GOLGA4	golgi autoantigen, golgin subfamily a, 4	NM_002078
P01083_C04	-1.1	1.2	1.2	-1.8	1.5	1.1	GOLPH2	golgi phosphoprotein 2	NM_016548
P01125_G10	1.8	-1.9	-1	1.6	-1.9	-1.1	GOLPH4	golgi phosphoprotein 4	NM_014498
P01131_F08	1.7	-2.3	-1.2	1.8	-1.6	-1.2	GOT1	glutamic-oxaloacetic transaminase 1, soluble (aspartate aminotransferase 1)	NM_002079
P01080_A01	-1.2	1.9	1.3	-3.6	1.8	1.2	GPM6B	glycoprotein M6B	NM_005278
P01082_E09	-2.2	2.3	1.2	-2.9	2.3	1	GPNMB	glycoprotein (transmembrane) nmb	NM_002510
P01087_G08	-3	2.3	1	-4.9	4	-1	GPNMB	glycoprotein (transmembrane) nmb	NM_002510
P01140_E04	-1.8	1.6	1.3	-2.5	1.8	1.1	GPRK5	G protein-coupled receptor	NM_005308

								kinase 5	
P01068_E08	-3.2	1.8	-1.1	-1.9	2.9	1.1	GSTM1	glutathione S-transferase M1	NM_000561
P01068_E09	-1.8	1.5	1.1	-1.7	2.3	1.1	GSTM3	glutathione S-transferase M3 (brain)	NM_000849
P01086_A10	-2.4	1.5	1.1	-1.9	2.7	1.1	GSTM5	glutathione S-transferase M5	NM_000851
P01080_C03	1.7	-1.8	1.1	2.1	-1.9	-1	GTPBP2	GTP binding protein 2	NM_019096
P01108_A05	-1.2	1.5	1.2	-1.9	1.7	1.1	GYPC	glycophorin C (Gerbich blood group)	NM_002101
P01121_B02	-1.6	1.2	-1	-1.9	1.9	1.1	HAGE	DEAD-box protein	NM_018665
P01133_H11	-1.2	1.8	1.4	-2.1	1.7	1.2	HAS2	hyaluronan synthase 2	NM_005328
P01101_C10	-1.8	1.6	-1	-1.4	1.5	-1	HEBP1	heme binding protein 1	NM_015987
P01137_B02	1.8	-1.5	1	1.6	-1.3	1	HERPUD1	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	NM_014685
P01136_A05	2	-2.1	1.1	2	-1.8	-1	HERPUD1	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	NM_014685
P01083_G12	1.6	-1.1	1.1	2.2	-1.5	1.2	HEYL	hairy/enhancer-of-split related with YRPW motif-like	NM_014571
P01126_B01	-1.3	1.4	1.1	-1.8	1.8	-1	HFL1	H factor (complement)-like 1	NM_002113
P01075_H10	-3.6	6.2	1.3	-5.3	3.8	1.3	HGF	hepatocyte growth factor (hepapoietin A; scatter factor)	NM_000601
P01110_C10	1.9	-1.6	1.3	1.3	-1.2	1.1	HMGCR	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	NM_000859
P01112_G07	2	-1.7	1.3	-1	-1.1	-1	HMGCS1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	NM_002130
P01064_F02	-2	2.6	1.1	-3.1	3	1	HNMT	histamine N-methyltransferase	NM_006895
P01078_F05	-2.1	2.4	1.2	-2.1	2.6	1.1	HPN	hepsin (transmembrane protease, serine 1)	NM_002151
P01107_H06	1.8	-1.9	-1.2	1.4	-1.7	-1.3	IARS	isoleucine-tRNA synthetase	NM_002161
P01100_C10	-1.2	1.5	1.3	-1.8	1.7	1.1	ICOS	inducible T-cell co-stimulator	NM_012092
P01124_A06	-1.7	2	1.3	-1.8	1.7	-1	ID2	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	NM_002166
P01072_H03	1.8	-1.6	1.1	1.6	-1.6	1.2	ID4	inhibitor of DNA binding 4, dominant negative helix-loop-helix protein	NM_001546
P01088_C01	-2.4	2.2	1	-2.5	2.2	-1	IDH2	isocitrate dehydrogenase 2 (NADP+), mitochondrial	NM_002168
P01130_F01	4.5	-2.9	1.3	1.8	-1.7	1	IGF1	insulin-like growth factor 1 (somatomedin C)	NM_000618
P01063_D10	2.1	1	1.2	3.8	-1.9	1.3	IGF1	insulin-like growth factor 1 (somatomedin C)	NM_000618
P00777_D09	-2.6	2.2	1.1	-2.9	3.2	1.2	IGFBP4	insulin-like growth factor binding protein 4	NM_001552
P01130_B02	12.3	-11.1	1.2	6.1	-5.4	1.1	IL11	interleukin 11	NM_000641
P01088_D05	-2	2	1.2	-1.8	1.4	1.1	IL1B	interleukin 1, beta	NM_000576
P01063_E06	-3	3.3	1.1	-6.7	6.1	1.1	IL1R1	interleukin 1 receptor, type I	NM_000877
P01110_E12	-1.4	2.3	1.3	-2.5	1.5	1.1	IL1R1	interleukin 1 receptor, type I	NM_000877
P01145_A04	-3	2.4	-1	-4.2	2.7	-1	IL6ST	interleukin 6 signal transducer (gp130, oncostatin M receptor)	NM_002184
P01091_B03	-1.9	1.9	-1	-1.3	1.3	1	IMPA2	inositol(myo)-1(or 4)-monophosphatase 2	NM_014214
P01063_E03	1.7	-1.7	-1.2	2.4	-1.7	1.1	INDO	indoleamine-pyrrole 2,3 dioxygenase	NM_002164
P01082_F07	2.1	-2.6	-1.1	2.2	-1.5	1.2	INHBA	inhibin, beta A (activin A, activin AB alpha polypeptide)	NM_002192

P01130_D09	2.1	-1.7	-1	1.7	-1.7	-1.1	INPP4B	inositol polyphosphate-4-phosphatase, type II, 105kDa	NM_003866
P01067_B04	2	-1.7	1.2	1.6	-1.3	1.1	INSIG1	insulin induced gene 1	NM_005542
P01074_G10	-1.7	1.7	-1	-4.7	3.1	-1.1	IQGAP2	IQ motif containing GTPase activating protein 2	NM_006633
P01061_E02	2.6	-2.6	1	2.4	-2.5	-1	ISGF3G	interferon-stimulated transcription factor 3, gamma 48kDa	NM_006084
P01140_B08	1.8	-1.7	1.2	3	-1.8	1	ITGA11	integrin, alpha 11	NM_012211
P01088_C11	-1.5	1.8	1.2	-1.8	1.9	1.1	ITGAM	integrin, alpha M (complement component receptor 3, alpha; also known as CD11b (p170), macrophage antigen alpha polypeptide)	NM_000632
P01081_E02	2.3	-1.8	1.2	2.2	-2.2	1	JUNB	jun B proto-oncogene	NM_002229
P01072_G01	1.6	-1.5	1.2	1.9	-1.6	1.1	JUP	junction plakoglobin	NM_002230
P01079_A01	-1.9	2.1	-1.1	-1.5	1.5	-1	JWA	vitamin A responsive; cytoskeleton related	NM_006407
P01122_A09	1.1	1.3	1.2	-1.9	1.6	1.1	KCNE3	potassium voltage-gated channel, Isk-related family, member 3	NM_005472
P01113_F02	-1.8	1.9	1.2	-2.4	2.3	1.1	KHDRBS3	KH domain containing, RNA binding, signal transduction associated 3	NM_006558
P01074_B01	-1.6	1.2	1.1	-1.9	1.7	1	KIAA0102	KIAA0102 gene product	NM_014752
P01104_A04	-3.2	3.8	-1	-3	3.4	-1	KIAA1049	KIAA1049 protein	NM_014972
P01120_B02	-1.6	1.5	1.1	-1.8	1.7	1	KIF1B	kinesin family member 1B	NM_015074
P01088_C06	-1.6	1.6	1.2	-1.9	1.8	1	KRT4	keratin 4	NM_002272
P01085_D06	-1.8	1.7	1.2	-3.8	4.1	1	LAMA4	laminin, alpha 4	NM_002290
P01131_H02	-1.4	1.4	1.1	-2	1.9	-1.1	LAMC1	laminin, gamma 1 (formerly LAMB2)	NM_002293
P01131_H10	-2.4	1.8	-1.1	-2.1	1.5	1	LCN2	lipocalin 2 (oncogene 24p3)	NM_005564
P01100_H05	-2.8	2.7	1.2	-5	2.7	1	LEPR	leptin receptor	NM_002303
P01088_B02	-2.3	2.4	1.1	-2.6	2.1	-1	LGALS3	lectin, galactoside-binding, soluble, 3 (galectin 3)	NM_002306
P01081_B11	-3.5	1.3	1.1	-4.6	4.4	1	LHFP	lipoma HMGIC fusion partner	NM_005780
P01107_D06	2.2	-2	-1	1.7	-1.8	-1.1	LIMK2	LIM domain kinase 2	NM_005569
P01085_G06	1.2	-1.4	-1.1	1.9	-2.1	-1	LMO7	LIM domain only 7	NM_005358
P01085_D05	-2.1	2.2	1.2	-3.9	3.7	1.1	LOC56270	hypothetical protein 628	NM_019613
P01082_E01	2.1	-1.5	1.2	1.8	-1.6	1.2	LOX	lysyl oxidase	NM_002317
P01083_H02	-1.4	1.5	1.1	-2	2	1	LPHN2	latrophilin 2	NM_012302
P01131_D06	-1.6	1.7	1.2	-2.4	1.8	1.2	LRP4	low density lipoprotein receptor-related protein 4	AB011540
P01072_F03	1.8	-1.2	-1	2.2	-1.6	-1	LTBP2	latent transforming growth factor beta binding protein 2	NM_000428
P01088_C04	-2.3	2.3	1.1	-4.4	4.7	1.1	LTF	lactotransferrin	NM_002343
P01063_A11	-2.3	2.4	-1	-4.8	3.9	-1	LUM	lumican	NM_002345
P01135_G05	-2.4	2.4	1.2	-1.7	1.6	-1	LY96	lymphocyte antigen 96	NM_015364
P01085_C04	-2	1.8	1.2	-2	1.5	1	MADH3	MAD, mothers against decapentaplegic homolog 3 (Drosophila)	NM_005902
P01091_G10	1.8	-1.4	1.2	2.2	-2.1	1.2	MADH7	MAD, mothers against decapentaplegic homolog 7 (Drosophila)	NM_005904
P01089_C01	1.2	-1.2	-1	1.8	-1.6	-1.2	MAGP2	Microfibril-associated glycoprotein-2	NM_003480
P01084_A09	1.8	-1.6	1.2	1.4	-1.6	-1	MAP3K2	mitogen-activated protein kinase kinase kinase 2	NM_006609
P01073_E08	-2	2.4	-1	-2.3	1.8	-1	MAP3K5	mitogen-activated protein kinase kinase kinase 5	NM_005923

P01066_F10	2	-2	1.1	1.9	-1.7	1.1	MAPK7	mitogen-activated protein kinase 7	NM_002749
P01076_B12	1.9	-2.1	-1.1	1.7	-1.7	-1.1	MAPRE2	microtubule-associated protein, RP/EB family, member 2	NM_014268
P01134_C04	3.1	-2.1	1.1	2.8	-3.3	-1	MATN3	matrilin 3	NM_002381
P01145_A05	-1.7	1.9	-1	-2.6	2.1	-1	ME1	malic enzyme 1, NADP(+)-dependent, cytosolic	NM_002395
P01072_D11	-3.3	3.7	-1	-3.5	3.1	1	MEST	mesoderm specific transcript homolog (mouse)	NM_002402
P01121_F04	-1.9	2.1	1.3	-2.1	1.8	1	MGC1203	hypothetical protein MGC1203	NM_024296
P01068_F12	-2.9	2.8	1.1	-2.6	2.4	-1	MGST1	microsomal glutathione S-transferase 1	NM_020300
P01091_B06	-1.8	1.6	-1	-1.5	1.6	-1.1	MGST2	microsomal glutathione S-transferase 2	NM_002413
P01099_H09	-2.4	2.3	1.1	-2.4	2	1.2	MID1	midline 1 (Opitz/BBB syndrome)	NM_000381
P01062_H05	-1.4	2.2	1.3	-2.4	2.4	1.3	MME	membrane metallo-endopeptidase (neutral endopeptidase, enkephalinase, CALLA, CD10)	NM_000902
P01125_H08	1	1	1.1	2.6	-2.1	-1	MMP11	matrix metalloproteinase 11 (stromelysin 3)	NM_005940
P01072_D02	2.8	-2.6	-1.3	1.7	-2	-1.2	MTHFD2	methylene tetrahydrofolate dehydrogenase (NAD+ dependent), methenyltetrahydrofolate cyclohydrolase	NM_006636
P01125_A10	-1.6	1.6	1.2	-1.8	1.5	1.1	MTMR4	myotubularin related protein 4	NM_004687
P01130_C09	1.9	-1.7	1.3	1.1	-1.1	1.1	MUCDHL	mucin and cadherin-like	NM_017717
P01102_A12	1.4	-1.3	1.2	2.5	-1.6	1.1	MVK	mevalonate kinase (mevalonic aciduria)	NM_000431
P01133_F05	1.9	-1.8	1	1.4	-1.4	-1.1	MYH9	myosin, heavy polypeptide 9, non-muscle	NM_002473
P01100_B07	-2	2.4	1.1	-5.5	2.6	-1.1	MYOZ2	myozenin 2	NM_016599
P01072_C06	-1.6	1.6	1	-2.6	2.6	1.1	NCK1	NCK adaptor protein 1	NM_006153
P01086_B12	-1.2	1.4	-1	-1.8	1.6	-1.1	NCOA3	nuclear receptor coactivator 3	NM_006534
P01135_C12	3.3	-3	1.3	3.1	-2.1	1.2	NEDD9	neural precursor cell expressed, developmentally down-regulated 9	NM_006403
P01112_A08	2.5	-2.1	1.2	1.7	-1.8	1.1	NET-6	transmembrane 4 superfamily member tetraspan NET-6	NM_014399
P01103_E02	-1.7	2.1	1.2	-2.5	2.1	-1	NFIA	nuclear factor I/A	AL096888
P01073_E06	-1.9	1.9	1	-2.1	1.8	-1.1	NFIB	nuclear factor I/B	NM_005596
P01064_C02	-1.9	2	1.2	-3.3	2.5	1	NID2	nidogen 2 (osteonidogen)	NM_007361
P01131_E08	2.3	-1.6	1.3	5.1	-3	1.3	NINJ2	ninjurin 2	NM_016533
P01072_D01	2.2	-2.2	1.1	2.2	-2.1	-1	NK4	natural killer cell transcript 4	NM_004221
P01121_G06	-2.2	2.1	-1.1	-2.5	2.2	-1.1	NOL3	nucleolar protein 3 (apoptosis repressor with CARD domain)	NM_003946
P01104_C08	6.9	-6.1	1.1	5.8	-5.8	1.1	NOX4	NADPH oxidase 4	NM_016931
P01107_D11	-1.7	1.6	-1	-1.8	1.8	1	NPC2	Niemann-Pick disease, type C2	NM_006432
P01132_G06	2.4	-2	1.3	1.5	-1.6	1.1	NPR3	natriuretic peptide receptor C/guanylate cyclase C (atrionatriuretic peptide receptor C)	NM_000908
P01096_F08	-1.5	1.6	1.2	-2.1	2	1.1	NPTX2	neuronal pentraxin II	NM_002523
P01126_E07	-1.5	2	1.2	-2	1.7	1.1	NR2F2	nuclear receptor subfamily 2, group F, member 2	NM_021005
P01064_G11	-1.5	1.6	1	-2.1	1.5	-1	NRCAM	neuronal cell adhesion molecule	NM_005010

P01097_E11	1.9	-1.8	1.1	1.6	-1.8	-1	NS1-BP	NS1-binding protein	NM_006469
P01103_C04	2.4	-2.2	-1	1.3	-1.5	-1	NUDT3	nudix (nucleoside diphosphate linked moiety X)-type motif 3	NM_006703
P01072_B11	2.6	-2.6	-1.1	2.3	-2.3	-1.2	ODC1	ornithine decarboxylase 1	NM_002539
P01082_E10	-1.4	2	1.3	-5.7	1.9	1.2	OGN	osteoglycin (osteoinductive factor, mimecan)	NM_014057
P01119_G07	-2.1	2.2	1.1	-2.2	1.6	-1	OSBPL1A	oxysterol binding protein-like 1A	NM_018030
P01075_F01	2.3	-1.6	-1	3.9	-3.7	-1	OSF-2	osteoblast specific factor 2 (fasciclin I-like)	NM_006475
P01129_A10	2.2	-1.6	-1	4.1	-3.6	-1	OSF-2	osteoblast specific factor 2 (fasciclin I-like)	NM_006475
P01126_B11	-2	1.5	-1.2	1.1	1.7	1.2	OXA1L	oxidase (cytochrome c) assembly 1-like	NM_005015
P01071_D09	-1.9	1.6	-1.1	1.1	1.7	1.3	OXA1L	oxidase (cytochrome c) assembly 1-like	NM_005015
P01085_C08	-1.3	1.3	1.1	-2.2	1.6	-1	OXTR	oxytocin receptor	NM_000916
P01125_D04	2.1	-1.7	1.2	4.2	-2.3	1.3	PACE4	paired basic amino acid cleaving system 4	NM_002570
P01090_D03	-1.4	1.2	-1	-2.4	1.8	-1.2	PARG1	PTPL1-associated RhoGAP 1	NM_004815
P01122_G06	2.6	-2.4	1.1	1.9	-2	-1	PAWR	PRKC, apoptosis, WT1, regulator	NM_002583
P01120_F04	-1.8	2.3	1.3	-2	1.7	1.1	PBF	papillomavirus regulatory factor PRF-1	NM_018660
P01071_G08	-2	1.5	-1	-1.4	1.7	1	PBP	prostatic binding protein	NM_002567
P01064_A09	1.1	-1.2	1.2	1.9	-1.5	1.2	PCDH1	protocadherin 1 (cadherin-like 1)	NM_002587
P01066_G05	-1.4	1.6	1.3	-3.2	2.4	1.2	PDE1A	phosphodiesterase 1A, calmodulin-dependent	NM_005019
P01128_B03	1.8	-1.7	1.1	-1.1	-1	-1	PDE5A	phosphodiesterase 5A, cGMP-specific	NM_001083
P01087_E02	3.4	-2.4	1.1	3	-3.7	-1	PDGFA	platelet-derived growth factor alpha polypeptide	NM_002607
P01081_F07	-2.3	2.1	1.1	-2.2	2.1	1.1	PDGFRA	platelet-derived growth factor receptor, alpha polypeptide	NM_006206
P01142_D01	-1.1	-1.8	1.2	-2.2	1.9	1.2	PDGFRL	platelet-derived growth factor receptor-like	NM_006207
P01064_G02	1.3	-1.1	1.3	2.3	-2	1.2	PDGFRL	platelet-derived growth factor receptor-like	NM_006207
P01137_F04	-1.8	2	1.1	-2	1.4	1.1	PDP	pyruvate dehydrogenase phosphatase	NM_018444
P01071_H07	1.8	-1.9	1	1.3	-1	1.1	PFKP	phosphofructokinase, platelet	NM_002627
P01064_H07	-1.8	1.7	1	-1.6	1.5	1	PHF3	PHD finger protein 3	NM_015153
P01131_G12	1.2	-1	1.2	1.8	-1.7	1.2	PIGB	phosphatidylinositol glycan, class B	NM_004855
P01074_H07	-1.8	1.9	1.2	-1.9	1.6	1	PIK3R1	phosphoinositide-3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	AF279367
P01068_A02	-2.4	1.7	-1.1	-1.4	2.1	1	PIR	Pirin	NM_003662
P01112_H01	1.8	-1.6	1.4	1.3	-1.4	-1	PIST	PDZ/coiled-coil domain binding partner for the rho-family GTPase TC10	NM_020399
P01118_H09	-2.4	2.1	-1	-1.8	1.9	1	PITPNM	phosphatidylinositol transfer protein, membrane-associated	NM_004910
P01110_G02	-1.3	1.6	1.3	-4	2	1	PKIB	protein kinase (cAMP-dependent, catalytic) inhibitor beta	NM_032471
P01146_A11	1.4	-1.5	-1	1.8	-1.9	-1	PLA2G4C	phospholipase A2, group IVC (cytosolic, calcium-independent)	NM_003706
P01124_G10	3	-2.5	1	2.5	-3.2	-1.2	PLA2R1	phospholipase A2 receptor 1, 180kDa	NM_007366

P01070_G08	1.8	-1.7	-1	1.9	-2.3	-1.1	PLAU	plasminogen activator, urokinase	NM_002658
P01064_F01	-1.8	2.3	1.2	-1.7	1.9	1.2	PLCL1	phospholipase C-like 1	NM_006226
P01118_E04	2.4	-1.8	1.3	2.3	-1.9	1.1	PLEK2	pleckstrin 2	NM_016445
P01072_A03	5.2	-5.1	1.3	2.1	-1.6	1.2	PLN	phospholamban	NM_002667
P01084_A08	2.8	-2.2	1.1	1.9	-1.8	1.1	PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase (lysine hydroxylase) 2	NM_000935
P01063_E04	1.6	-1.7	-1.2	2.4	-1.6	1.1	PLP2	proteolipid protein 2 (colonic epithelium-enriched)	NM_002668
P01130_B04	-3.6	4.3	1	-5.6	5.1	1.1	PMP2	peripheral myelin protein 2	NM_002677
P01131_C08	-2.2	1.6	1.1	-3.4	2.3	1.1	PNUTL2	peanut-like 2 (Drosophila)	NM_004574
P01106_F02	1.5	-1.3	1.2	1.8	-1.7	1.1	PODXL	podocalyxin-like	NM_005397
P01074_B08	2.8	-1.8	1.2	2.2	-2.8	1.1	POLD3	polymerase (DNA directed), delta 3	BC020587
P01080_A04	-1.3	1.4	1	-1.8	1.5	1.1	PP	pyrophosphatase (inorganic)	NM_021129
P01123_E01	-2.9	3.2	1.3	-3.1	2.8	1.3	PPAP2B	phosphatidic acid phosphatase type 2B	NM_003713
P01064_B12	-1.5	1.7	1.2	-2.2	1.5	-1	PPARG	peroxisome proliferative activated receptor, gamma	NM_005037
P01136_D03	-5.4	3.3	1.1	-5.3	4.2	-1	PPL	periplakin	NM_002705
P01131_H04	1.2	-1.4	-1.2	2	-1.6	-1.2	PPP2R4	protein phosphatase 2A, regulatory subunit B' (PR 53)	NM_021131
P01087_D04	-1.2	1.3	-1.1	-1.9	1.5	-1.1	PRKCM	protein kinase C, mu	NM_002742
P01128_H07	2.3	-2.2	1.3	1.4	-1.4	1.1	PRPS1	phosphoribosyl pyrophosphate synthetase 1	NM_002764
P01062_F06	-1.6	1.5	1.1	-4.4	3.6	1	PSG1	pregnancy specific beta-1-glycoprotein 1	NM_006905
P01133_G04	-2	1.9	1.2	-5.5	4.8	-1	PSG1	pregnancy specific beta-1-glycoprotein 1	NM_006905
P01131_G08	-1.4	1.4	1.2	-2.6	2.6	1.1	PSG11	pregnancy specific beta-1-glycoprotein 11	NM_002785
P01141_B07	-1.4	1.8	1.3	-4.1	4	1.1	PSG4	pregnancy specific beta-1-glycoprotein 4	NM_002780
P01079_F07	-1.5	1.5	1.1	-2	1.5	-1	PTGER4	prostaglandin E receptor 4 (subtype EP4)	NM_000958
P01131_C07	-2.8	1.7	-1.1	-2.2	1.8	-1.1	PTGIS	prostaglandin I2 (prostacyclin) synthase	NM_000961
P01102_D10	2.3	-2.8	-1.1	1.1	-1.2	-1.1	PTGS1	prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	NM_000962
P01087_D05	3	-2.6	1.1	1.3	-1.2	-1.1	PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	NM_000963
P01106_G06	1.8	-1.5	1.2	3.1	-1.5	1.1	PTHLH	parathyroid hormone-like hormone	NM_002820
P01071_G12	-1.9	1.4	-1	-3.7	3.6	-1.1	PTN	pleiotrophin (heparin binding growth factor 8, neurite growth-promoting factor 1)	NM_002825
P01128_H08	-2.3	2.4	1.1	-1.5	1.3	-1	PTTG1	pituitary tumor-transforming 1	NM_004219
P01095_A03	-2.4	2.4	1.2	-1.4	1.2	1	PTTG1	pituitary tumor-transforming 1	NM_004219
P01097_G06	-1.7	1.7	-1	-2.2	1.6	-1	PUS1	pseudouridylate synthase 1	NM_025215
P01076_C04	2.5	-1.7	1.2	3.1	-2.6	1.2	QPCT	glutaminyl-peptide cyclotransferase (glutaminyl cyclase)	NM_012413
P01129_C05	-2.1	1.8	-1	-1.5	2.1	1.2	RAB13	RAB13, member RAS oncogene family	NM_002870
P01115_G01	-1.8	1.5	1.1	-1.6	2.2	1.2	RAB13	RAB13, member RAS oncogene family	NM_002870
P01110_E09	1.4	-1.2	1.2	1.8	-1.6	1	RAI	RelA-associated inhibitor	NM_006663
P01100_E02	-1.5	1.5	1.1	-3.3	2.7	1	RAI3	retinoic acid induced 3	NM_003979

P01082_A01	-2.4	1.8	1.1	-2.6	2.3	1.1	RARRES3	retinoic acid receptor responder (tazarotene induced) 3	NM_004585
P01117_H10	-1.8	1.6	1.1	-2.5	2	1	RASSF5	Ras association (RalGDS/AF-6) domain family 5	NM_031437
P01108_C07	1.4	-1.4	1.1	2.5	-1.9	1.2	RBP1	retinol binding protein 1, cellular	NM_002899
P01136_C04	2.6	-1.8	1.1	2.3	-1.6	1.2	RGS2	regulator of G-protein signalling 2, 24kDa	NM_002923
P01145_A10	-1.2	1.2	-1.1	-2	1.6	-1.1	RGS4	regulator of G-protein signalling 4	NM_005613
P01090_D02	-1.3	1.2	-1.1	-3	1.9	-1.3	RGS4	regulator of G-protein signalling 4	NM_005613
P01081_H10	-2.2	1.6	1	-6.7	4.7	-1	RGS5	regulator of G-protein signalling 5	NM_003617
P01071_E04	-1.9	1.4	-1.1	-3.8	3.2	-1.1	RNASE1	ribonuclease, RNase A family, 1 (pancreatic)	NM_002933
P01088_G09	-1	1	1	-1.8	1.8	-1.1	RPL5	ribosomal protein L5	NM_000969
P01127_E10	1.8	-1.6	1.1	1.7	-1.5	-1	RRAS	related RAS viral (r-ras) oncogene homolog	NM_006270
P01122_B03	-2	2	1.1	-2.4	3.1	1.2	RRP4	homolog of Yeast RRP4 (ribosomal RNA processing 4), 3'-5'-exoribonuclease	NM_014285
P01104_D09	2.1	-1.7	1.1	2	-1.8	1	RTP801	HIF-1 responsive RTP801	NM_019058
P01121_G04	2.1	-1.8	1.1	4.1	-3.2	1.1	RUVBL2	RuvB-like 2 (E. coli)	NM_006666
P01087_B06	-1.4	1	-1.2	-1.9	2.4	-1.2	S100A10	S100 calcium binding protein A10 (annexin II ligand, calpactin I, light polypeptide (p11))	NM_002966
P01064_F10	1.5	-1.5	-1.3	1.8	-1.5	-1.2	S100A11	S100 calcium binding protein A11 (calgizzarin)	NM_005620
P00777_A05	-1.9	1.7	1.1	-2.3	2.4	1.1	S100A4	S100 calcium binding protein A4 (calcium protein, calvasculin, metastasin, murine placental homolog)	NM_002961
P00777_A06	-1.9	1.8	1.1	-2.6	2.7	1.1	S100A4	S100 calcium binding protein A4 (calcium protein, calvasculin, metastasin, murine placental homolog)	NM_002961
P01143_A11	-1.7	1.7	1.1	-2.4	2.4	1.1	S100A4	S100 calcium binding protein A4 (calcium protein, calvasculin, metastasin, murine placental homolog)	NM_002961
P01141_F03	1.5	-1.2	1.3	3.9	-1.7	1.3	SAA2	serum amyloid A2	NM_030754
P01061_F04	-3.1	4	1.3	-2.2	2.8	1.3	SAT	spermidine/spermine N1-acetyltransferase	NM_002970
P01124_B03	-2.9	3.7	1.4	-2.1	2.5	1.4	SAT	spermidine/spermine N1-acetyltransferase	NM_002970
P01140_G05	2	-2.1	1.1	1.3	-1.3	-1	SC5DL	sterol-C5-desaturase (ERG3 delta-5-desaturase homolog, fungal)-like	NM_006918
P01066_H04	4.1	-2.7	1.2	3	-2	1.2	SCD	stearoyl-CoA desaturase (delta-9-desaturase)	NM_005063
P01140_D11	4.7	-3.8	1.2	3.5	-2.4	1	SCD	stearoyl-CoA desaturase (delta-9-desaturase)	NM_005063
P01119_B12	-1.6	1.9	1.2	-3.9	2.3	1.1	SCDGF-B	spinal cord-derived growth factor-B	NM_025208
P01087_A04	-1.1	1.3	1.2	-4	2	1.2	SCG2	secretogranin II (chromogranin C)	NM_003469
P01096_B12	2.6	-1.9	1.2	2.8	-2.5	-1	SCRG1	scrapie responsive protein 1	NM_007281
P01071_B04	-1.7	1.7	-1.1	-2.6	2.3	1	SDC4	syndecan 4 (amphiglycan, ryudocan)	NM_002999
P01063_H09	-1.8	1.7	1	-1.8	1.6	-1	SDCBP	syndecan binding protein (syntenin)	NM_005625

P01076_C05	1.8	-1.5	1.2	1	-1.2	-1	SEC23A	Sec23 homolog A (S. cerevisiae)	NM_006364
P01096_G04	-3.6	2.5	-1	-3	6	1.3	SELENBP1	selenium binding protein 1	NM_003944
P01119_G09	-3.2	2.4	1.1	-2.5	5.8	1.4	SELENBP1	selenium binding protein 1	NM_003944
P01076_B03	-1.6	1.4	-1	-2	1.5	-1.1	SEPP1	selenoprotein P, plasma, 1	NM_005410
P01062_D11	3	-2.9	-1	4.3	-3.3	-1	SERPINE1	serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	NM_000602
P01090_H11	-1.3	1.2	1.2	-1.9	2.1	1.3	SFRP1	secreted frizzled-related protein 1	NM_003012
P01078_F01	-1.8	2.4	-1.1	-1.6	1.6	1.1	SFRP4	secreted frizzled-related protein 4	NM_003014
P01087_A06	-2.9	1.9	-1.2	-3	2.2	-1.3	SGNE1	secretory granule, neuroendocrine protein 1 (7B2 protein)	NM_003020
P01106_G05	1.8	-1.7	1.3	2.9	-2.2	1.2	SKIL	SKI-like	NM_005414
P01102_A06	-1.8	2	1.3	-3.2	2.8	1.3	SLC11A3	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 3	NM_014585
P01105_A03	1.9	-1.7	1.1	1.5	-1.4	-1	SLC1A4	solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	NM_003038
P01143_D11	-2.7	2.5	1.3	-2.1	2.8	1.1	SLC25A11	solute carrier family 25 (mitochondrial carrier; oxoglutarate carrier), member 11	NM_003562
P01111_H03	1.8	-1.7	1	1.9	-2	-1	SLC7A11	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	NM_014331
P01138_A08	3	-2.9	-1	2.3	-2.4	-1.1	SLC7A5	solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	NM_003486
P01088_E10	3.1	-2.7	1.1	2.6	-2.3	1	SLC7A5	solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	NM_003486
P01112_E05	-1.6	1.3	1	-3	2.2	-1	SLIT3	slit homolog 3 (Drosophila)	NM_003062
P01136_F07	-1.1	1.4	1.2	-2.1	1.9	1.1	SLIT3	slit homolog 3 (Drosophila)	NM_003062
P01079_G03	-3.1	3.4	-1	-3.9	3.5	-1	SNAI2	snail homolog 2 (Drosophila)	NM_003068
P01140_F07	2.9	-2.6	1.1	2.2	-2.5	1.1	SNF1LK	SNF1-like kinase	
P01083_A04	-3.2	3.2	1	-9.3	7	-1.1	SNK	serum-inducible kinase	NM_006622
P01085_F06	-1.2	1.2	1.1	-2.6	1.7	1.1	SOD3	superoxide dismutase 3, extracellular	NM_003102
P01074_H12	1	1.1	1.1	-2.6	1.5	1.1	SPINT2	serine protease inhibitor, Kunitz type, 2	NM_021102
P01108_B02	-2.5	2.6	1.2	-4.2	2.2	-1	SPRY1	sprouty homolog 1, antagonist of FGF signaling (Drosophila)	AF041037
P01095_F04	-2.6	2	-1.1	-1.8	1.8	-1.1	SQRDL	sulfide quinone reductase-like (yeast)	NM_021199
P01128_E07	1.9	-2	1	2.6	-2.7	-1	SRPUL	sushi-repeat protein	NM_014467
P01073_B02	-1.7	1.7	1.2	-2.5	1.9	-1	SRPX	sushi-repeat-containing protein, X chromosome	NM_006307
P01104_F12	-2.1	2.5	1.2	-2.2	2.3	-1.1	SSBP2	single-stranded DNA binding protein 2	NM_012446
P01069_C06	1.9	-1.3	-1	2.7	-2.6	-1	SSR1	signal sequence receptor, alpha (translocon-associated protein alpha)	NM_003144
P01130_F10	-1.3	1.6	1.1	-2.3	2.3	-1.1	STC1	stanniocalcin 1	NM_003155
P01130_B11	2.1	-2	-1	1.7	-1.8	-1.1	STCH	stress 70 protein chaperone, microsome-associated, 60kDa	NM_006948
P01074_E03	1.7	-1.3	-1	-1.9	1.5	-1.1	STE	sulfotransferase, estrogen-	NM_005420

								preferring	
P01127_G01	-1.4	1.5	1.2	-1.9	1.5	1.2	STK17B	serine/threonine kinase 17b (apoptosis-inducing)	NM_004226
P01125_C11	-2	1.6	-1	-2.2	2.1	-1	STK25	serine/threonine kinase 25 (STE20 homolog, yeast)	NM_006374
P01076_D03	-2.7	2.9	1.1	-2.1	1.8	1	STK38	serine/threonine kinase 38	NM_007271
P01105_E03	-2.8	2.7	-1.1	-2.7	2.5	-1.1	STMN1	stathmin 1/oncoprotein 18	NM_005563
P01069_A08	-1.5	1.5	1.1	-1.8	1.5	1	STOM	stomatin	NM_004099
P01102_E10	-1.5	1.6	1.1	-2.3	1.5	1	SVIL	supervillin	NM_003174
P01062_H06	-1.5	2.1	1.2	-2.9	2.7	1.2	TACSTD2	tumor-associated calcium signal transducer 2	NM_002353
P01098_E05	1.9	-1.8	1.2	1.2	-1.3	1.1	TAF13	TAF13 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 18kDa	NM_005645
P01101_B02	-1.9	1.4	1.1	-2	1.8	1	TCF7L1	transcription factor 7-like 1 (T-cell specific, HMG-box)	NM_031283
P01061_C01	-1.7	1.6	1.3	-2	2.4	1.1	TF	transferrin	NM_001063
P01144_C03	-3.4	3.6	1.2	-4	2.9	1.1	TFPI	tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)	NM_006287
P01071_A04	-1.4	1.6	1.4	-2.3	2.3	1.1	TFPI2	tissue factor pathway inhibitor 2	NM_006528
P01085_B12	-1.4	1.4	1.2	-2.1	1.7	1.1	TGFB2	transforming growth factor, beta 2	NM_003238
P01061_C08	-3.5	3.8	1.2	-4.7	4	1.2	TGFBR3	transforming growth factor, beta receptor III (betaglycan, 300kDa)	NM_003243
P01078_B04	1.8	-1.7	-1	1.8	-1.9	-1.2	THBS2	thrombospondin 2	NM_003247
P01124_G04	2.8	-2.5	-1	2.4	-3.1	-1.3	TIMP3	tissue inhibitor of metalloproteinase 3 (Sorsby fundus dystrophy, pseudoinflammatory)	NM_000362
P01086_F06	2.1	-2.4	-1	2.6	-3.1	-1.2	TIMP3	tissue inhibitor of metalloproteinase 3 (Sorsby fundus dystrophy, pseudoinflammatory)	NM_000362
P01071_A06	-3	3	-1	-2.5	2.6	-1	TM4SF1	transmembrane 4 superfamily member 1	NM_014220
P01099_E08	-1.6	1.8	1	-1.8	1.5	-1.1	TncRNA	trophoblast-derived noncoding RNA	
P01126_E09	-1.7	2	1	-3.7	4.2	1.1	TNFAIP2	tumor necrosis factor, alpha-induced protein 2	NM_006291
P01085_A06	-1.5	1.6	-1	-2.4	1.9	1.1	TNFAIP3	tumor necrosis factor, alpha-induced protein 3	NM_006290
P01138_G10	1.8	-1.7	1.2	2.1	-2	1	TNFRSF12A	tumor necrosis factor receptor superfamily, member 12A	NM_016639
P01078_E05	-2.1	3	1.4	-2.6	2.5	1.2	TNFSF10	tumor necrosis factor (ligand) superfamily, member 10	NM_003810
P01144_C11	-2	2	1	-1.9	1.4	1.2	TOP2A	topoisomerase (DNA) II alpha 170kDa	NM_001067
P01140_D03	2.1	-1.7	-1	1.2	-1.6	-1	TTID	titin immunoglobulin domain protein (myotilin)	NM_006790
P01070_H07	-2.9	2.3	1.1	-3	2.6	-1.1	TXNRD1	thioredoxin reductase 1	NM_003330
P01089_D01	1.7	-3	1.1	2.5	-2.3	-1	UCHL1	ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	NM_004181
P01123_D07	-1.9	2.4	1.1	-2	1.8	1.1	UGCG	UDP-glucose ceramide glucosyltransferase	NM_003358
P01089_F07	1.5	-2.6	1.2	2.4	-1.7	1.2	UMPK	uridine monophosphate kinase	NM_012474

P01070_F11	2.1	-3.1	1.1	2.4	-1.9	1	UMPS	uridine monophosphate synthetase (orotate phosphoribosyl transferase and orotidine-5'-decarboxylase)	NM_000373
P01061_B02	-2.7	2.4	1	-4.2	2.3	-1.3	VCAM1	vascular cell adhesion molecule 1	NM_001078
P01141_C06	2.7	-1.8	1.3	1.4	-1.2	1.2	WISP1	WNT1 inducible signaling pathway protein 1	NM_003882
P00777_C09	-1.6	1.8	1.1	-5	4	-1	WISP2	WNT1 inducible signaling pathway protein 2	NM_003881
P00777_C10	-2.2	2.1	1.1	-5.6	4.4	-1	WISP2	WNT1 inducible signaling pathway protein 2	NM_003881
P01126_H07	-1.8	1.6	1.1	-3	3.9	-1	WISP2	WNT1 inducible signaling pathway protein 2	NM_003881
P01142_D08	3.7	-3	1.3	3.6	-2.8	1.1	XRCC4	X-ray repair complementing defective repair in Chinese hamster cells 4	NM_003401
P01104_H07	-1.7	1.7	1.2	-1.9	1.5	1.1	ZFPM2	zinc finger protein, multitype 2	NM_012082
P01064_H12	-1.4	1.5	1.1	-1.8	1.5	-1	ZNF142	zinc finger protein 142 (clone pHZ-49)	NM_005081
P01075_E02	1.5	-1.2	1.1	1.9	-1.9	1	ZNF193	zinc finger protein 193	NM_006299

Validation of Microarray by Real-time RT-PCR and Western Blot Analyses

Representative microarray data was validated using real-time RT-PCR and Western analyses. TGFβ induced *Collagen 1* mRNA levels in human cardiac fibroblasts at 6, 24, and 48 h; this induction was blocked by BNP at all 3 time points (Fig. 5A). Collagen 1 protein synthesis was also induced (~3-fold) at 48 h, and BNP inhibited this stimulation by ~75% (Fig. 5B). BNP also inhibited TGFβ-induced *Fibronectin* mRNA and protein expression at 48 h (Fig. 5C,D). These data corroborate the microarray results, with the exception of *Fibronectin,* which did not exceed the array differential expression threshold value, most likely due to the lower sensitivity of the microarray compared to real-time RT-PCR. The effects of BNP on TGFβ stimulation of pro-fibrotic genes *CTGF, PAI-1, TIMP3, IL11,* and *ACTA2* were also confirmed by real-time RT-PCR (Fig. 6). Additional verification was obtained for the pro-inflammatory genes *COX2* and *IL6* at 6, 24, and 48 h (Fig. 6). Again, most likely due

to sensitivity issues, *IL6* was not included in Fig. 4C, since it did not exceed the array differential expression threshold value.

In addition, real-time RT-PCR assays were performed for 9 genes on primary cultures of human cardiac fibroblasts from a second independent donor lot of fibroblasts (see Table 3). The effects of BNP on TGFβ-induced gene expression in both donors were similar, although donor lot 2 was slightly less responsive to TGFβ. Taken together, these results confirm the microarray data using independent assay methods, as well as, multiple human cardiac fibroblast donors.

Table 3. Real-time RT-PCR validation of microarray data using human cardiac fibroblasts from two separate donors (lot 1 and lot 2). Expression levels are normalized to 18s RNA and are shown relative to the control samples. Standard deviations reflect duplicate biological replicates; real-time RT-PCR reactions were performed in triplicate.

Gene	Control	BNP	TGFβ	TGFβ +BNP	Time (h)	Lot
Collagen 1	1.0±0.05	1.0±0.05	1.9±0.04	1.2±0.01	6	1
	1.0±0.06	1.1±0.13	3.3±0.05	1.3±0.26	24	1
	1.0±0.11	1.0±0.26	1.5±0.09	1.2±0.01	24	2
	1.0±0.13	1.2±0.03	3.8±0.38	1.3±0.03	48	1
	1.0±0.20	1.0±0.01	2.5±0.32	1.3±0.18	48	2
Fibronectin	1.0±0.04	0.9±0.19	1.1±0.17	1.0±0.29	6	1
	1.0±0.21	1.0±0.10	1.0±0.05	1.0±0.18	24	1
	1.0±0.19	0.9±0.24	1.0±0.02	1.0±0.12	24	2
	1.0±0.04	1.1±0.04	2.2±0.38	1.3±0.35	48	1
	1.0±0.01	1.0±0.11	2.0±0.39	1.5±0.02	48	2
SERPINE1/PAI-1	1.0±0.07	0.7±0.08	7.3±0.44	1.7±0.37	6	1
	1.0±0.01	0.7±0.01	8.5±0.08	0.7±0.10	24	1
	1.0±0.10	0.7±0.11	2.4±0.06	1.1±0.10	24	2
	1.0±0.22	0.9±0.00	8.4±1.33	0.9±0.13	48	1
	1.0±0.17	0.8±0.03	2.6±0.03	0.9±0.06	48	2
CTGF	1.0±0.15	0.9±0.24	3.5±0.08	0.9±0.03	6	1
	1.0±0.28	1.0±0.29	3.3±0.25	0.7±0.25	24	1
	1.0±0.09	1.5±0.44	2.2±0.16	1.5±0.04	24	2
	1.0±0.45	1.4±0.13	3.1±0.01	1.1±0.01	48	1
	1.0±0.32	1.3±0.12	2.1±0.14	1.0±0.24	48	2
IL11	1.0±0.20	1.1±0.04	13.3±0.89	2.1±0.06	6	1

	1.0±0.13	1.2±0.07	32.3±0.82	1.1±0.14	24	1
	1.0±0.06	1.0±0.05	7.7±0.81	2.1±0.18	24	2
	1.0±0.23	0.7±0.10	17.6±0.22	1.0±0.08	48	1
	1.0±0.09	0.8±0.09	5.9±0.18	1.2±0.10	48	2
TIMP3	1.0±0.01	0.9±0.11	1.4±0.03	1.0±0.12	6	1
	1.0±0.31	1.0±0.12	2.6±0.26	1.0±0.23	24	1
	1.0±0.13	0.7±0.09	1.5±0.12	1.3±0.14	24	2
	1.0±0.26	0.9±0.00	3.0±0.34	1.0±0.09	48	1
	1.0±0.08	0.6±0.00	1.7±0.13	0.8±0.01	48	2
IL6	1.0±0.06	0.9±0.02	3.6±0.27	1.3±0.14	6	1
	1.0±0.13	0.9±0.21	1.7±0.14	0.8±0.03	24	1
	1.0±0.09	0.9±0.07	1.4±0.05	1.0±0.11	24	2
	1.0±0.13	0.9±0.03	1.6±0.12	0.9±0.05	48	1
	1.0±0.17	0.9±0.06	1.4±0.17	0.9±0.17	48	2
PTGS2/COX-2	1.0±0.01	1.2±0.22	9.0±1.49	1.8±0.05	6	1
	1.0±0.08	1.2±0.38	3.5±0.67	1.2±0.19	24	1
	1.0±0.07	1.1±0.05	4.9±0.36	1.4±0.18	24	2
	1.0±0.10	1.0±0.12	2.2±0.12	1.3±0.03	48	1
	1.0±0.19	1.0±0.06	5.4±0.92	1.2±0.01	48	2
ACTA2	1.0±0.03	0.8±0.12	1.1±0.11	0.9±0.20	6	1
	1.0±0.14	0.9±0.11	2.2±0.00	0.9±0.07	24	1
	1.0±0.04	0.9±0.25	2.3±0.12	1.6±0.41	24	2
	1.0±0.17	1.0±0.03	1.0±0.19	1.0±0.21	48	1
	1.0±0.05	0.7±0.11	2.5±0.13	1.0±0.12	48	2

In a related study, a gene microassay profile of rat heart tissue was conducted. The results of this study are shown in Figure 12. Fibrotic and extracellular matrix associated genes were stimulated in vivo by L-NAME plus angiotensin II. MRNA expression for collagen I, collagen III, and fibronectin was markedly reduced by the administration of BNP.

MEK/ERK pathway involved in BNP's Anti-Fibrotic Role

Natriuretic peptides were previously shown to stimulate ERK activity in cardiac myocytes and vascular endothelial cells. The MEK/ERK pathway has been linked to the repression of TGFβ/Smad signaling. To determine whether PKG or ERK

signaling is involved in BNP-dependent attenuation of TGFβ signaling, cultured cells were treated with BNP and/or TGFβ in the presence of a PKG inhibitor (KT5823) or two different MEK inhibitors (U0126, PD98059). BNP induced ERK phosphorylation was completely blocked by KT5823 and U0126, indicating that BNP activates ERK via PKG and MEK signaling cascades (Fig. 7a). Both MEK inhibitors (U0126, PD98059) reversed BNP inhibition of TGFβ–induced Collagen-1 expression analyzed by Western blot (Fig. 7b) and real-time RT-PCR (Fig. 7c). A similar result was demonstrated for *PAI-1* using real-time RT-PCR. These findings suggest that the ERK pathway plays an important role in BNP-dependent inhibition of the fibrotic response induced by TGFβ in human cardiac fibroblasts.

Fibrosis and ECM

One of the key features of cardiac fibrosis is the increased deposition of the ECM. The dynamic turnover of ECM proteins is controlled by several regulatory mechanisms: de novo biosynthesis of ECM components, proteolytic degradation of ECMs by matrix metalloproteinases (MMPs), and inhibition of MMP activities by endogenous inhibitors, TIMPs. All of these processes have been shown to be profoundly affected by TGFβ. The results provided herein suggest that TGFβ-induced ECM deposition in human cardiac fibroblasts occurs largely by increasing ECM gene expression, including *Fibronectin*, *COL1A2*, *COL15A*, *COL7A1*, *MAGP2, MATN3, FBN1,* and *COMP*. Fibronectin and collagen expression in cardiac fibroblasts has been well-established in the fibrotic response, however, this is the first report of TGFβ induction of other ECM genes including *MAGP2, MATN3, FBN1* and *COMP*, further corroborating TGFβ's role in ECM induction. Interestingly, *COMP*, which is a member of the thrombospondin family, has been shown to have a direct interaction

with Fibronectin,[25] supporting its role in fibrotic processes. We also found *Thombospondin 2,* which is involved in the activation of latent TGFβ[26] regulated by TGFβ in our studies and opposed by BNP (Table 2). Also sharing close identity with the latent TGFβ family of binding proteins is *FBN1*, a component of extracellular microfibrils. The opposing effects of BNP on these gene regulatory events, suggests that BNP modulates cardiac fibrosis.

In addition to the suppression of TGFβ–induced ECM biosynthesis, BNP may also modulate the degradation of ECM proteins by opposing elevated *TIMP3* levels in TGFβ–stimulated cells. The TIMP family of proteins is believed to play significant roles in controlling extracellular matrix remodeling. Elevation of *TIMP3* expression has been observed in animal models of myocardial infarction, suggesting that it may be a contributor to matrix remodeling in the failing heart.

Another hallmark of the fibrotic process is the transformation of cardiac fibroblasts to myofibroblasts and the induction of pro-fibrotic mediators. Myofibroblasts acquire contractile properties similar to smooth muscle cells. The results provided above demonstrate that BNP inhibited TGFβ-induction of several myofibroblast markers including *ACTA2* and *MYH9*. BNP also inhibited TGFβ pro-fibrotic mediators, such as, *CTGF, PAI-1, and IL11*. *CTGF* and *PAI-1* are well-established downstream signaling genes of the TGFβ pathway, and IL11 has been associated with tissue remodeling and fibrosis. IL11 expression in cardiac fibroblasts also seems to contribute to TGFβ-mediated fibrosis. The use of BNP to suppress this response should result in a protective effect.

Collectively, these effects of BNP on gene expression in TGFβ–stimulated cells demonstrate a role for BNP in anti-fibrotic processes in cardiac fibroblasts. In striking contrast to TGFβ-treated cells, BNP had no significant effects in unstimulated

fibroblasts. This is consistent with the physiological actions of BNP, working only in opposition to other hormonal systems such as the renin-angiotensin-aldosterone system.

Changes in Cell Proliferation

The effects of TGFβ on cell growth is cell-type dependent. As provided above, TGFβ stimulated cardiac fibroblast proliferation. Whether TGFβ has a direct effect on cell cycle or an indirect effect through other mechanisms is unclear. However, cDNA microarray analysis revealed that BNP markedly inhibits the expression of a number of TGFβ-induced growth factors or growth factor-like genes including *PDGFA, IGF1, FGF18,* and *IGFBP10* (*CYR61*). The up-regulation of these genes by TGFβ could partially explain the induction of cell proliferation, suggesting that it may be mediated indirectly through the stimulation of growth factor productions. TGFβ also induced the expression of *PTHLH (PTHrP)*, which has known chronotropic and vasodilatory effects. In osteoblast-like cells PTHrP can induce cell proliferation. Interestingly, in the myocardium, PTHrP levels are increased in congestive heart failure (CHF).

The growth inhibitory effects of natriuretic peptides have previously been reported. Cao and Gardner first demonstrated that natriuretic peptides inhibit PDGF, FGF2, and mechanical stretch-induced DNA synthesis in neonatal rat cardiac fibroblasts. Consistent with these findings, natriuretic peptides and cyclic GMP have been reported to inhibit cell proliferation induced by angiontensin II, endothelin-1, and norepinephrine in many cell types including cardiac fibroblasts, vascular smooth muscle cells, endothelial cells, and mesangial cells. The results provided herein

suggest an important role for BNP in regulating fibroblast growth during cardiac remodeling.

Changes in Inflammatory Genes

Cardiac expression of cytokines is thought to contribute to a decrease in left ventricle contractile performance and deleterious remodeling. Although similar effects have been observed with ANP, reported herein for the first time is that *brain natriuretic peptide* blocks TGFβ stimulation of several pro-inflammatory genes including *COX2, IL6, TNFAIP6,* and *TNFSF4.*

TGFβ has a dual effect in the regulation of inflammatory processes. For example, it increases COX2 expression and prostaglandin E2 release in pulmonary artery smooth muscle cells, airway smooth muscle cells, and intestinal epithelial cells. On the other hand, TGFβ down-regulates the production of MCP-1 and complement components (C3 and C4) in human proximal tubular epithelial cells and macrophages. The results provided herein corroborates the dual effect of TGFβ in the modulation of inflammatory gene expression in cardiac fibroblasts. From these results, it was found that while TGFβ induced some inflammatory genes, it down-regulated others, such as, *IL1b, MCP1-R, GRO1, GRO3,* and *MCP4.* Both effects are reversed by BNP. However, in the absence of TGFβ stimulation, BNP had no significant effect on the expression of inflammatory genes. It is likely that a balance of pro- and anti-inflammatory stimuli is important in the process of cardiac remodeling.

Signaling Mechanism Underlying BNP's Anti-Fibrotic Role

Studies aimed at elucidating the mechanism of BNP's inhibition of a fibrotic response indicate that the ERK signaling pathway plays an important role. The results

provided herein demonstrate that BNP phosphorylates ERK via PKG-dependent signaling in primary human cardiac fibroblasts. Moreover, this activation attenuates the TGFβ-induced fibrotic response as measured by Collagen 1 expression. This is consistent with previous studies showing that ERK activation is required for both the anti-hypertrophic effect of ANP in cardiac myocytes, and the inhibition of TGFβ signaling in mammary and lung epithelial cells.

In vivo studies

In a related study, an *in vivo* model for acute myocardial injury was used to explore the effects of BNP. Male Sprague Dawley rats ranging in weight from 225 to 250 gm were utilized. Acute myocardial injury was induced by administration of *N*ω-nitro-L-arginine methyl ester (L-NAME, 40 mg/kg/day)salt (1%NaCl) plus angiotensin II (AngII, 0.5 mg/kg/day) in the rats. The L-NAME was administered in drinking water from day 1 to day 14. Angiotensin II was continuously infused subcutaneously with an osmotic pump from day 11 to day 14. Rat BNP (400 mg/kg/min) was intravenously infused through an external infusion pump from day 10 to day 14.

Systolic blood pressure, plasma level of aldosterone, cardiac function heart/body weight ration and gene expression in the heart were analyzed. Systolic blood pressure was monitored via tail cuff technique with an IITC blood pressure recording system. Cardiac function was monitored via a Millar ARIA Pressure Volume Conductance System with an 1.4 F catheter. Gene expression as referenced above with results provided in Figure 12 were monitored by RT-PCR with an ABI Prism TM 7700 sequence detection system.

It was observed that BNP had no effect on systolic blood pressure raised by L-NAME+AngII but significantly attenuated aldosterone(1.25.2±0.2 vs. 6.6±0.16 ng/ml,

$p<0.05$). See Figure 10. As shown in Figure 13, BNP improved cardiac function by significantly increase in stroke volume (2.68 ± 0.23 vs. 4.74 ± 0.73 ul, $p<0.05$), ejection fraction ($13.6\pm$ 1.1 vs. $20.4\pm2.4\%$ $p<0.05$), and diastolic volume (19.0 ± 0.9 vs 22.4 ± 1.1ul, $p<0.05$) and stroke work (223.0 ± 29.4 vs 531.5 ± 99.1mmH*ul,$p<0.05$), and decrease in arterial elastance (6.50 ± 5.7 vs 42.6 ± 5.1 mmHg/ul, $p<0.01$). As shown in Figure 11, BNP significantly reduced the heart/body weigh ratio (0.0039 ± 0.002 vs. 0.0029 ± 0.001, $p<o.05$) and as referenced above, abolished the profibrotic phenotype indicated by decreasing expression of collagen I ($p<0.01$), collagen III ($p<0.05$) and fibronectin ($p<0.05$).

Summary

Along with the endothelin pathway, the renin-angiotensin and aldosterone system, the fibrosis-promoting TGFβ pathwayis important in the pathophysiology of heart failure. BNP appears to oppose TGFβ-regulated gene expression related to fibrosis and myofibroblast conversion. Furthermore, BNP's opposition to the TGFβ-stimulated fibrotic response is dependent on the PKG and the MEK/ERK pathways. This finding is consistent with the observation that BNP deficient mice show increased fibrosis and *Collagen 1* expression. In addition to BNP's global effects on fibrosis, it may also have effects on other processes, such as inflammation and proliferation (Fig. 8). These findings support a beneficial role for BNP in the prevention of cardiac fibrosis and the treatment of cardiac diseases. They also provide the first demonstration that BNP has a direct effect on cardiac fibroblasts to oppose a TGFβ-induced fibrotic response, suggesting that BNP functions as an anti-fibrotic factor in the heart to prevent cardiac remodeling in pathological conditions.

Independent from the antifibrotic effect, the in vivo studies as provided herein indicate that BNP may be used to reduce cardiac remodeling and prevent subsequent heart failure. BNP may also be useful as a cardioprotective agent to improve cardiac function post acute myocardial injury such as myocardial infarction.

All references cited throughout the specification are expressly incorporated herein by reference. While the present invention has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, and the like. All such modifications are within the scope of the claims appended hereto.

What is claimed is:

1. A method for treating cardiac remodeling in a subject that has undergone myocardial injury, said method comprising administering a therapeutically effective amount of natriuretic peptide to said subject.

2. A method for treating cardiac dysfunction in a subject that has undergone myocardial injury, said method comprising administering a therapeutically effective amount of natriuretic peptide to said subject.

3. A method for treating cardiac fibrosis in a subject who has undergone myocardial injury, said method comprising administering a therapeutically effective amount of natriuretic peptide to said subject.

4. The method of claims 1 or 2 wherein said natriuretic peptide is brain natriuretic peptide.

5. A method of inhibiting the production of Collagen 1, Collagen 3 or Fibronectin protein in a subject who has undergone myocardial injury, said method comprising administering a therapeutically effective amount of brain natriuretic peptide to said subject.

6. A method of alleviating or reversing the effect of TGFβ mediated cell activation in cardiac tissue on the expression of one or more genes associated with fibrosis, comprising contacting one or more cells or tissues in which the expression of said genes is altered as a result of TGFβ mediated activation, with brain natriuretic peptide.

7. The method of claim 5 wherein said genes are selected from the group consisting essentially of Collagen1, Collagen 3, Fibronectin, CTGF, PAI-1, and TIMP3.

8. A method of inhibiting the transformation of cardiac fibroblast cells into myofibroblast cells in a subject that has undergone myocardial injury, said method comprising administering a therapeutically effective amount of brain natriuretic peptide to said subject.

Figure 1

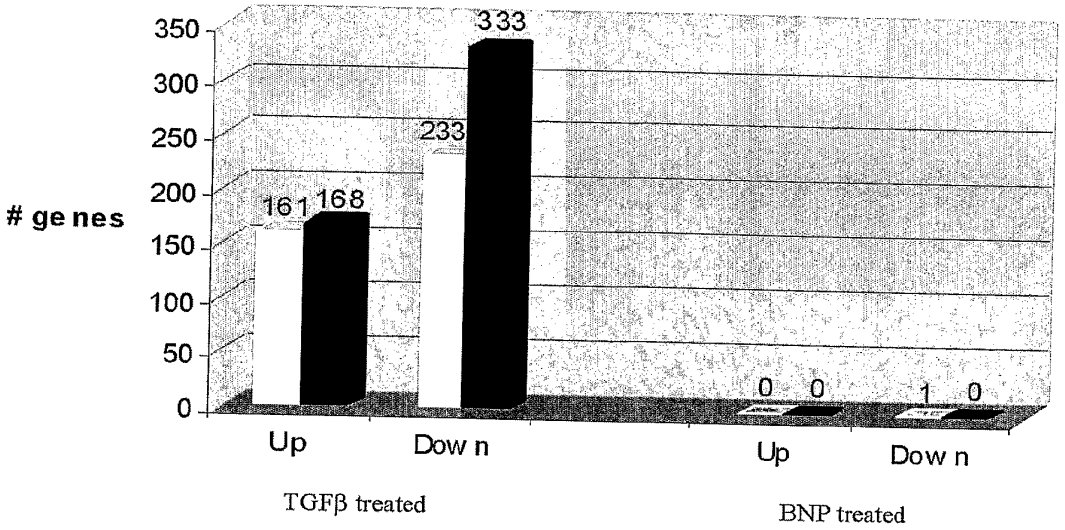

350
300
250
200
150
100
50
0
genes
161
168
233
333
0
0
1
0
Up
Down
Up
Down
TGFβ treated
BNP treated

Figure 2

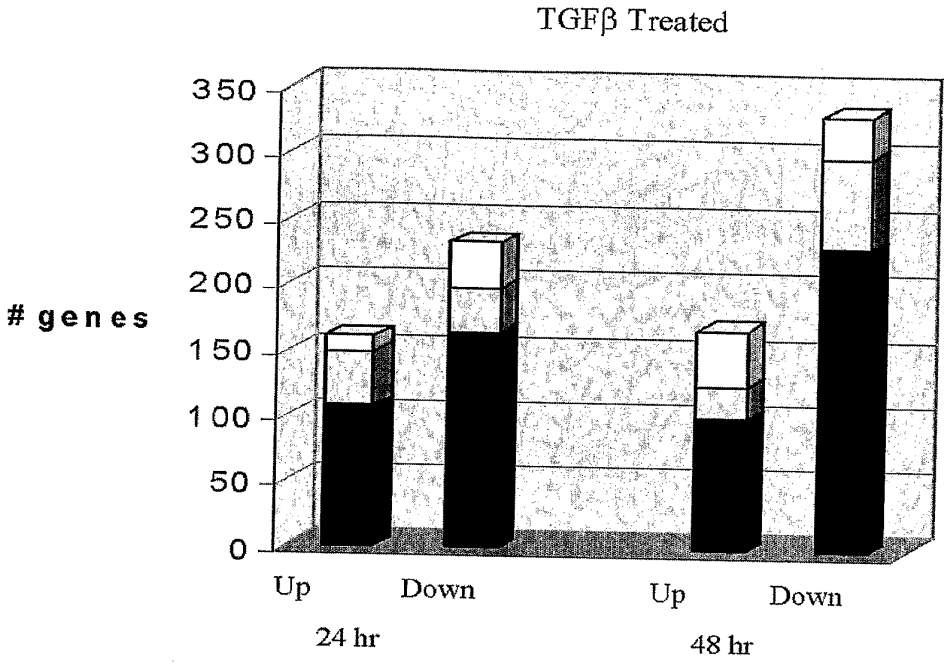

TGFβ Treated
genes
350
300
250
200
150
100
50
0
Up
Down
Up
Down
24 hr
48 hr

Figure 3

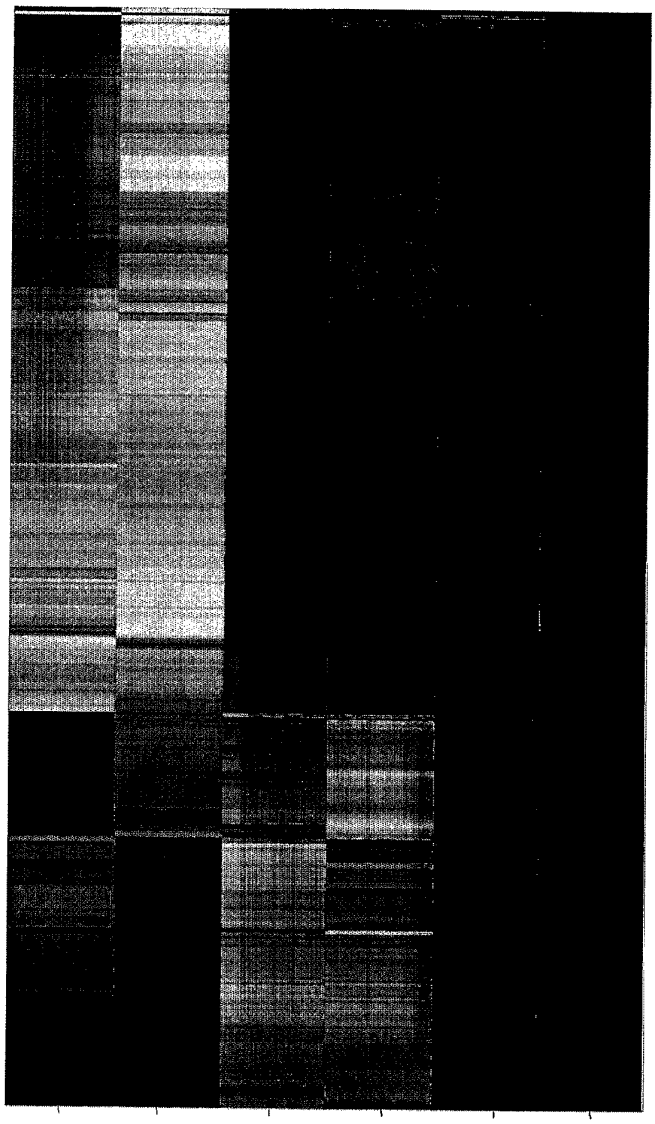

TGFβ 24h
TGFβ 48h
TGFβ BNP 24h
TGFβ BNP 48h
BNP 24h
BNP 48h
A
B
C
D
E
F

Figure 4

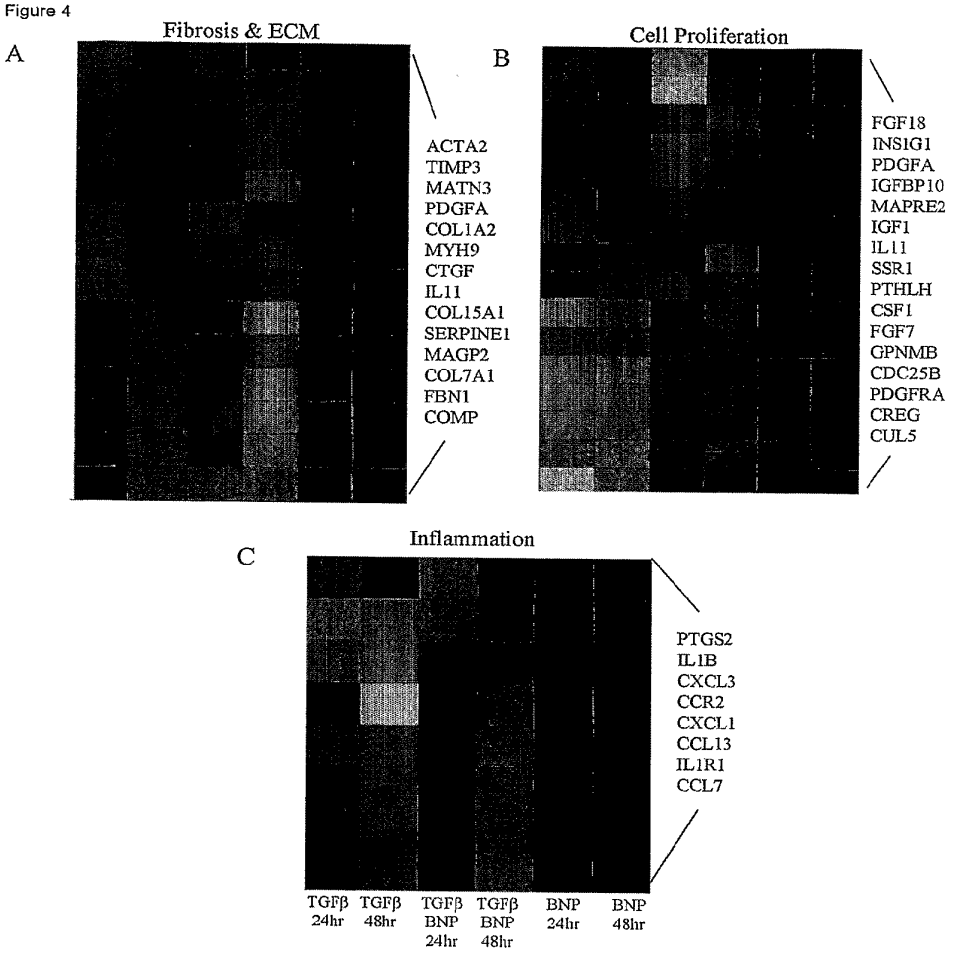

A
Fibrosis & ECM
ACTA2
TIMP3
MATN3
PDGFA
COL1A2
MYH9
CTGF
IL11
COL15A1
SERPINE1
MAGP2
COL7A1
FBN1
COMP
B
Cell Proliferation
FGF18
INSIG1
PDGFA
IGFBP10
MAPRE2
IGF1
IL11
SSR1
PTHLH
CSF1
FGF7
GPNMB
CDC25B
PDGFRA
CREG
CUL5
C
Inflammation
PTGS2
IL1B
CXCL3
CCR2
CXCL1
CCL13
IL1R1
CCL7
TGFβ 24hr
TGFβ 48hr
TGFβ BNP 24hr
TGFβ BNP 48hr
BNP 24hr
BNP 48hr

Figure 5

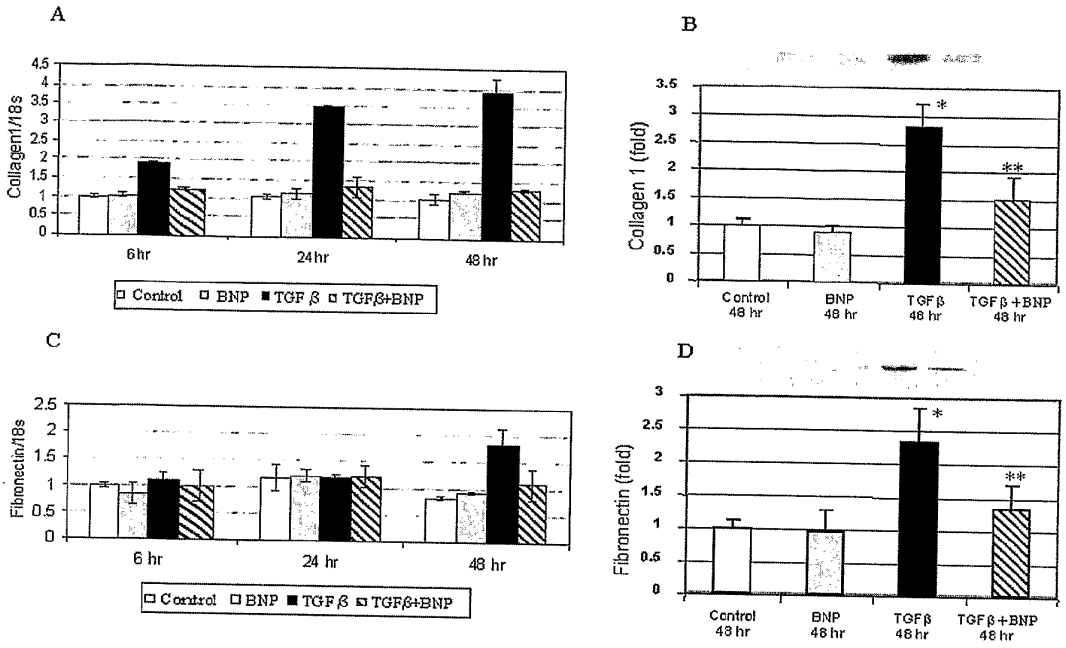

A
Collagen1/18s
6hr
24hr
48 hr
Control BNP TGF β TGFβ+BNP
B
Collagen 1 (fold)
Control 48 hr
BNP 48 hr
TGFβ 48 hr
TGFβ+BNP 48 hr
C
Fibronectin/18s
6 hr
24 hr
48 hr
Control BNP TGFβ TGFβ+BNP
D
Fibronectin (fold)
Control 48 hr
BNP 48 hr
TGFβ 48 hr
TGFβ+BNP 48 hr

Figure 6

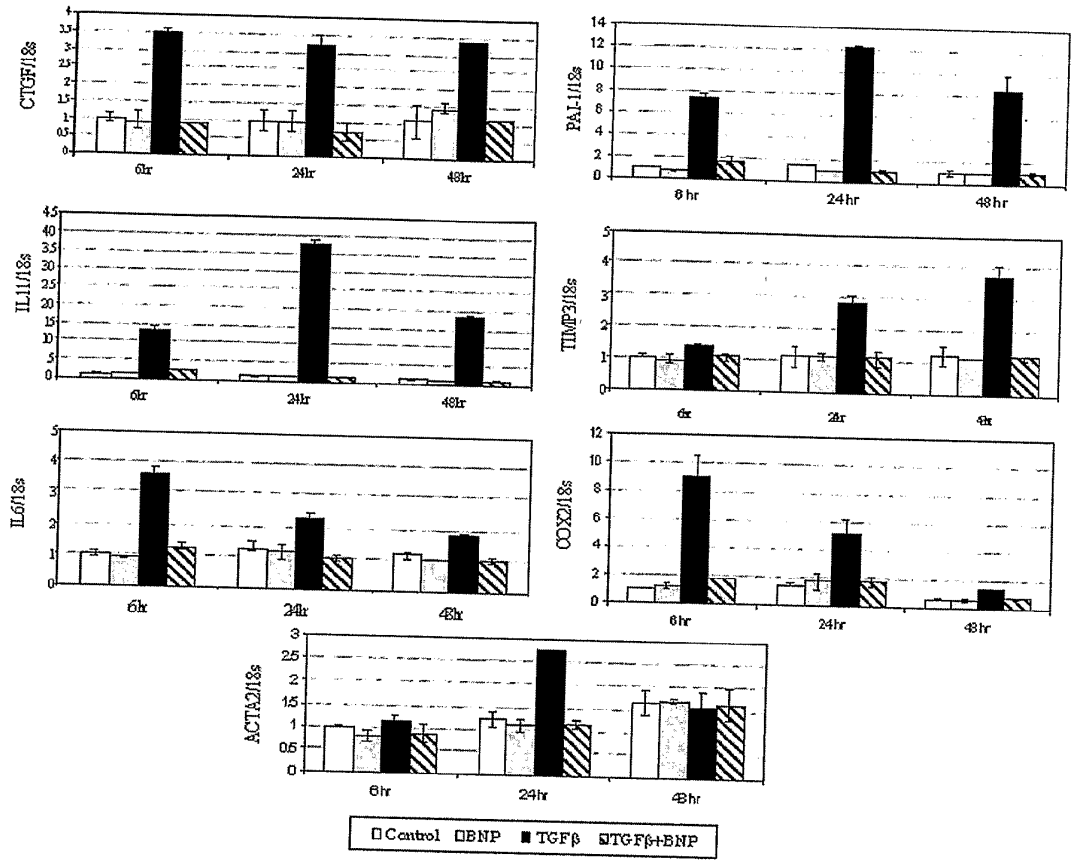

CTGF/18s
PAI-1/18s
IL11/18s
TIMP3/18s
IL6/18s
COX2/18s
ACTA2/18s
6hr
24hr
48hr
8 hr
4hr
Control
BNP
TGFβ
TGFβ+BNP

Figure 7

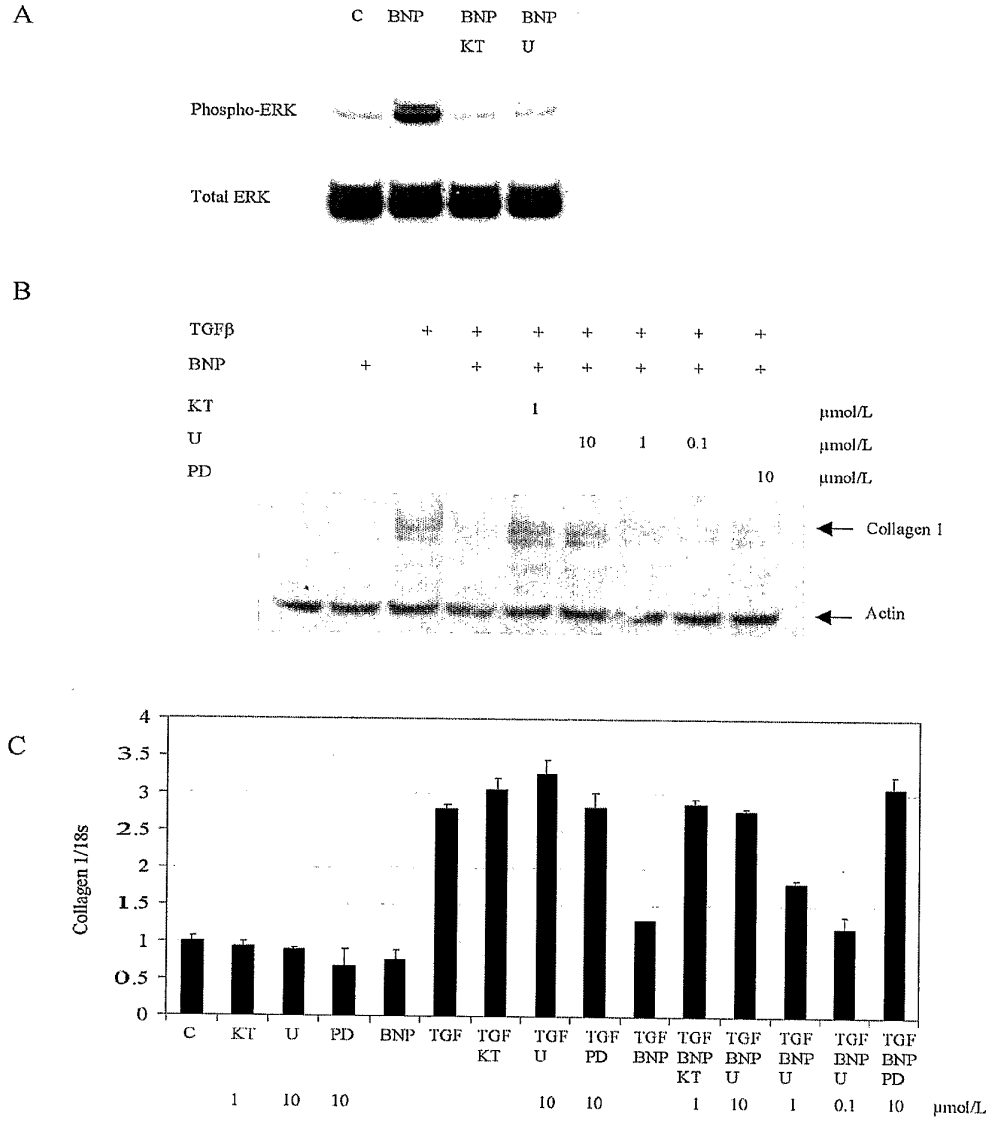

A
C
BNP
BNP KT
BNP U
Phospho-ERK
Total ERK
B
TGFβ
BNP
KT
U
PD
1
10
1
0.1
10
μmol/L
μmol/L
μmol/L
Collagen 1
Actin
C
Collagen 1/18s
4
3.5
3
2.5
2
1.5
1
0.5
0
C
KT
U
PD
BNP
TGF
TGF KT
TGF U
TGF PD
TGF BNP
TGF BNP KT
TGF BNP U
TGF BNP U
TGF BNP U
TGF BNP PD
1
10
10
10
10
1
10
1
0.1
10
μmol/L

Figure 8

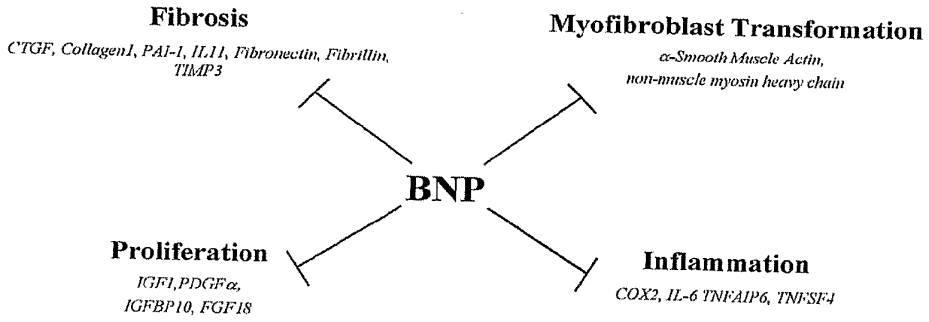

Fibrosis
CTGF, Collagen1, PAI-1, IL11, Fibronectin, Fibrillin, TIMP3
Myofibroblast Transformation
α-Smooth Muscle Actin, non-muscle myosin heavy chain
BNP
Proliferation
IGF1, PDGFα, IGFBP10, FGF18
Inflammation
COX2, IL-6 TNFAIP6, TNFSF4

Figure 9

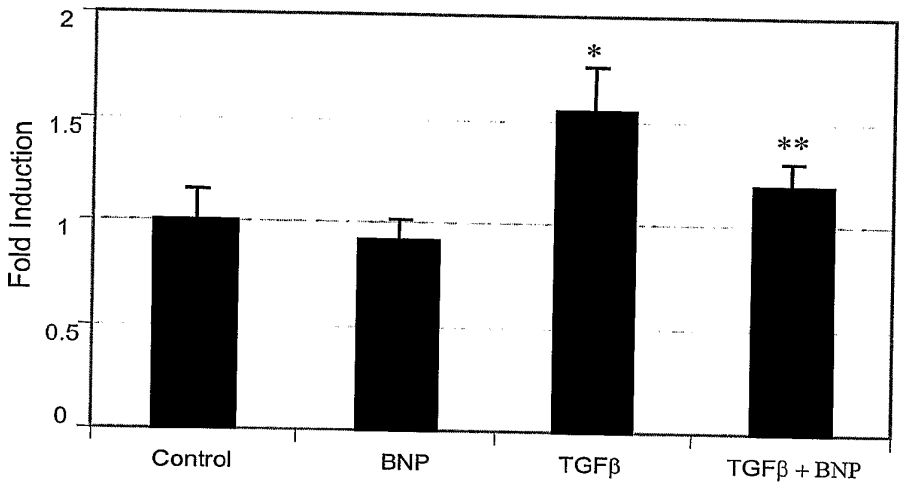


Figure 10

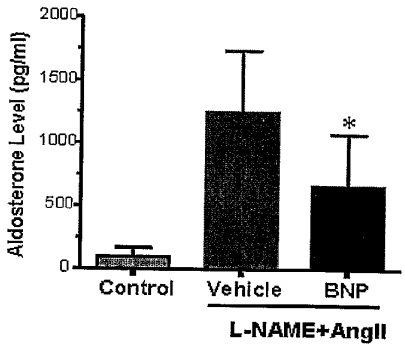

Aldosterone Level (pg/ml)
2000
1500
1000
500
0
*
Control
Vehicle
BNP
L-NAME+AngII

Figure 11

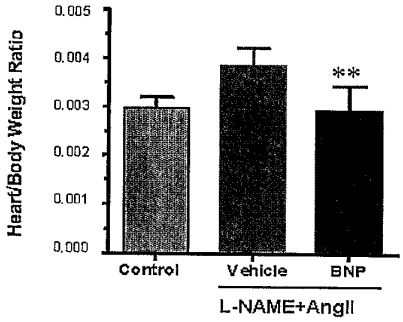

Heart/Body Weight Ratio
0.005
0.004
0.003
0.002
0.001
0.000
**
Control
Vehicle
BNP
L-NAME+AngII

Figure 12

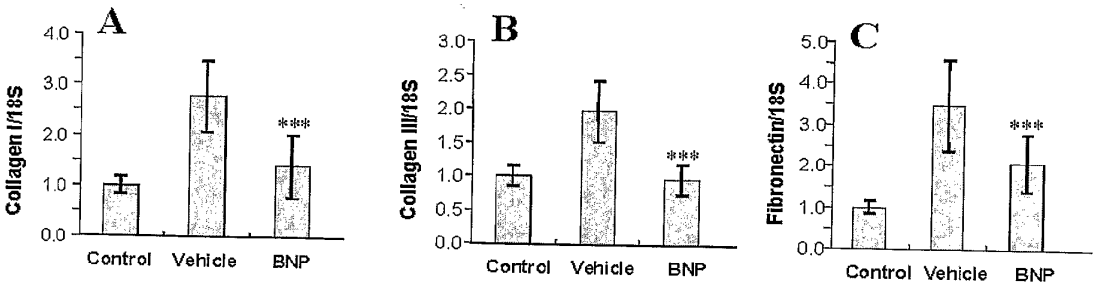
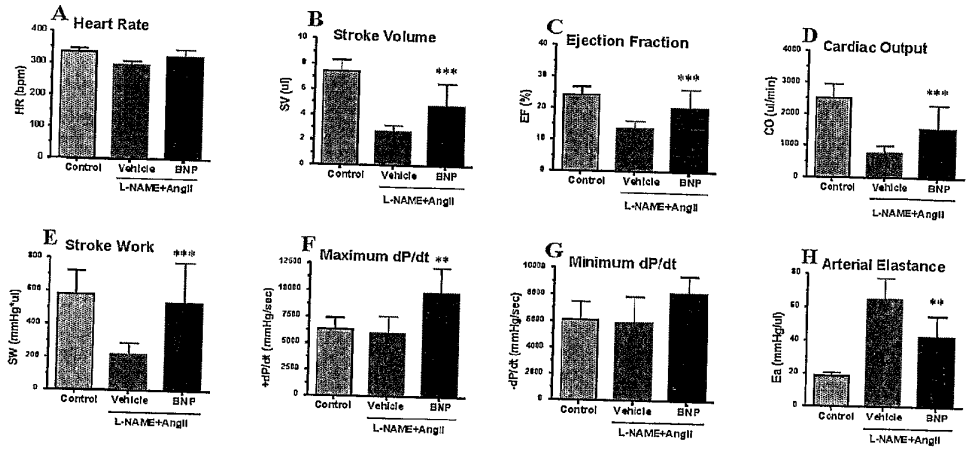

A
Collagen I/18S
4.0
3.0
2.0
1.0
0.0
***
Control
Vehicle
BNP
B
Collagen III/18S
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
Control
Vehicle
BNP
C
Fibronectin/18S
5.0
4.0
3.0
2.0
1.0
0.0
***
Control
Vehicle
BNP

Figure 13


A Heart Rate
HR (bpm)
Control
Vehicle
BNP
L-NAME+AngII
B Stroke Volume
SV (ul)
***
C Ejection Fraction
EF (%)
D Cardiac Output
CO (ul/min)
E Stroke Work
SW (mmHg*ul)
F Maximum dP/dt **
+dP/dt (mmHg/sec)
G Minimum dP/dt
-dP/dt (mmHg/sec)
H Arterial Elastance
Ea (mmHg/ul)
**

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US05/01480

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A61K 38/00
US CL : 514/12
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
STN EAST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Hayashi et al. Intravenous Atrial Natriuretic Peptide Prevents Left Ventricular Remodeling in Patients With Anterior Acute Myocardial Infarction. Journal of the American College of Cardiology, Feb. 2000. Vol. 35, No. 2 suppl. A, page 345A. See entire document.	1-2 ---------- 4
X	Fernándes et al. Cardiac remodeling in patients with systemic sclerosis with no signs or symptoms of heart failure: An endomyocardial biopsy study. J. Cardiac Failure, August 2003, Vol.9, No.4, abstract only, page I. See last paragraph.	3
Y	Diez et al. Losartan-Dependent Regression of Myocardial Fibrosis Is Associated With Reduction of Left Ventricular Chamber Stiffness in Hypertensive Patients. Circulation, 2002. Vol. 105: pages 2512-2517.	5-8
X,P --- Y,P	Tsuneyoshi et al. Atrial Natriuretic Peptide Helps Prevent Late Remodeling After Left Ventricular Aneurysm Repair. Circulation. 2004: 110:II-174-II179 (abstract attached, pages I-2). See entire document, e.g., page 2, last paragraph.	I-4

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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16 March 2005 (16.03.2005)	07 JUN 2005
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703) 305-3230	Authorized officer Marcela M Cordero Garcia Telephone No. (571) 272-1600

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US05/01480

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	Kapoun et al. B-Type Natriuretic Peptide Exerts Broad Functional Opposition to Transforming Growth Factor-B in Primary Human Cardiac Fibroblasts. Fibroses, Myofibroblast Conversion, Proliferation, and Inflammation. Circulation Research, March 5, 2004. Vol. 94. No. 4, pages 453-461. See entire document, e.g., abstract and pages 459-460.	1-8

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(54) Title: DETECTION AND TREATMENT OF FIBROTIC DISORDERS

(57) Abstract: The present invention provides a method for detecting a fibrotic disorder in a subject by: (a) providing a biological sample obtained from the subject (such as endometrium, peritoneal fluid, and/or smooth muscle cells); (b) analyzing the expression of at least one gene that is differentially expressed in the fibrotic disorder of interest; and (c) correlating the expression of the genes) with the presence or absence of the fibrotic disorder in the subject. The present invention also provides a method and compositions for modulating the expression of genes that are differentially expressed in fibrotic tissues, compared to normal tissues. Restoration of gene expression to levels associated with normal tissue is expected to ameliorate at least some of the symptoms of the fibrotic disorder. This method includes the step of contacting the tissue with an agent that modulates e:pression of one or more differentially expressed genes in the tissue. The present invention also includes arrays, such as microfluidic cards, for detecting differential gene expression in samples of fibrotic tissue.

WO 2005/098041 A2

DESCRIPTION

DETECTION AND TREATMENT OF FIBROTIC DISORDERS

Cross-Reference to Related Applications

The present application claims the benefit of U.S. Application Serial Numbers 60/556,546, filed March 26, 2004, 60/620,444, filed October 19, 2004, and 60/636,240, filed December 15, 2004, each of which is hereby incorporated by reference herein in its entirety, including any figures, tables, nucleic acid sequences, amino acid sequences, and drawings.

Government Support

The subject invention was made with government support under a research project supported by the National Institutes of Health Grant No. HD37432.

The Sequence Listing for this application is four compact discs labeled "Copy 1", "Copy 2", "Copy 3", and "CRF". Each copy contains only one file named "03-28-05.ST25.txt" which was created on March 28, 2005. The file is 9,994 KB. The entire contents of each of the computer discs are incorporated herein by reference in their entireties.

Background of Invention

Leiomyomas are benign uterine smooth muscle tumors, accounting for more than 30% of hysterectomies performed in the United States annually. Leiomyomas consist mainly of smooth muscle cells of myometrial origin and a network of connective tissue (Anderson, *Semin. Reprod. Endocrinol.*, 1996, 14:269-282; Chegini, *Cytokines and Reproduction*, 1999, 133-162).

Abnormal vaginal bleeding, pelvic pain and pelvic masses are among the major symptoms associated with leiomyomas. Leiomyomas are considered to originate from cellular transformation of myometrial smooth muscle cells and/or connective tissue fibroblasts during the reproductive years. The identity of factors that initiate such cellular transformation is not known; however, ovarian steroids are essential for leiomyoma

growth, and GnRH anolog (GnRHa) therapy, creating a hypoestrogenic condition, is often used for their medical management (Chegini, N "Implication of growth factor and cytokine networks in leiomyomas" In Cytokines in human reproduction. J. Hill ed. New York, Wiley & Sons Publisher, 2000, 133-162; Maruo, T *et al. Hum Reprod Update*, 2004, 10:207-20; Takeuchi, H *et al. J Obstet Gynaecol Res*, 2000, 26:325-331; Steinauer, J *et al. Obstet Gynecol*, 2004, 103:1331-6; Palomba, S *et al. Hum Reprod*, 2002, 17:3213-3219; DeManno, D *et al. Steroids*, 2003, 68:1019-32; Carr, BR *et al. J Clin Endocrinol Metab*, 1993, 76:1217-1223).

Hypoestrogenic conditions created by GnRHa therapy affect both leiomyoma and myometrium; however, clinical observations indicate a difference in their response to changes in the hormonal environment (Carr, BR *et al. J Clin Endocrinol Metab*, 1993, 76:1217-1223). In addition to GnRHa therapy, clinical and preclinical assessments of selective estrogen and progesterone receptor modulators, either alone or in combination with GnRHa therapy, have shown efficacy in leiomyoma regression (Steinauer, J *et al. Obstet Gynecol*, 2004, 103:1331-6; Palomba, S *et al. Hum Reprod*, 2002, 17:3213-3219; DeManno, D *et al. Steroids*, 2003, 68:1019-32).

GnRHa-induced leiomyoma regression is accompanied by alterations in uterine arteriole size, blood flow, and cellular content as well as changes in the expression of several growth factors, cytokines, extracellular matrix, proteases, and protease inhibitors (reviewed in Chegini, *Cytokines in Human Reproduction,* 2000, 133-162; Nowak, *Bailliere Best Pract Res. Clin Obstet. Gynaecol.,* 1999, 13:223-238). Differential expression and autocrine/paracrine action of many of these molecules are considered to play a central role in leiomyoma growth and GnRHa-induced regression (Chegini, *Cytokines in Human Reproduction,* 2000, 133-162; Nowak, *Bailliere Best Pract Res. Clin Obstet. Gynaecol.,* 1999, 13:223-238).

At the cellular level, a combination of mitotic activity, cellular hypertrophy, and accumulation of extracellular matrix (ECM) are considered to participate in leiomyoma growth (Anderson, *Semin. Reprod. Endocrinol.,* 1996, 14:269-282; Chegini, *Cytokines and Reproduction,* 1999, 133-162; Stewart *et al., J. Clin. Endocrinal Metab.,* 1994, 79:900-906; Wolanska *et al., Mol Cell Biochem.,* 1998, 189:145-152). Compared to myometrium, leiomyomas are reported to overexpress estrogen and progesterone receptors, and GnRHa therapy lowers their content in both tissues (Stewart *et al., Semin, Reprod. Endocrinol.,* 1995, 10:344-357; Englund *et al., J. Clin. Endocrinol Metab.,* 1998,

83:4092-4092). Clinical and basic science research shows that GnRHa acting through suppression of the pituitary–gonadal axis cause leiomyoma to regress by affecting uterine arteriole size, blood flow at the tumor level. But its effect at cellular and molecular levels in leiomyoma has not been investigated.

With respect to the leiomyoma molecular environment, several genome-wide allel-typing studies have evaluated the association between genomic instability and the pathogenesis of leiomyoma (for review; Ligon, AH and Morton, CC *Hum Reprod Update*, 2001, 7:8-14). These studies have led to the identification of several candidate genes, however in the majority of cases evidence of genomic instability is either lacking or inconsistent (Ligon, AH and Morton, CC *Hum Reprod Update*, 2001, 7:8-14), implying the existence of unrecognized pathways that can lead to the development of leiomyoma. Further studies have provided support for various autocrine/paracrine regulators in the pathogenesis of leiomyoma including local estrogen production, growth factors, cytokines, chemokines and their receptors, whose expression are regulated by ovarian steroids (Chegini, N "Implication of growth factor and cytokine networks in leiomyomas" In Cytokines in human reproduction. J. Hill ed. New York, Wiley & Sons Publisher, 2000, 133-162; Maruo, T *et al. Hum Reprod Update*, 2004, 10:207-20). These studies in many instances demonstrated altered expression of these factors and/or their receptors in leiomyoma compared to normal myometrium. In recent years cDNA microarray has been utilized as a high throughput method to identify a large number of differentially expressed and regulated genes in various tissues and cells. Using this approach, several recent studies have further assisted in fingerprinting the gene expression profile of leiomyoma and myometrium during the menstrual cycle (Tsibris, JCM *et al. Fertil Steril*, 2002, 78:114-121; Chegini, N *et al. J Soc Gynecol Investig*, 2003, 10:161-71; Wang, H *et al. Fertil Steril*, 2003, 80:266-76; Weston, G *et al. Mol Hum Reprod*, 2003, 9:541-9; Ahn, WS *et al. Int J Exp Pathol*, 2003, 84:267-79; Quade, BJ *et al. Genes Chromosomes Cancer*, 2004, 40:97-108). However, only the expression of a few of these newly identified genes has been validated, and their regulation and correlation with pathogenesis of leiomyoma remains to be investigated.

With respect to GnRHa therapeutic action, it is traditionally believed to act primarily at the level of the pituitary-gonadal axis, and by suppressing ovarian steroid production causes leiomyoma regression. However, the identification of GnRH and GnRH receptor expression in several peripheral tissues, including the uterus, has

implicated an autocrine/paracrine role for GnRH and additional sites of action for GnRHa therapy (Chegini, N *et al. J Clin Endocrinol Metab*, 1996, 81:3215-3221; Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557; Chegini, N *et al. Mol Cell Endocrinol*, 2003, 209:9-16; Xu, J *et al. J Clin Endocrinol Metab*, 2003, 88:1350-61; Chegini, N and Kornberg, L *J Soc Gynecol Investig*, 2003, 10:21-6; Chegini, N *et al. Mol Hum Reprod*, 2002, 8:1071-8). Demonstration of the expression of GnRH, as well as GnRH I and II receptors mRNA in leiomyoma and myometrium and their isolated smooth muscle cells has provided support for this concept (Chegini, N *et al. J Clin Endocrinol Metab*, 1996, 81:3215-3221; Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557). Several *in vitro* studies have also demonstrated GnRHa direct action on various cell types derived from peripheral tissues resulting in alteration of cell growth, apoptosis, the expression of cell cycle proteins, growth factors, pro- and anti-inflammatory cytokines, proteases, and protease inhibitors (Chegini, N "Implication of growth factor and cytokine networks in leiomyomas" In Cytokines in human reproduction. J. Hill ed. New York, Wiley & Sons Publisher, 2000, 133-162; Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557; Chegini, N *et al. Mol Cell Endocrinol*, 2003, 209:9-16; Xu, J *et al. J Clin Endocrinol Metab*, 2003, 88:1350-61; Chegini, N and Kornberg, L *J Soc Gynecol Investig*, 2003, 10:21-6; Chegini, N *et al. Mol Hum Reprod*, 2002, 8:1071-8; Klausen, C *et al. Prog Brain Res*, 2002, 141:111-128; Mizutani, T *et al. J Clin Endocrinol Metab*, 1998, 83:1253-1255; Wu, X *et al. Acta Obstet Gynecol Scand*, 2001, 80:497-504). Local expression and differential regulation of these genes influences cell proliferation, differentiation, migration, inflammatory response, angiogenesis, expression of adhesion molecules, ECM turnover and apoptosis, *etc.*, processes that are central to leiomyoma growth and regression (Chegini, N "Implication of growth factor and cytokine networks in leiomyomas" In Cytokines in human reproduction. J. Hill ed. New York, Wiley & Sons Publisher, 2000, 133-162; Maruo, T *et al. Hum Reprod Update*, 2004, 10:207-20; Chegini, N *et al. J Clin Endocrinol Metab*, 1996, 81:3215-3221; Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557; Chegini, N *et al. Mol Cell Endocrinol*, 2003, 209:9-16; Xu, J *et al. J Clin Endocrinol Metab*, 2003, 88:1350-61; Chegini, N and Kornberg, L *J Soc Gynecol Investig*, 2003, 10:21-6; Chegini, N *et al. Mol Hum Reprod*, 2002, 8:1071-8; Klausen, C *et al. Prog Brain Res*, 2002, 141:111-128; Mizutani, T *et al. J Clin Endocrinol Metab*, 1998, 83:1253-1255; Wu, X *et al. Acta Obstet Gynecol Scand*, 2001, 80:497-504; Dou, Q *et al. Mol Hum Reprod*, 1997, 3:1005-1014; Chegini, N *et al. J*

Clin Endocrinol Metab, 1999, 84:4138-4143; Senturk, LM *et al. Am J Obstet Gynecol*, 2001, 184:559-566; Sozen, I *et al. Fertil Steril*, 1998, 69:1095-1102; Gustavsson, I *et al. Mol Hum Reprod*, 2000, 6:55-59; Orii, A *et al. J Clin Endocrinol Metab*, 2002, 87:3754-9; Fukuhara, K *et al. J Clin Endocrinol Metab*, 2002, 87:1729-36; Zhai, YL *et al. Int J Cancer*, 1999, 84:244-50; Ma, C and Chegini, N *Mol Hum Repord*, 1999, 5:950-954). Microarray studies, including a small-scaled array, have also identified the expression profile of additional genes targeted by GnRHa in murine gonadotrope tumor cell line LβT2, human breast tumor cell line MCF-7 and leiomyoma and myometrium (Chegini, N *et al. J Soc Gynecol Investig*, 2003, 10:161-71; Ma, C and Chegini, N *Mol Hum Repord*, 1999, 5:950-954; Kakar, SS *et al. Gene*, 2003, 308:67-77).

Transforming growth factors beta (TGF-β) is a multifunctional cytokine and key regulator of cell growth and differentiation, inflammation, apoptosis and tissue remodeling (Blobe, GC *et al. N Engl J Med*, 2000, 342:1350-1358; Flanders, KC *Int J Exp Pathol*, 2004, 85:47-64; Schnaper, HW *et al. Am J Physiol Renal Physiol*, 2003, 284:F243-252; Clancy, RM and Buyon, JP *J Leukoc Biol*, 2003, 74:959-960; Olman, MA and Matthay, MA *Am J Physiol Lung Cell Mol Physiol*, 2003, 285:L522-6). While under normal physiological conditions the expression and autocrine/paracrine actions of TGF-β are highly regulated, alteration in TGF-β and TGF-β receptor expression and their signaling mechanisms often resulte in various pathological disorders, including fibrosis (Blobe, GC *et al. N Engl J Med*, 2000, 342:1350-1358; Flanders, KC *Int J Exp Pathol*, 2004, 85:47-64; Schnaper, HW *et al. Am J Physiol Renal Physiol*, 2003, 284:F243-252; Clancy, RM and Buyon, JP *J Leukoc Biol*, 2003, 74:959-960; Olman, MA and Matthay, MA *Am J Physiol Lung Cell Mol Physiol*, 2003, 285:L522-6). Altered expression of TGF-β isoforms (TGF-β1, β2 and β3) and TGF-β receptors (type I, II and III) in leiomyoma and their isolated smooth muscle cells (LSMC) compared to normal myometrium has been observed (Dou, Q *et al. J Clin Endocrinol Metab*, 1996, 81:3222-3230; Chegini, N *et al. J Clin Endocrinol Metab*, 1999, 84:4138-43; Chegini, N *et al. Mol Hum Reprod*, 2002, 8:1071-1078; Chegini, N *et al. Mol Cell Endocrinol*, 2003, 209:9-16). Recently, it has also been demonstrated that leiomyoma and LSMC cxpress elevated levels of Smads, components of the TGF-β receptor signaling pathway, compared to myometrium and MSMC (Chegini, N *et al. Mol Cell Endocrinol*, 2003, 209:9-16; Xu, J *et al. J Clin Endocrinol Metab*, 2003, 88:1350-1361). TGF-β regulates its own expression and the expression of Smad in LSMC and MSMC, and through downstream signaling

from this and MAPK pathways regulates the expression of c-fos, c- jun, fibronectin, type I collagen and plasminogen activator inhibitor 1 in these cells (Chegini, N *et al. J Clin Endocrinol Metab*, 1999, 84:4138-43; Chegini, N *et al. Mol Hum Reprod*, 2002, 8:1071-1078; Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557). Additionally, data have demonstrated the ability of TGF-β to regulate LSMC and MSMC cell growth (Tang, XM *et al. Mol Hum Reprod*, 1997, 3:233-40; Arici, A and Sozen, I *Am J Obstet Gynecol*, 2003, 188:76-83; Lee, BS and Nowak, RA *J Clin Endocrinol Metab*, 2001, 86:913-920; Arici, A and Sozen, I *Fertil Steril*, 2000, 73:1006-1011).

Because leiomyoma growth is dependent on ovarian steroids, GnRHa therapy and most recently selective estrogen and progesterone receptors modulators are used for their medical management (Steinauer, J *et al. Obstet Gynecol*, 2004, 103:1331-6; Palomba, S *et al. Hum Reprod*, 2002, 17:3213-3219; DeManno, D *et al. Steroids*, 2003, 68:1019-32). It has been demonstrated that GnRHa therapy results in a marked down-regulation of TGF-β isoforms and TGF-β receptors expression and alters the expression and activation of Smads in leiomyoma as well as LSMC (Dou, Q *et al. J Clin Endocrinol Metab*, 1996, 81:3222-3230; Chegini, N *et al. Mol Hum Reprod*, 2002, 8:1071-1078; Chegini, N *et al. Mol Cell Endocrinol*, 2003, 209:9-16). It has also been shown that TGF-β expression in LSMC and MSMC is inversely regulated by ovarian steroid compared to their antagonists, ICI-182780, ZK98299, and RU486 (Chegini, N *et al. Mol Hum Reprod*, 2002, 8:1071-1078). In addition, it has been shown that other cytokines such as GM-CSF, IL-13 and IL-15, which promotes myofibroblast transition, granulation tissue formation and inflammatory response, respectively, may mediate their action either directly or through induction of TGF-β expression in LSMC and MSMC (Chegini, N *et al. J Clin Endocrinol Metab*, 1999, 84:4138-43; Chegini, N *et al. Mol Cell Endocrinol*, 2003, 209:9-16; Ding, L *et al. J Soc Gyncol Invest*, 2004, 00, 00). From these observations, it was proposed that the TGF-β system serves as a major autocrine/paracrine regulator of fibrosis in leiomyoma (Dou, Q *et al. J Clin Endocrinol Metab*, 1996, Chegini, N *et al. J Clin Endocrinol Metab*, 1999; 81:3222-3230; Chegini, N *et al. Mol Hum Reprod*, 2002, 8:1071-1078; Chegini, N *et al. Mol Cell Endocrinol*, 2003, 209:9-16; Xu, J *et al. J Clin Endocrinol Metab*, 2003, 88:1350-1361; Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557; Tang, XM *et al. Mol Hum Reprod*, 1997, 3:233-40). Evidence has been developed reflecting the molecular environments directed by

GnRHa therapy in leiomyoma and myometrium, as well as by GnRHa direct action in LSMC and MSMC (Chegini, N *et al. J Soc Gynecol Investig*, 2003, 10:161-71).

Brief Summary of Invention

The present invention provides a method for detecting a fibrotic disorder in a subject by: (a) providing a biological sample obtained from the subject (such as endometrium, peritoneal fluid, and/or smooth muscle cells); (b) analyzing the expression of at least one gene that is differentially expressed in the fibrotic disorder of interest as compared to normal tissue (such as myometrium); and (c) correlating the expression of the gene(s) with the presence or absence of the fibrotic disorder in the subject. Preferably, the fibrotic disorder is a fibrotic disorder of the female reproductive tract. Examples of reproductive tract disorders include, but are not limited to, leiomyoma, endometriosis, ovarian hyperstimulation syndrome, adhesions, endometrial cancer, and other tissue fibroses. Fibrosis involves the deposition of large amounts of extracellular matrix molecules, notably collagen. Fibrosis is involved in normal physiological responses (*e.g.*, wound healing) as well as pathophysiological conditions such as renal failure, liver cirrhosis and heart disease. The compositions and methods of the present invention are useful for detecting or treating abnormal fibrotic changes in the tissue of a subject.

Differentially expressed genes include those that are differentially expressed in a given fibrotic disorder (such as leiomyoma), including but not limited to, docking protein 1, 62 kD (downstream of tyrosine kinase 1); centromere protein A (17 kD); catenin (cadherin-associated protein), beta 1 (88 kD); nuclear receptor subfamily 1, group I, member 2; v-rel avian reticuloendotheliosis viral oncogene homolog A; LGN Protein; CDC28 protein kinase 1; hypothetical protein; solute carrier family 17 (sodium phosphate), member 1; FOS-like antigen-1; nuclear matrix protein p84; LERK-6 (EPLG6); visinin-like 1; phosphodiesterase 10A; KH-type splicing regulatory protein (FUSE binding protein 2); Polyposis locus (DP1 gene) mRNA; microtubule-associated protein 2; CDC5 (cell division cycle 5, S pombe, homolog)-like; Centromere autoantigen C (CENPC) mRNA; RNA guanylyltransferase and 5'-phosphatase; Nijmegen breakage syndrome 1 (nibrin); ribonuclease, RNase A family, 4; keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris); basic helix-loop-helix domain containing, class B, 2; dual specificity phosphatase 1; annexin A11; putative receptor protein; Human

endogenous retrovirus HERV-K(HML6); mitogen-activated protein kinase kinase kinase 12; TXK tyrosine kinase; kynureninase (L-kynurenine hydrolase); ubiquitin specific protease 4 (proto-oncogene); peroxisome biogenesis factor 13; olfactory receptor, family 2, subfamily F, member 1; membrane protein, palmitoylated 3 (MAGUK p55 subfamily member 3); origin recognition complex, subunit 1 (yeast homolog)-like; dTDP-D-glucose 4,6-dehydratase; cytochrome c oxidase subunit VIa polypeptide 2; gamma-tubulin complex protein 2; Monocyte chemotactic protein-3; myelin transcription factor 1; inhibitor of growth family, member 1-like; thyroid hormone receptor, alpha myosin-binding protein C, slow-type; fragile X mental retardation 2; sonic hedgehog (Drosophila) homolog; 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2; SFRS protein kinase 2; excision repair cross-complementing rodent repair deficiency; cyclin-dependent kinase 5, regulatory subunit 1 (p35); poly(A)-specific ribonuclease (deadenylation nuclease); solute carrier family 12 (potassium/chloride transporters), member 4; Pseudogene for metallothionein; natriuretic peptide precursor A; intercellular adhesion molecule 2; apoptosis antagonizing transcription factor; similar to rat HREV107; major histocompatibility complex, class II, DP beta 1; MpV17 transgene, murine homolog, glomerulosclerosis; uroporphyrinogen decarboxylase; proteasome (prosome, macropain) 26S subunit, ATPase, 1; fms-related tyrosine kinase 3 ligand; actin, gamma 1; Protein Kinase Pitslre, Alpha, Alt. Splice 1-Feb; nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; pyruvate kinase, muscle; telomeric repeat binding factor 2; cell division cycle 2, G1 to S and G2 to M; ADP-ribosylation factor 3; NRF1 Protein; H factor (complement)-like 3; serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 6; mRNA of muscle specific gene M9; solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3; ribosomal protein L36a; suppressor of Ty (S. cerevisiae) 4 homolog 1; amino-terminal enhancer of split; ubiquitin A-52 residue ribosomal protein fusion product 1; hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase; chaperonin containing TCP1, subunit 2 (beta); tyrosine kinase with immunoglobulin and epidermal growth factor homology; domains; Fc fragment of IgG, receptor, transporter, alpha; NRD1 convertase; ADP-ribosylation factor 5; transcription elongation factor A (SII), 1; like mouse brain protein E46; titin; fibromodulin; Abi-interactor 2 (Abi-2); and other differentially expressed genes disclosed herein.

In one embodiment, the differentially expressed gene is at least one of CDKN1B, CDKN1C, CTGF, fibromodulin, and Abi-2.

In another embodiment, the differentially expressed gene is at least one of IL-11, IL-13, EGR1, EGR2, EGR3, CITED2, P300, E2F1, E2F2, E2F3, E2F4, E2F5, MCP3, CXCL5, CCL7, SMAD3, TYMS, GT198, SMAD7, NCOR2, TIMP-1, and ADAM17, wherein elevated expression of IL-11, IL-13, EGR1, EGR2, EGR3, CITED2, P300, E2F1, E2F2, E2F3, E2F4, E2F5, MCP3, CXCL5, CCL7, SMAD3, TYMS, and/or GT198 is indicative of a fibrotic disorder; and wherein reduced expression of SMAD7, NCOR2, TIMP-1, and/or ADAM17 is indicative of a fibrotic disorder.

In another embodiment, the differentially expressed gene is at least one listed in Table 9 herein.

The step of analyzing expression of the differentially expressed gene can be performed by quantifying the amount of differentially expressed gene product present in the sample, *e.g.*, by contacting the sample with an antibody that specifically binds the gene product. This step can also be performed by quantifying the amount of a nucleic acid (*e.g.*, DNA or RNA) that encodes the gene product present in the sample, *e.g.*, by contacting the sample with a polynucleotide that hybridizes under stringent conditions to the nucleic acid that encodes the gene product. The latter can also be performed using a polymerase chain reaction (PCR), for example.

Preferably, expression of a plurality of differentially expressed genes is analyzed. In this case, step (c) of correlating the expression of the differentially expressed gene with the presence or absence of the fibrotic disorder in the subject can include determining the ratio of two or more differentially expressed gene products in the sample.

In another aspect, the invention features a method for modulating gene expression in fibrotic tissue. This method includes contacting the fibrotic tissue *in vitro* or *in vivo* with an agent that modulates expression of a differentially expressed gene in the tissue. Preferably, the fibrotic tissue is tissue from a subject with leiomyoma, endometriosis, ovarian hyperstimulation syndrome, adhesions, or other tissue fibroses of the female reproductive tract, for example. The agent can be one that specifically binds the product that is expressed by a differentially expressed gene. The agent can also be a nucleic acid that modulates (*i.e.*, increases or decreases) expression of one or more differentially expressed genes in a cell. The agent can also be one that modulates transcription or translation of a nucleic acid encoding the product of one or more differentially expressed

genes, such as antisense oligonucleotide, ribozyme, or small interfering RNA (siRNA). Nucleic acid molecules that are modulators of differentially expressed genes in fibrotic tissue can be administered, for example, in a viral vector (such as lentivirus) or non-viral vector (such as a liposome). In other variations of this method, the agent can be an ovarian steroid, such as estradiol and medroxyprogesterone actetate. However, the agent is preferably not a hormone, but is nonetheless capable of modulating the expression of one or more genes that are differentially expressed in a fibrotic disorder, such as those genes that are differentially expressed upon GnRHa therapy.

In a preferred embodiment, the agent that modulates expression of a differentially expressed gene in fibrotic tissue is one that decreases or down-regulates the action or expression of one or more genes selected from the group consisting of IL-11, IL-13, EGR1, EGR2, EGR3, CITED2, P300, E2F1, E2F2, E2F3, E2F4, E2F5, MCP3, CXCL5, CCL7, SMAD3, TYMS, and/or GT198. In a another preferred embodiment, the agent that modulates expression of a differentially expressed gene in fibrotic tissue is one that increases or up-regulates the action or expression of one or more genes selected from the group consisting of SMAD-7, NCOR2, TIMP-1, and ADAM17. More preferably, the agent decreases or down-regulates the action or expression of one or more genes selected from the group consisting of IL-11, IL-13, EGR1, EGR2, EGR3, CITED2, P300, E2F1, E2F2, E2F3, E2F4, E2F5, MCP3, CXCL5, CCL7, SMAD3, TYMS, and/or GT198, and increases or up-regulates the action or expression of one or more genes selected from the group consisting of SMAD-7, NCOR2, TIMP-1, and ADAM17.

In one embodiment, the agent that modulates expression of a differentially expressed gene in fibrotic tissue is selected from the group consisting of a selective estrogen receptor modulator (such as Roloxifene or other SERM), a selective progesterone receptor modulator (such as Asoprisnil (J867), RU486, or other SPRM), SB-505124, SB-431542, a mast cell inhibitor (such as Tranlist), Cystatin C (CystC), SD-208, LY550410, LY580276, Pitavastatin, 1,5 naphthyridine amiothiazole derivative, 1,5 naphthyridine pyrazole derivative, and ursolic acid (see, for example, Yingling, J. *et al.*, *Nat. Rev. Drug Discov.*, 2004, Dec.;3(12):1011-22, which is incorporated herein by reference in its entirety). In another embodiment, the agent is one based on a pyrazolopyridine scaffold (Beight, D.W. *et al.*, WO 2004/026871), a pyrazole scaffold (Gellibert, F. *et al.*, *J. Med. Chem.*, 2004, 47:4494-4506), an imidazopyridine scaffold (Lee, W.C. *et al.*, Wo 2004/021989), triazole scaffold (Blumberg, L.C. *et al.*, WO

2004/026307), a pyridopyrimidine scaffold (Chakravarty, S. *et al.*, WO 2000/012497), or an isothiazole scaffold (Munchhof, M.J., WO 2004/147574), each of which is incorporated herein by reference in its entirety. In another embodiment, the agent is a GnRhH agonist or antagonist, such as those disclosed herein.

Preferably, the agent administered to the subject for treatment or prevention of fibrosis is one that inhibits (reduces) TGF-beta signaling (signal transduction). More preferably, the agent administered to the subject is one that inhibits (reduces) TGF-beta II signaling (signal transduction). Preferably, the inhibition is selective, as opposed to "upstream" of TGF-beta II.

In another aspect of the method of the invention, the subject invention includes a method for treating (alleviating symptoms associated with) fibrotic tissue or reducing the likelihood of fibrotic tissue formation, by administering GnRH analog (*e.g.*, GnRH agonist or antagonist) locally to the target site. For example, the GnRH analog can be administered directly to a fibroid to reduce the size of the fibroid.

In another aspect, the present invention includes a method for identifying a modulator of a gene that is differentially-expressed in fibrotic tissue and/or during fibrogenesis, or a polypeptide encoded by the differentially-expressed gene, in a cell population, comprising: contacting the cell population with a test agent under conditions effective for the test agent to modulate a differentially-expressed gene disclosed herein, to modulate the biological activity of a polypeptide encoded by the differentially-expressed gene; and determining whether the test agent modulates the expression of the gene or biological activity of the polypeptide encoded by the gene. In one embodiment, the determining step is carried out by detecting mRNA or the polypeptide of the differentially expressed gene. Preferably, the cell population comprises mammalian cells (such as human cells) of the female reproductive tract (such as endometrial cells). In one embodiment, the differentially expressed gene is selected from the group consisting of IL-11, IL-13, EGR1, EGR2, EGR3, CITED2, P300, E2F1, E2F2, E2F3, E2F4, E2F5, MCP3, CXCL5, CCL7, SMAD3, TYMS, GT198, SMAD-7, NCOR2, TIMP-1, and ADAM17. Preferred modulators are those that decrease the activity of or down-regulate the expression of one or more of IL-11, IL-13, EGR1, EGR2, EGR3, CITED2, P300, E2F1, E2F2, E2F3, E2F4, E2F5, MCP3, CXCL5, CCL7, SMAD3, TYMS, and GT198, or increase the activity of or up-regulate the expression of one or more of SMAD-7, NCOR2, TIMP-1, and ADAM17. More preferably, the modulator decreases the activity

of or down-regulates the expression of one or more of IL-11, IL-13, EGR1, EGR2, EGR3, CITED2, P300, E2F1, E2F2, E2F3, E2F4, E2F5, MCP3, CXCL5, CCL7, SMAD3, TYMS, and GT198; and increases the activity of or up-regulates the expression of one or more of SMAD-7, NCOR2, TIMP-1, and ADAM17. In one embodiment, the identified modulator modulates one or more genes (up to and including all the genes) listed in Table 9 herein.

The present invention also includes arrays, such as microfluidic cards, for detecting differential gene expression in samples of fibrotic tissue.

Brief Description of Drawings

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

Figures 1A-1J show the expression profile of a selected group of genes representing growth factors/cytokines/polypeptide hormones/receptors (Figures 1A-1B), intracellular signal transduction pathways (Figures 1C-1D), transcription factors (Figures 1E-1F), cell cycle (Figures 1G-1H) and cell adhesion/ ECM/cytoskeletons (Figures 1I-1J) in response to time-dependent action of GnRHa in LSMC and MSMC. Values on the x-axis represent an arbitrary unit derived from the mean gene expression value for each factor after supervised analysis, statistical analysis in R programming environment and ANOVA, with gene expression values for the untreated controls (Ctrl) set at 1.

Figures 2A-2J show comparative analysis of the expression profile of 10 genes identified as differentially expressed in response to GnRH therapy in leiomyoma and matched myometrium and untreated group by microarray and Realtime PCR. Values on the x-axis represent an arbitrary unit derived from the mean expression value for each gene with values for the untreated controls (Crtl) set at 1. Total RNA isolated from these tissues was used for both microarray analysis and Realtime PCR validating the expression of IL-11, EGR3, CITED2, Nur77, TEIG, TGIF, p27, p57, Gas1 and GPRK5. On the Y-axis untreated myometrium and leiomyoma are designated as Unt-MM and Un-LM, and GnRH- treated as GnRH-Trt MM and GnRH-Trt LM.

Figures 3A-3T show comparative analysis of the expression profile of 10 genes identified as differentially expressed and regulated in response to GnRHa time-dependent action in LSMC and MSMC by microarray and Realtime PCR. Values on the x-axis

represent an arbitrary unit derived from the mean expression value for each gene, and y-axis represent the time course of GnRHa (0.1μM) treatment (2, 6 and 12 hours) with untreated control (Crtl) gene expression values set at 1. Total RNA isolated from these cells used for both microarray analysis and Realtime PCR for validating the expression of IL-11, EGR3, TEIG, TGIF, CITED2, Nur77, CDKN1B (p27), CDKN1C (p57), Gas1 and GPRK5.

Figures 4A-4E show immunohistochemical localization of IL-11, TGIF, TIEG, Nur77, EGR3, CITED2, p27, p57 and Gas1 in leiomyoma and myometrium. Note the presence of immunoreactive IL-11, TGIF, TIEG, Nur77, EGR3, CITED2, p27, p57 and Gas1 in association with leiomyoma and myometrial smooth muscle cells, and cellular components of connective tissue and vasculature. Both nuclear (EGR3, Nur77, p27, p57) and cytoplasmic (IL-11) staining is observed. Incubation of tissue sections with non-immune mouse (A), rabbit (B) and goat (figure not shown) IgGs instead of primary antibodies during immunostaining served as controls (Ctrl) reduced the staining intensity. Mag: X150 and X300.

Figures 5A-5N show the expression profile of a group of genes representing growth factors/cytokines/polypeptide hormones/receptors (Figures 5A-5B), intracellular signal transduction pathways (Figures 5C-5D), transcription factors (Figures 5E-5F), cell cycle (Figures 5G-5H) and cell adhesion/ ECM/cytoskeletons (Figures 5I-5J) in response to time-dependent action of TGF-β in LSMC and MSMC. Values on the x-axis represent an arbitrary unit derived from the mean gene expression value for each factor after supervised analysis, statistical analysis in R programming environment and ANOVA, with gene expression values for the untreated controls (Ctrl) set at 1.

Figures 6A-6R show comparative analysis of the expression profile of 12 genes identified as differentially expressed and regulated in response to time-dependent action of TGF-β1 in LSMC and matched MSMC by microarray and Realtime PCR. Values on the x-axis represent an arbitrary unit derived from the mean expression value for each gene and y-axis represent the time course of TGF-β (2.5 ng/ml) treatment (2, 6 and 12 hours) with untreated control (Crtl) gene expression values set at 1. Total RNA isolated from these cells was used for both microarray analysis and Realtime PCR validating the expression of IL-11, EGR3, CITED2, Nur77, TEIG, TGIF, Runx1, Runx2, p27, p57, Gas1 and GPRK5.

Figures 7A-7E show a comparative analysis of the expression profile of Runx1 and Runx2 genes in leiomyoma (LM) and matched myometrium (MM) from untreated (un-Trt) and women who received GnRHa therapy (GnRHa-Trt) as well as in leiomyoma and myometrial smooth muscle cells (LSMC and MSMC) in response to GnRHa (0.1 μM) time dependent action (2, 6 and 12 hours) and in response to time-dependent (2, 6 and 12 hours) action of TGF-β1 (2.5 ng/ml) determined by Realtime PCR. In microarray analysis Runx2 expression was not included since its expression value did not reach the study standard. Values on the x-axis represent an arbitrary unit derived from the mean expression value for each gene and y-axis represents the time course of TGF-beta and GnRHa treatments, with untreated control (Crtl) gene expression values set at 1. Total RNA isolated from these cells was used for both microarray analysis and Realtime PCR validation.

Figures 8A-8E are bar graphs showing mean ± SEM of relative mRNA expression levels of CCN2, CCN3, CCN4, fibulin-1C and S100A4 in leiomyoma (LM) and matched myometrium (MM) from untreated (Un-Trt) and GnRH treated (GnRH-Trt) groups (N=12) determined by Real-time PCR. Values on the Y-axis represent an arbitrary unit derived from the mean expression value for each gene with values for the untreated MM (Un-TrtMM) set at 1. Total RNA isolated from tissues including tissues used for microarray analysis (Luo X. *et al.*, *Endocrinology* 146:1074-1095). For CCN2, denotes b, c and d are statistically different from a, and d is different from c. For CCN3 and S100A4 denotes b, c and d are different from a. For CCN4, denotes b and c are different from a. For fibulin-1C, denotes c and d are different from a and b. All with $p<0.05$.

Figure 9 shows Western blot analysis of CCN2, CCN3, CCN4 and fibulin-1C in 9 paired myometrium (M) and leiomyoma (L) from proliferative (N=3) and secretory (N=3) phases of the menstrual cycle, and from women who received GnRHa therapy (GnRHa-treated, N=3). Total protein was isolated from these tissues and equal amount of protein was subjected to immunoblotting using antibodies specific to CCN2, CCN3, CCN4 and fibulin-1C.

Figures 10A-10L show immunohistochemical localization of CCN2 (Figures 10A and 10B), CCN3 (Figures 10C and 10D), CCN4 (Figures 10E and 10F), fibulin-1C (Figures 10G and 10H) and S100A4 (Figures 10I and 10J) in leiomyoma and myometrium with immunoreactive proteins in association with leiomyoma and

myometrial smooth muscle cells, and cellular components of connective tissue and vasculature. Incubation of tissue sections with non-immune rabbit (Figures 10K) and goat (Figures 10L) IgGs, instead of primary antibodies during immunostaining served as controls reduced the staining intensity. Mag: X60.

Figure 11A and 11B are bar graphs showing the mean ± SEM of relative mRNA expression of TGF-β1 and TGF-β3 in leiomyoma and matched myometrium. Total RNA was isolated from paired tissues (N=12) and subjected to Realtime PCR. Total protein isolated from these tissues and equal amount of protein was subjected to ELISA before and after activation. Denotes a and b are significantly different from c and d, respectively; and denotes a and c are statistically different from b and d with $P<0.05$. Arrows point out the significant differences between the expression of TGF-β1 and TGF-β3 mRNA expression and total and active TGF-β1 in leiomyoma and myometrium.

Figures 12A-12E are bar graphs whowing relative mRNA expression of CCN2, CCN3, CCN4, fibulin-1C and S100A4 in leiomyoma (LSMC) and myometrial (MSMC) smooth muscle cells following treatment with TGF-β1 (2.5 ng/ml) for 2, 6 and 12 hrs. Total RNA was isolated from treated and untreated control (Ctrl) cells and subjected to Realtime PCR. Results are the mean ± SEM of three experiments performed using independent cell cultures from different tissues. For CCN2, denotes b, b', c, c', d and d'; for CCN3 denotes b, b', c, c', and d; for CCN4, denotes b, c, c', d and d'; for fibulin-1C, denotes b and d; and for S100A4 denote c', d and d' are statistically different from a and a' respectively, with $P<0.05$. Arrows point out the significant differences between the expression of CCNs, fibulin-1C and S100A4 in LSMC and MSMC.

Figures 13A-13E are bar graphs showing the relative mRNA expression of CCN2, CCN3, CCN4, fibulin-1C and S100A4 in leiomyoma (LSMC) and myometrial (MSMC) smooth muscle cells following treatment with GnRHa (0.1μM) for 2, 6 and 12 hrs. Total RNA was isolated from treated and untreated control (Ctrl) cells and subjected to Realtime PCR. Results are the mean ± SEM of three experiments performed using independent cell cultures from different tissues. For CCN2, denotes b, c', d and d'; for CCN3 denotes b, b', c, c', d and d'; for CCN4, denotes b, b', c, and d'; for fibulin-1C, denotes b, b', c, c', d and d'; and for S100A4 denote b, b', c, c', d and d' are statistically different from a and a', respectively with $P<0.05$. Arrows point out the significant

differences between the expression of CCNs, fibulin-1C and S100A4 in LSMC and MSMC.

Figures 14A-14E are bar graphs showing the relative mRNA expression of CCN2, CCN3, CCN4, fibulin-1C and S100A4 in leiomyoma (LSMC) and myometrial (MSMC) smooth muscle cells pretreated with U0126 (U) MEK1/2MAPK inhibitor followed by treatment with GnRHa and TGF-β1. Serum-starved cells were pretreated with U0126 at 20 μM for 2 hrs, washed and then treated with 2.5 ng/ml of TGF-β1, or 0.1 μM of GnRH for 2 hrs. Total RNA was isolated from treated and untreated controls (Ctrl) and subjected to Realtime PCR. Results are the mean ± SEM of three experiments performed using independent cell cultures from different tissues. Denotes * are significantly different from control and **, and denotes *** are significantly different from * and control with $P<0.05$, respectively. Arrows point out the significant differences between the expression of CCNs, fibulin-1C and S100A4 in LSMC as compared with MSMC.

Figures 15A-15E are bar graphs showing relative mRNA expression of CCN2, CCN3, CCN4, fibulin-1C and S100A4 in leiomyoma (LSMC) and myometrial (MSMC) smooth muscle cells transfected with Smad SiRNA (SmadSi) and treatment with TGF-β1. The cells were transfected with Smad3 SiRNA or scrambled SiRNA for 48 hrs washed and then treated with 2.5 ng/ml of TGF-β1 for 2 hrs. Total RNA was isolated from treated and untreated controls (Ctrl) and subjected to Realtime PCR. Results are the mean ± SEM of three experiments performed using independent cell cultures from different tissues. Denotes * are significantly different from ** and ***, as well as *** are significantly different from ** with $P<0.05$, respectively. Arrows point out the significant differences between the expression of CCNs, fibulin-1C and S100A4 in LSMC as compared with MSMC.

Figure 16 is a bar graph showing the relative expression of fibromodulin mRNA in leiomyoma (LM) and matched myometrium (MM) from untreated (Un-Trt) and GnRH treated (GnRH-Trt) groups determined by real-time PCR. Values on the Y-axis represent an arbitrary unit derived from the mean expression value for each gene with values for the untreated MM (Un-TrtMM) set at 1. Total RNA isolated from tissues used for both microarray analysis (Luo, X. *et al. Endocrinology*, 2005, 146:1074-1096) is included in the results. Denotes * are statistically different from ** and UnTrt-MM (P) with $p<0.05$. Results are the mean ± SEM of mRNA expression in leiomyoma and matched

myometrium form proliferative (N=8) and secretory (N=12) phases of the menstrual and GnRHa-treated group (N=7).

Figure 17 shows Western blot analysis of fibromodulin in 14 paired myometrium (M) and leiomyoma (L) from proliferative (N=7) and secretory (N=7) phases of the menstrual cycle, and from women who received GnRHa therapy (GnRHa-treated; N=6). Total protein was isolated from these tissues and equal amount of protein was subjected to immunoblotting using antibodies specific to fibromodulin.

Figures 18A-18D show immunohistochemical localization of fibromodulin in leiomyoma (A) and myometrium (B) with immunoreactive proteins in association with leiomyoma and myometrial smooth muscle cells, and cellular components of connective tissue and vasculature. Incubation of tissue sections with non-immune and goat IgGs instead of primary antibodies (C and D) during immunostaining served as controls (Ctrl) reduced the staining intensity. Mag: X60.

Figures 19A-19D are bar graphs showing relative mRNA expression of fibromodulin in leiomyoma (LSMC) and myometrial (MSMC) smooth muscle cells following treatment with TGF-β1 (2.5 ng/ml) and GnRHa (0.1mM) for 2, 6 and 12 hrs; or in cells pretreated with 20 μM of U0126 (U) MEK1/2MAPK inhibitor followed by 2hrs of treatment with TGF-β1 (T) or GnRHa (G). Serum-starved cells were pretreated with U0126 at for 2 hrs, washed and then treated with 2.5 ng/ml of TGF-β1 for 2 hrs. Additionally LSMC and MSMC were transfected with Smad3 SiRNA or scrambled SiRNA for 48 hrs washed and then treated with 2.5 ng/ml of TGF-β1 (T/Si) for 2 hrs Total RNA was isolated from treated and untreated control (Ctrl) cells and subjected to Realtime PCR. Results are the mean ± SEM of three experiments performed using independent cell cultures from different tissues. Denotes *, ** and *** are statistically different from untreated control. In Smad SiRNA-treated cells * is different from ** and *** with $P<0.05$, respectively. Arrows point out the significant differences between the expression of fibromodulin in LSMC and MSMC.

Detailed Disclosure

The study disclosed herein was designed to further define the molecular environments of leiomyoma and matched myometrium during the early-mid luteal phase of the menstrual cycle, which is characterized by elevated production of ovarian steroids,

compared with tissues obtained from hormonally suppressed patients on GnRHa therapy. The present inventors further evaluated the direct action of GnRHa on global gene expression and their regulation in leiomyoma and myometrial cells isolated from the untreated tissue cohort. These approaches enabled the identification of expression profiles of genes targeted by GnRHa. The present inventors validated the expression of 10 of these genes in these cohorts, and concluded that local expression and activation of these genes may represent features differentiating leiomyoma and myometrial molecular environments during growth as well as GnRHa-induced regression.

Microarrays have been shown to be of great value in understanding the molecular biology of many diseases, and they have been successfully used to classify various tumors based on their clinical phenotype or genetic background. In this experiment, the present inventors have used gene expression profiling to define the biological relationship between TGF-β and GnRH in tumor growth and regression, and try to unveil the complexity of leiomyoma genesis and development. The present inventors have evaluated the underlying differences between molecular responses directed by TGF-β autocrine/paracrine actions in LSMC and MSMC, and following interference with these actions using TGF-β receptor type II antisense oligomers treatment. Since TGF-β receptors expression is targeted by GnRHa in leiomyoma and myometrium, the present inventors further evaluated the gene expression profiles in response to TGF-β type II receptor antisense treatment and GnRHa-treated LSMC and MSMC to identify the genes whose expression are the specific target of these treatments. Using this approach, several differentially expressed and regulated genes targeted by TGF-β autocrine/paracrine action were evaluated, and the expression of 12 genes in LSMC and MSMC in response to the time-dependent action of TGF-β was validated using Realtime PCR.

Methods involving conventional molecular biology techniques are described herein. Such techniques are generally known in the art and are described in detail in methodology treatises such as Molecular Cloning: A Laboratory Manual, 2nd ed., vol. 1-3, ed. Sambrook *et al.*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989; and Current Protocols in Molecular Biology, ed. Ausubel *et al.*, Greene Publishing and Wiley-Interscience, New York, 1992 (with periodic updates). Various techniques using polymerase chain reaction (PCR) are described, *e.g.*, in Innis *et al.*, PCR Protocols: A Guide to Methods and Applications, Academic Press: San Diego, 1990. Methods for chemical synthesis of nucleic acids are discussed, for example, in Beaucage and

Carruthers, Tetra. Letts. 22:1859-1862, 1981, and Matteucci *et al.*, J. Am. Chem. Soc. 103:3185, 1981. Chemical synthesis of nucleic acids can be performed, for example, on commercial automated oligonucleotide synthesizers. Immunological methods (*e.g.*, preparation of antigen-specific antibodies, immunoprecipitation, and immunoblotting) are described, *e.g.*, in Current Protocols in Immunology, ed. Coligan *et al.*, John Wiley & Sons, New York, 1991; and Methods of Immunological Analysis, ed. Masseyeff *et al.*, John Wiley & Sons, New York, 1992. Conventional methods of gene transfer and gene therapy can also be adapted for use in the present invention. See, *e.g.*, Gene Therapy: Principles and Applications, ed. T. Blackenstein, Springer Verlag, 1999; Gene Therapy Protocols (Methods in Molecular Medicine), ed. P. D. Robbins, Humana Press, 1997; and Retro-vectors for Human Gene Therapy, ed. C. P. Hodgson, Springer Verlag, 1996.

The following publications are specifically incorporated herein by reference in their entirety, including all figures, tables, and sequences, to the extent they are not inconsistent with the explicit teachings of this specification: U.S. patent publication US 2003/0032044 (Chegini *et al.*), filed July 17, 2002; international publication WO 03/007685 (Chegini *et al.*), filed July 17, 2002; international publication WO 00/20642 (Chegini *et al.*), filed October 1, 1999; U.S. patent publication US 2003/0077589 (Hess-Stumpp *et al.*), filed September 25, 2001; and U.S. patent publication US 2001/0002393 (Palmer *et al.*), filed December 20, 2000.

1. Detecting Fibrotic Disorders

The invention provides a method for detecting a fibrotic disorder in the tissue of a subject. This method includes the steps of: (a) providing a biological sample obtained (*i.e.*, derived) from the subject (such as endometrium or peritoneal fluid); (b) analyzing the expression of a differentially expressed gene in the sample; and (c) correlating the expression of the differentially expressed gene with the presence or absence of the fibrotic disorder in the subject.

Examples of reproductive tract disorders include, but are not limited to, leiomyoma, endometriosis, ovarian hyperstimulation syndrome, adhesions, and other tissue fibroses (*e.g.*, fibroids) (Smits G. *et al.*, N. Engl. J. Med., 2003, 349(8):760-766; Elchalal U. *et al.*, Human Reproduction, 1997, 12(6):1129-1137; Stewart E. *et al.*, Human Reproduction Update, 1996, 2(4):295-306; Shozu M. *et al.*, The Journal of Clinical Endocrinology & Metabolism, 86(11):5405-5411; Estaban J. *et al.*, Arch. Pathol. Lab.

Med., 1999, 123:960-962; Lee W. *et al.*, The Korean Journal of Pathology, 2003, 37:71-73; and Kurioka H. *et al.*, Human Reproduction, 1998, 13(5):1357-1360).

Differentially expressed genes include those which are differentially expressed in a given fibrotic disorder, including but not limited to, docking protein 1, 62 kD (downstream of tyrosine kinase 1); centromere protein A (17 kD); catenin (cadherin-associated protein), beta 1 (88 kD); nuclear receptor subfamily 1, group I, member 2; v-rel avian reticuloendotheliosis viral oncogene homolog A; LGN Protein; CDC28 protein kinase 1; hypothetical protein; solute carrier family 17 (sodium phosphate), member 1; FOS-like antigen-1; nuclear matrix protein p84; LERK-6 (EPLG6); visinin-like 1; phosphodiesterase 10A; KH-type splicing regulatory protein (FUSE binding protein 2); Polyposis locus (DP1 gene) mRNA; microtubule-associated protein 2; CDC5 (cell division cycle 5, S pombe, homolog)-like; Centromere autoantigen C (CENPC) mRNA; RNA guanylyltransferase and 5'-phosphatase; Nijmegen breakage syndrome 1 (nibrin); ribonuclease, RNase A family, 4; keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris); basic helix-loop-helix domain containing, class B, 2; dual specificity phosphatase 1; annexin A11; putative receptor protein; Human endogenous retrovirus HERV-K(HML6); mitogen-activated protein kinase kinase kinase 12; TXK tyrosine kinase; kynureninase (L-kynurenine hydrolase); ubiquitin specific protease 4 (proto-oncogene); peroxisome biogenesis factor 13; olfactory receptor, family 2, subfamily F, member 1; membrane protein, palmitoylated 3 (MAGUK p55 subfamily member 3); origin recognition complex, subunit 1 (yeast homolog)-like; dTDP-D-glucose 4,6-dehydratase; cytochrome c oxidase subunit VIa polypeptide 2; gamma-tubulin complex protein 2; Monocyte chemotactic protein-3; myelin transcription factor 1; inhibitor of growth family, member 1-like; thyroid hormone receptor, alpha myosin-binding protein C, slow-type; fragile X mental retardation 2; sonic hedgehog (Drosophila) homolog; 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2; SFRS protein kinase 2; excision repair cross-complementing rodent repair deficiency; cyclin-dependent kinase 5, regulatory subunit 1 (p35); poly(A)-specific ribonuclease (deadenylation nuclease); solute carrier family 12 (potassium/chloride transporters), member 4; Pseudogene for metallothionein; natriuretic peptide precursor A; intercellular adhesion molecule 2; apoptosis antagonizing transcription factor; similar to rat HREV107; major histocompatibility complex, class II, DP beta 1; MpV17 transgene, murine homolog, glomerulosclerosis; uroporphyrinogen decarboxylase; proteasome (prosome, macropain)

26S subunit, ATPase, 1; fms-related tyrosine kinase 3 ligand; actin, gamma 1; Protein Kinase Pitslre, Alpha, Alt. Splice 1-Feb; nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; pyruvate kinase, muscle; telomeric repeat binding factor 2; cell division cycle 2, G1 to S and G2 to M; ADP-ribosylation factor 3; NRF1 Protein; H factor (complement)-like 3; serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 6; mRNA of muscle specific gene M9; solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3; ribosomal protein L36a; suppressor of Ty (S. cerevisiae) 4 homolog 1; amino-terminal enhancer of split; ubiquitin A-52 residue ribosomal protein fusion product 1; hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase; chaperonin containing TCP1, subunit 2 (beta); tyrosine kinase with immunoglobulin and epidermal growth factor homology; domains; Fc fragment of IgG, receptor, transporter, alpha; NRD1 convertase; ADP-ribosylation factor 5; transcription elongation factor A (SII), 1; like mouse brain protein E46; titin; fibromodulin; Abl-interactor 2 (Abi-2); and other differentially expressed genes disclosed herein. In one embodiment, the differentially expressed gene includes one or more of the genes listed in Table 9. The number of differentially expressed genes analyzed in the sample can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more.

In another embodiment, the differentially expressed gene is at least one of CDKN1B, CDKN1C, CTGF, fibromodulin, and Abl-interactor 2 (Abi-2).

Suitable subjects for use in the invention can be any human or non-human animal. For example, the subject can be a female animal, such as mammal, like a dog, cat, horse, cow, pig, sheep, goat, chicken, primate, rat, or mouse. Because the experiments presented herein relate to human subjects, a preferred subject for the methods of the invention is a human, such as a human female. Particularly preferred are female subjects suspected of having or at risk for developing a fibrotic disorder within the reproductive tract, *e.g.*, a woman suspected of having or at risk for developing leiomyoma, endometriosis, or peritoneal adhesions based on clinical findings or other diagnostic test results.

The step of providing a biological sample obtained from the subject can be performed by conventional medical techniques. For example, an endometrial tissue sample can be taken from the subject by biopsy. As another example, a sample of peritoneal fluid can be taken from a subject by conventional techniques. Suitable methods are described in more detail in the Examples sections presented below.

The step of analyzing the expression of a differentially expressed gene in the sample can be performed in a variety of different ways. Numerous suitable techniques are known for analyzing gene expression. For example, gene expression can be determined directly by assessing protein expression of cells or fluid of a biological sample (*e.g.*, endometrial tissue or peritoneal fluid). Proteins can be detected using immunological techniques, *e.g.*, using antibodies that specifically bind the protein in assays such as immunofluorescence or immunohistochemical staining and analysis, enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), immunoblotting (*e.g.*, Western blotting), and like techniques. Expression of differentially expressed genes can also be determined by directly or indirectly measuring the amount of mRNA encoding protein in a cellular sample using known techniques such as Northern blotting and PCR-based methods such as competitive quantitative reverse transcriptase PCR (Q-RT-PCR). Suitable methods for analyzing expression of differentially expressed genes are described below; nonetheless, other suitable methods might also be employed.

The step of correlating the expression of the gene with the presence or absence of the fibrotic disorder in the subject involves comparing the level of gene expression in the test biological sample with levels of gene expression in control samples, *e.g.*, those derived from subjects known to have or not to have the particular disorder. Thus, after quantifying gene expression in a biological sample from a test subject, the test result is compared to levels of gene expression determined from (a) a panel of cells or tissues derived from subjects (preferably matched to the test subject by age, species, strain or ethnicity, and/or other medically relevant criteria) known to have a particular disorder and (b) a panel of cells or tissues derived from subjects (preferably also matched as above) known not to have a particular disorder. If the test result is closer to the levels (*e.g.*, mean or arithmetic average) from the panel of cells or tissues derived from subjects known to have a particular disorder, then the test result correlates with the test subject having the particular disorder. On the other hand, if the test result is closer to the levels (*e.g.*, mean or arithmetic average) from the panel of cells or tissues derived from subjects known not to have a particular disorder, then the test result correlates with the test subject not having the particular disorder. Optionally, the method further comprises selecting and administering a therapy or therapies to the patient to treat for the correlated disorder(s).

II. Modulating Gene Expression

The present invention also provides a method for modulating the expression of genes that are differentially expressed in fibrotic tissues (such as leiomyoma), compared to normal tissues. Restoration of gene expression to levels associated with normal tissue is expected to amcliorate at least some of the symptoms associated with the fibrotic disorder. This method includes the step of contacting the tissue with an agent that modulates expression of one or more differentially expressed genes in the tissue. Optionally, the method includes the step of diagnosing the subject with the fibrotic disorder prior to contacting the tissue with the agent that modulates expression of one or more differentially expressed genes in the fibrotic tissue.

Differentially expressed genes include those which are differentially expressed in a given fibrotic disorder, including but not limited to, docking protein 1, 62 kD (downstream of tyrosine kinase 1); centromere protein A (17 kD); catenin (cadherin-associated protein), beta 1 (88 kD); nuclear receptor subfamily 1, group I, member 2; v-rel avian reticuloendotheliosis viral oncogene homolog A; LGN Protein; CDC28 protein kinase 1; hypothetical protein; solute carrier family 17 (sodium phosphate), member 1; FOS-like antigen-1; nuclear matrix protein p84; LERK-6 (EPLG6); visinin-like 1; phosphodiesterase 10A; KH-type splicing regulatory protein (FUSE binding protein 2); Polyposis locus (DP1 gene) mRNA; microtubule-associated protein 2; CDC5 (cell division cycle 5, S pombe, homolog)-like; Centromere autoantigen C (CENPC) mRNA; RNA guanylyltransferase and 5'-phosphatase; Nijmegen breakage syndrome 1 (nibrin); ribonuclease, RNase A family, 4; keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris); basic helix-loop-helix domain containing, class B, 2; dual specificity phosphatase 1; annexin A11; putative receptor protein; Human endogenous retrovirus HERV-K(HML6); mitogen-activated protein kinase kinase kinase 12; TXK tyrosine kinase; kynureninase (L-kynurenine hydrolase); ubiquitin specific protease 4 (proto-oncogene); peroxisome biogenesis factor 13; olfactory receptor, family 2, subfamily F, member 1; membrane protein, palmitoylated 3 (MAGUK p55 subfamily member 3); origin recognition complex, subunit 1 (yeast homolog)-like; dTDP-D-glucose 4,6-dehydratase; cytochrome c oxidase subunit Vla polypeptide 2; gamma-tubulin complex protein 2; Monocyte chemotactic protein-3; myelin transcription factor 1; inhibitor of growth family, member 1-like; thyroid hormone receptor, alpha myosin-binding protein C, slow-type; fragile X mental retardation 2; sonic hedgehog (Drosophila)

homolog; 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2; SFRS protein kinase 2; excision repair cross-complementing rodent repair deficiency; cyclin-dependent kinase 5, regulatory subunit 1 (p35); poly(A)-specific ribonuclease (deadenylation nuclease); solute carrier family 12 (potassium/chloride transporters), member 4; Pseudogene for metallothionein; natriuretic peptide precursor A; intercellular adhesion molecule 2; apoptosis antagonizing transcription factor; similar to rat HREV107; major histocompatibility complex, class II, DP beta 1; MpV17 transgene, murine homolog, glomerulosclerosis; uroporphyrinogen decarboxylase; proteasome (prosome, macropain) 26S subunit, ATPase, 1; fms-related tyrosine kinase 3 ligand; actin, gamma 1; Protein Kinase Pitslre, Alpha, Alt. Splice 1-Feb; nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; pyruvate kinase, muscle; telomeric repeat binding factor 2; cell division cycle 2, G1 to S and G2 to M; ADP-ribosylation factor 3; NRF1 Protein; H factor (complement)-like 3; serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 6; mRNA of muscle specific gene M9; solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3; ribosomal protein L36a; suppressor of Ty (*S. cerevisiae*) 4 homolog 1; amino-terminal enhancer of split; ubiquitin A-52 residue ribosomal protein fusion product 1; hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase; chaperonin containing TCP1, subunit 2 (beta); tyrosine kinase with immunoglobulin and epidermal growth factor homology; domains; Fc fragment of IgG, receptor, transporter, alpha; NRD1 convertase; ADP-ribosylation factor 5; transcription elongation factor A (SII), 1; like mouse brain protein E46; titin; fibromodulin; Abl-interactor 2 (Abi-2); and other differentially expressed genes disclosed herein. In one embodiment, the differentially expressed gene includes one or more of the genes listed in Table 9.

In another embodiment, the differentially expressed gene is at least one of CDKN1B, CDKN1C, CTGF, fibromodulin, and Abl-interactor 2 (Abi-2).

In a preferred embodiment, the agent that modulates expression of a differentially expressed gene in fibrotic tissue (such as leiomyoma) is one that decreases or down-regulates the action or expression of one or more genes selected from the group consisting of IL-11, IL-13, EGR1, EGR2, EGR3, CITED2, P300, E2F1, E2F2, E2F3, E2F4, E2F5, MCP3, CXCL5, CCL7, SMAD3, TYMS, and/or GT198. In a another preferred embodiment, the agent that modulates expression of a differentially expressed gene in fibrotic tissue is one that increases or up-regulates the action or expression of one or more

genes selected from the group consisting of SMAD-7, NCOR2, TIMP-1, and ADAM17. More preferably, the agent decreases or down-regulates the action or expression of one or more genes selected from the group consisting of IL-11, IL-13, EGR1, EGR2, EGR3, CITED2, P300, E2F1, E2F2, E2F3, E2F4, E2F5, MCP3, CXCL5, CCL7, SMAD3, TYMS, and/or GT198, and increases or up-regulates the action or expression of one or more genes selected from the group consisting of SMAD-7, NCOR2, TIMP-1, and ADAM17.

In one embodiment, the agent that modulates expression of a differentially expressed gene in fibrotic tissue (such as leiomyoma) is selected from the group consisting of a selective estrogen receptor modulator (such as Roloxifene or other SERM), a selective progesterone receptor modulator (such as Asoprisnil (J867), RU486, or other SPRM), SB-505124, SB-431542, a mast cell inhibitor (such as Tranlist), Cystatin C (CystC), SD-208, LY550410, LY580276, Pitavastatin, 1,5 naphthyridine amiothiazole derivative, 1,5 naphthyridine pyrazole derivative, and ursolic acid (see, for example, Yingling, J. *et al.*, *Nat. Rev. Drug Discov.*, 2004, Dec.;3(12):1011-22; Chwalisz, K. *et al.*, *Semin. Reprod. Med.*, 2004, 22(2):113-119; Hodl, C. *et al.*, *Bioconjug. Che.*, 2004, 15(2):359-365; Dubey, R.K. *et al.*, *J. Clin. Endocrinol. Metab.*, 2004, 89(2):852-859; DeManno, D. *et al.*, *Steroids*, 2003, 68(10-13):1019-1032; DaCosta, B.S. *et al.*, *Mol. Pharmacol.*, 65(3):744-752; Sokol, J.P. *et al.*, *Mol. Cancer Res.*, 2004, 2(3):183-195; Wanatabe, T. *et al.*, *Journal of Cell Biology*, 2003, 163(6):1303-1311, and Hjelmeland, M.D. *et al.*, *Mol. Cancer Ther.*, 2004, 3(6):737-745), which are incorporated herein by reference in their entirety). In another embodiment, the agent is one based on a pyrazolopyridine scaffold (Beight, D.W. *et al.*, WO 2004/026871), a pyrazole scaffold (Gellibert, F. *et al.*, *J. Med. Chem.*, 2004, 47:4494-4506), an imidazopyridine scaffold (Lee, W.C. *et al.*, Wo 2004/021989), triazole scaffold (Blumberg, L.C. *et al.*, WO 2004/026307), a pyridopyrimidine scaffold (Chakravarty, S. *et al.*, WO 2000/012497), or an isothiazole scaffold (Munchhof, M.J., WO 2004/147574), each of which is incorporated herein by reference in its entirety.

Preferably, the agent administered to the subject for treatment or prevention of fibrosis is one that inhibits (reduces) TGF-beta signaling (signal transduction). More preferably, the agent administered to the subject that inhibits (reduces) TGF-beta II signaling (signal transduction).

In another aspect of the method of the invention, the subject invention includes a method for treating (alleviating symptoms associated with) fibrotic tissue or reducing the likelihood of fibrotic tissue formation, by administering GnRH analog locally to the target site. For example, the GnRH analog can be administered directly to a fibroid to reduce the size of the fibroid.

The tissue for use in this method can be any derived from a human or non-human animal. In some embodiments, the tissue is derived from a female reproductive system, *e.g.*, endometrium, or tissue derived from the uterus, cervix, vagina, fallopian tube, or ovary. Because the experiments presented herein relate to human subjects, a preferred tissue sample for the methods of the invention is one derived from a human. Particularly preferred is tissue derived from a subject suspected of having or at risk for developing a fibrotic disorder (such as a woman suspected of having or at risk for developing leoimyoma, endometriosis, ovarian hyperstimulation syndrome, peritoneal adhesions, or other tissue fibroses) based on clinical findings or other diagnostic test results.

The method of the present invention utilizes one or more agents that modulate expression one or more differentially expressed genes in the tissue. Numerous agents for modulating expression of such genes in a tissue are known. Any of those suitable for the particular system being used may be employed. Typical agents for modulating expression of such genes are proteins, nucleic acids, and small organic or inorganic molecules such as hormones (*e.g.*, natural or synthetic steroids). Preferably, the agent is not a hormone.

An example of a protein that can modulate gene expression is an antibody that specifically binds to the gene product. Such an antibody can be used to interfere with the interaction of the gene product and other molecules that bind the gene product. Products of the differentially expressed genes (or immunogenic fragments or analogs thereof) can be used to raise antibodies useful in the invention. Such gene products (*e.g.*, proteins) can be produced by purification from cells/tissues, recombinant techniques or chemical synthesis as described above. Antibodies for use in the invention include polyclonal antibodies, monoclonal antibodies, single chain antibodies, Fab fragments, $F(ab')_2$ fragments, and molecules produced using a Fab expression library. See, for example, Kohler *et al.*, *Nature*, 1975, 256:495; Kohler *et al.*, *Eur. J. Immunol.*, 1976, 6:511; Kohler *et al.*, *Eur. J. Immunol.*, 1976, 6:292; Hammerling *et al.*, "Monoclonal Antibodies and T Cell Hybridomas," Elsevier, N.Y., 1981; Ausubel *et al.*, supra; U.S. Patent Nos.

4,376,110, 4,704,692, and 4, 946,778; Kosbor *et al.*, *Immunology Today*, 1983, 4:72; Cole *et al.*, *Proc. Natl. Acad. Sci. USA*, 1983, 80:2026; Cole *et al.*, "Monoclonal Antibodies and Cancer Therapy," Alan R. Liss, Inc., pp. 77-96, 1983; and Huse *et al.*, *Science*, 1989, 246:1275.

Other proteins that can modulate gene expression include variants of the gene products that can compete with the native gene products for binding ligands such as naturally occurring receptors of these gene products. Such variants can be generated through various techniques known in the art. For example, protein variants can be made by mutagenesis, such as by introducing discrete point mutation(s), or by truncation. Mutation can give rise to a protein variant having substantially the same, or merely a subset of the functional activity of a native protein. Alternatively, antagonistic forms of the protein can be generated which are able to inhibit the function of the naturally occurring form of the protein, such as by competitively binding to another molecule that interacts with the protein. In addition, agonistic (or superagonistic) forms of the protein may be generated that constitutively express one or more functional activities of the protein. Other variants of the gene products that can be generated include those that are resistant to proteolytic cleavage, as for example, due to mutations which alter protease target sequences. Whether a change in the amino acid sequence of a peptide results in a protein variant having one or more functional activities of a native protein can be readily determined by testing the variant for a native protein functional activity (*e.g.*, binding a receptor or inducing a cellular response).

Another agent that can modulate gene expression is a non-peptide mimetic or chemically modified form of the gene product that disrupts binding of the encoded protein to other proteins or molecules with which the native protein interacts. See, *e.g.*, Freidinger *et al.* in Peptides: Chemistry and Biology, G. R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), azepine (*e.g.*, see Huffman *et al.* in Peptides: Chemistry and Biology, G. R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted gamma lactam rings (Garvey *et al.* in Peptides: Chemistry and Biology, G. R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), keto-methylene pseudopepitides (Ewenson *et al. J. Med. Chem.*, 1986, 29:295; and Ewenson *et al.* in Peptides: Structure and Function (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, Ill, 1985), beta-turn dipeptide cores (Nagai *et al. Tetrahedron Lett*, 1985, 26:647; and Sato *et al. J. Chem. Soc. Perkin. Trans.*, 1986,

1:1231), and beta-aminoalcohols (Gordon *et al. Biochem. Biophys. Res. Commun.*, 1985, 126:419; and Dann *et al. Biochem. Biophys. Res. Commun.*, 1986, 134:71). Proteins may also be chemically modified to create derivatives by forming covalent or aggregate conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like. Covalent derivatives of proteins encoded by differentially expressed genes can be prepared by linking the chemical moieties to functional groups on amino acid side chains of the protein or at the N-terminus or at the C-terminus of the polypeptide.

The agent that directly reduces expression of the differentially expressed gene can also be a nucleic acid molecule that reduces expression of the gene. For example, the nucleic acid molecule can be an antisense nucleic acid that hybridizes to mRNA encoding the protein. Antisense nucleic acid molecules for use within the invention are those that specifically hybridize (*e.g.* bind) under cellular conditions to cellular mRNA and/or genomic DNA encoding a protein in a manner that inhibits expression of the protein, *e.g.*, by inhibiting transcription and/or translation. The binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix.

In one embodiment, the nucleic acid molecule that directly reduces the expression of the differentially expressed gene is selected from the group consisting of antisense, short interfering RNA (siRNA), and a ribozyme. In a specific embodiment, the nucleic acid molecule is targed to the TGF-beta type II receptor, directly reducing its expression.

Vectors may be used to deliver the nucleic acid molecule to the target site (*e.g.*, the fibrotic tissue) *in vitro* or *in vivo*. The vector may be, for example, a viral vector (such as lentivirus) or a non-viral vector (such as a liposome or other cholesterol molecule); see, for example, Soutschek, J. *et al.*, *Nature*, 2004, 432(7014):173-178, which desribes therapeutic silencing of an endogenous gene by administration siRNAs, and which is incorporated herein by reference in its entirety.

Antisense constructs can be delivered as an expression plasmid which, when transcribed in the cell, produces RNA which is complementary to at least a unique portion of the cellular mRNA which encodes the protein. Alternatively, the antisense construct can take the form of an oligonucleotide probe generated *ex vivo* which, when introduced into a protein expressing cell, causes inhibition of protein expression by hybridizing with an mRNA and/or genomic sequences coding for the protein. Such oligonucleotide probes

are preferably modified oligonucleotides that are resistant to endogenous nucleases, *e.g.* exonucleases and/or endonucleases, and are therefore stable *in vivo*. Exemplary nucleic acid molecules for use as antisense oligonucleotides are phosphoramidate, phosphothioate and methylphosphonate analogs of DNA (see, *e.g.*, U.S. Patent Nos. 5,176,996; 5,264,564; and 5,256,775). Additionally, general approaches to constructing oligomers useful in antisense therapy have been reviewed, for example, by Van der Krol *et al.*, *Biotechniques*, 1988, 6:958-976; and Stein *et al.*, *Cancer Res.*, 1988, 48:2659-2668. With respect to antisense DNA, oligodeoxyribonucleotides derived from the translation initiation site, *e.g.*, between the -10 and +10 regions of a protein encoding nucleotide sequence, are preferred.

Antisense approaches involve the design of oligonucleotides (either DNA or RNA) that are complementary to mRNA encoding the protein to be inhibited. The antisense oligonucleotides will bind to mRNA transcripts and prevent translation. Absolute complementarity, although preferred, is not required. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an RNA it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

In one embodiment, the antisense oligonucleotides used in the subject invention are targeted to the TGF-beta type II receptor, such as those disclosed herein.

Oligonucleotides that are complementary to the 5' end of the message, *e.g.*, the 5' untranslated sequence up to and including the AUG initiation codon, should work most efficiently at inhibiting translation. However, sequences complementary to the 3' untranslated sequences of mRNAs have been shown to be effective at inhibiting translation of mRNAs as well (Wagner, R., *Nature*, 1994, 372:333). Therefore, oligonucleotides complementary to either the 5' or 3' untranslated, non-coding regions of a differentially expressed gene could be used in an antisense approach to inhibit translation of endogenous mRNA. Oligonucleotides complementary to the 5' untranslated region of the mRNA should include the complement of the AUG start codon. Antisense oligonucleotides complementary to mRNA coding regions are less efficient inhibitors of translation but could be used in accordance with the invention. Whether designed to hybridize to the 5', 3' or coding region of the mRNA, antisense nucleic acids

should be at least eighteen nucleotides in length, and are preferably less than about 100 and more preferably less than about 30, 25, 20, or 18 nucleotides in length.

Antisense oligonucleotides of the invention may comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxyethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouricil, beta-D-galactosylqueosin- e, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-idimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopenten- yladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine. Antisense oligonucleotides of the invention may also comprise at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose; and may additionally include at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet a further embodiment, the antisense oligonucleotide is an alpha-anomeric oligonucleotide. An alpha-anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual beta-units, the strands run parallel to each other (Gautier *et al.*, *Nucl. Acids Res.*, 1987, 15:6625-6641). Such oligonucleotide can be a 2'-0-methylribonucleotide (Inoue *et al.*, *Nucl. Acids Res.*, 1987, 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue *et al.*, *FEBS Lett.*, 1987, 215:327-330).

Oligonucleotides of the invention may be synthesized by standard methods known in the art, *e.g.* by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, *etc.*). As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein *et al. Nucl. Acids Res.*, 1988,

16:3209), methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 1988, 85:7448-7451).

The antisense molecules should be delivered into cells that express the differentially expressed (*e.g.*, overexpressed) genes *in vivo*. A number of methods have been developed for delivering antisense DNA or RNA into cells. For instance, antisense molecules can be introduced directly into the tissue site by such standard techniques as electroporation, liposome-mediated transfection, CaCl-mediated transfection, or the use of a gene gun. Alternatively, modified antisense molecules, designed to target the desired cells (*e.g.*, antisense linked to peptides or antibodies that specifically bind receptors or antigens expressed on the target cell surface) can be used.

However, because it is often difficult to achieve intracellular concentrations of the antisense sufficient to suppress translation of endogenous mRNAs, a preferred approach utilizes a recombinant DNA construct in which the antisense oligonucleotide is placed under the control of a strong promoter (*e.g.*, the CMV promoter). The use of such a construct to transform cells will result in the transcription of sufficient amounts of single stranded RNAs that will form complementary base pairs with the endogenous gene transcripts and thereby prevent translation of the mRNA.

Ribozyme molecules designed to catalytically cleave target mRNA transcripts can also be used to prevent translation of mRNA and expression of protein (see, *e.g.*, PCT Publication No. WO 90/11364, published Oct. 4, 1990; Sarver *et al.*, *Science*, 1990, 247:1222-1225 and U.S. Pat. No. 5,093,246). While ribozymes that cleave mRNA at site-specific recognition sequences can be used to destroy target mRNAs, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases: 5'-UG-3'. The construction and production of hammerhead ribozymes is well known in the art and is described more fully in Haseloff and Gerlach, *Nature*, 1988, 334:585-591. Preferably the ribozyme is engineered so that the cleavage recognition site is located near the 5' end of the mRNA; *i.e.*, to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts. Ribozymes within the invention can be delivered to a cell using a vector.

The expression of endogenous genes that are overexpressed in fibrotic disorders can also be reduced by inactivating or "knocking out" the gene or its promoter using

targeted homologous recombination. See, *e.g.*, Kempin *et al.*, *Nature*, 1997, 389:802; Smithies *et al.*, *Nature*, 1985, 317:230-234; Thomas and Capecchi, *Cell*, 1987, 51:503-512; and Thompson *et al.*, *Cell*, 1989, 5:313-321. For example, a mutant, non-functional gene variant (or a completely unrelated DNA sequence) flanked by DNA homologous to the endogenous gene (either the coding regions or regulatory regions of the gene) can be used, with or without a selectable marker and/or a negative selectable marker, to transfect cells that express the gene *in vivo*.

Alternatively, endogenous gene expression may be reduced by targeting deoxyribonucleotide sequences complementary to the regulatory region of the target gene(s) (*i.e.*, the gene promoter and/or enhancers) to form triple helical structures that prevent transcription of the gene in target cells. (See generally, Helene, C., *Anticancer Drug Des.*, 1991, 6(6):569-84; Helene, C., *et al.*, *Ann. N.Y. Acad. Sci.*, 1992, 660:27-36; and Maher, L. J., *Bioassays*, 1992, 14(12):807-15).

Antisense nucleic acid, ribozyme, and triple helix molecules of the invention may be prepared by any method known in the art for the synthesis of DNA and RNA molecules. These include techniques for chemically synthesizing oligodeoxyribonucleotides and oligoribonucleotides well known in the art such as for example solid phase phosphoramide chemical synthesis. Alternatively, RNA molecules may be generated by in vitro and in vivo transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which incorporate suitable RNA polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

Another agent that can be used to modulate gene expression in fibrotic tissue is a hormone. Numerous naturally occurring and synthetic hormones are known to cause physiological changes in such tissue and are available commercially. See, *e.g.*, PDR: Physician's Desk Reference, 2002. Those particular hormones which modulate expression of differentially expressed genes in a given sample tissue can be determined empirically by contacting a series of tissue samples with a panel of different hormones and analyzing the tissue samples for changes in phenotype over time. In experiments relating to the invention, it was shown that GnRHa therapy modulated the expression of 297 genes in leiomyoma and myometrium compared to untreated group ($P<0.02$). In addition, GnRHa, TGF-b and TGF-b receptor type II antisense treatments resulted in

differential regulation of 134, 144, and 154 specific genes, respectively ($P<0.005$ and 0.001). The products of these genes were functionally categorized as key regulators of cell cycle, transcription factors, signal transduction, ECM turnover and apoptosis. Based on (i) expression values, (ii) functional classification and (iii) regulation by GnRH and TGF-b mediated actions, we selected 10 of these genes and validated their expression in leiomyoma and myometrium, and in LSMC and MSMC using RealTime PCR, western blotting and immunohistochemistry. In conclusion, the results provide additional evidence for the difference in gene expression profile between leiomyoma and myometrium, and reveal the profile of previously unrecognized novel genes whose expression are the target of GnRH and TGF-β actions in leiomyoma and myometrium.

The agent that can be used to modulate gene expression in fibrotic tissue may be administered to non-human animals or humans in pharmaceutically acceptable carriers (*e.g.*, physiological saline) that are selected on the basis of mode and route of administration and standard pharmaceutical practice. For example, the pharmaceutical compositions of the invention might include suitable buffering agents such as acetic acid or its salt (1-2% w/v); citric acid or its salt (1-3% w/v); boric acid or its salt (0.5-2.5% w/v); succinic acid; or phosphoric acid or its salt (0.8-2% w/v); and suitable preservatives such as benzalkonium chloride (0.003-0.03% w/v); chlorobutanol (0.3-0.9% w/v); parabens (0.01-0.25% w/v) or thimerosal (0.004-0.02% w/v). Examples of compositions suitable for parenteral administration include sterile aqueous preparations such as water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils might be used as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed including synthetic mono-or di-glycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables. Carrier formulations suitable for local, subcutaneous, intramuscular, intraperitoneal or intravenous administrations may be found in Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, PA. The pharmaceutical compositions useful in the invention may be delivered in mixtures of more than one pharmaceutical composition.

The compositions of the invention (containing an agent that can be used to modulate gene expression in fibrotic tissue) may be administered to animals or humans by any conventional technique. Such administration might be parenteral (*e.g.*, intravenous, subcutaneous, intramuscular, or intraperitoneal introduction). Preferably, the compositions may also be administered directly to the target site (*e.g.*, a portion of the

reproductive tract or peritoneal cavity) by, for example, surgical delivery to an internal or external target site, or by catheter to a site accessible by a blood vessel. Other methods of delivery, *e.g.*, liposomal delivery or diffusion from a device impregnated with the composition, are known in the art. The composition may be administered in a single bolus, multiple injections, or by continuous infusion (*e.g.*, intravenously or by peritoneal dialysis).

The methods of this invention, generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that produces effective levels of response without causing clinically unacceptable adverse effects. Preferred modes of administration include parenteral, injection, infusion, deposition, implantation, anal or vaginal supposition, oral ingestion, inhalation, and topical administration. Injections can be intravenous, intradermal, subcutaneous, intramuscular, or interperitoneal. For example, the pharmaceutical composition can be injected directly into target site for the prevention of fibrotic disorders, such as leiomyoma, endometriosis, ovarian hyperstimulation syndrome, or adhesion formation. In some embodiments, the injections can be given at multiple locations. Implantation includes inserting implantable drug delivery systems, *e.g.*, microspheres, hydrogels, polymeric reservoirs, cholesterol matrixes, polymeric systems, *e.g.*, matrix erosion and/or diffusion systems and non-polymeric systems, *e.g.*, compressed, fused, or partially fused pellets. Inhalation includes administering the pharmaceutical composition with an aerosol in an inhaler, either alone or attached to a carrier that can be absorbed. For systemic administration, it may be preferred that the pharmaceutical composition is encapsulated in liposomes. The term "parenteral" includes subcutaneous injections, intravenous, intramuscular, intraperitoneal, intrastemal injection or infusion techniques. In certain preferred embodiments of the invention, the administration can be designed so as to result in sequential exposure of the pharmaceutical composition over some period of time, *e.g.*, hours, days, weeks, months or years. This can be accomplished by repeated administrations of the pharmaceutical composition, by one of the methods described above, or alternatively, by a sustained-release delivery system in which the pharmaceutical composition is delivered to the subject for a prolonged period without repeated administrations. By sustained-release delivery system, it is meant that total release of the pharmaceutical composition does not occur immediately upon administration, but rather is delayed for some period of time. Release can occur in bursts or it can occur gradually and continuously. Administration of

such a system can be, *e.g.*, by long-lasting oral dosage forms, bolus injections, transdermal patches, and subcutaneous implants.

A therapeutically effective amount is an amount that is capable of producing a medically desirable result in a treated animal or human. As is well known in the medical arts, dosage for any one animal or human depends on many factors, including the subject's size, body surface area, age, the particular composition to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. Toxicity and therapeutic efficacy of the compositions of the invention can be determined by standard pharmaceutical procedures, using cells in culture and/or experimental animals to determine the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Agents that exhibit large therapeutic indices are preferred. While agents that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of the tissues to be treated in order to minimize potential damage to uninvolved tissue and thereby reduce side effects. The data obtained from cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within the range of circulating concentrations that include an ED50 with little or no toxicity. The dosage may vary within this range depending on the dosage form employed and the route of administration utilized.

III. Methods for Identifying Agents that Modulate Fibrosis

The present invention also relates to methods of identifying agents, and the agents themselves, which modulate differentially-expressed genes or polypeptides expressed in endothelial or other fibrosis-forming (*e.g.*, leiomyoma-forming) cells, such as cells of the female reproductive tract. In one embodiment, the fibrosis is uterine fibrosis. These agents can be used to modulate the biological activity of the polypeptide encoded for the gene, or the gene, itself. Agents that regulate the gene or its product are useful in variety of different environments, including as medicinal agents to treat or prevent disorders associated with fibrosis and as research reagents to modify the function of tissues and cells.

The methods for identifying agents, in accordance with the present invention, generally comprise steps in which an agent is placed in contact with the gene, its transcription product, its translation product, or other target, and then a determination is performed to assess whether the agent "modulates" the target. The specific method utilized will depend upon a number of factors, including, *e.g.*, the target (*i.e.*, is it the gene or polypeptide encoded by it), the environment (*e.g.*, *in vitro* or *in vivo*), the composition of the agent, *etc.*

Differentially expressed genes include those which are differentially expressed in a given fibrotic disorder, including but not limited to, docking protein 1, 62 kD (downstream of tyrosine kinase 1); centromere protein A (17 kD); catenin (cadherin-associated protein), beta 1 (88 kD); nuclear receptor subfamily 1, group I, member 2; v-rel avian reticuloendotheliosis viral oncogene homolog A; LGN Protein; CDC28 protein kinase 1; hypothetical protein; solute carrier family 17 (sodium phosphate), member 1; FOS-like antigen-1; nuclear matrix protein p84; LERK-6 (EPLG6); visinin-like 1; phosphodiesterase 10A; KH-type splicing regulatory protein (FUSE binding protein 2); Polyposis locus (DP1 gene) mRNA; microtubule-associated protein 2; CDC5 (cell division cycle 5, S pombe, homolog)-like; Centromere autoantigen C (CENPC) mRNA; RNA guanylyltransferase and 5'-phosphatase; Nijmegen breakage syndrome 1 (nibrin); ribonuclease, RNase A family, 4; keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris); basic helix-loop-helix domain containing, class B, 2; dual specificity phosphatase 1; annexin A11; putative receptor protein; Human endogenous retrovirus HERV-K(HML6); mitogen-activated protein kinase kinase kinase 12; TXK tyrosine kinase; kynureninase (L-kynurenine hydrolase); ubiquitin specific protease 4 (proto-oncogene); peroxisome biogenesis factor 13; olfactory receptor, family 2, subfamily F, member 1; membrane protein, palmitoylated 3 (MAGUK p55 subfamily member 3); origin recognition complex, subunit 1 (yeast homolog)-like; dTDP-D-glucose 4,6-dehydratase; cytochrome c oxidase subunit VIa polypeptide 2; gamma-tubulin complex protein 2; Monocyte chemotactic protein-3; myelin transcription factor 1; inhibitor of growth family, member 1-like; thyroid hormone receptor, alpha myosin-binding protein C, slow-type; fragile X mental retardation 2; sonic hedgehog (Drosophila) homolog; 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2; SFRS protein kinase 2; excision repair cross-complementing rodent repair deficiency; cyclin-dependent kinase 5, regulatory subunit 1 (p35); poly(A)-specific ribonuclease (deadenylation nuclease); solute

carrier family 12 (potassium/chloride transporters), member 4; Pseudogene for metallothionein; natriuretic peptide precursor A; intercellular adhesion molecule 2; apoptosis antagonizing transcription factor; similar to rat HREV107; major histocompatibility complex, class II, DP beta 1; MpV17 transgene, murine homolog, glomerulosclerosis; uroporphyrinogen decarboxylase; proteasome (prosome, macropain) 26S subunit, ATPase, 1; fms-related tyrosine kinase 3 ligand; actin, gamma 1; Protein Kinase Pitslre, Alpha, Alt. Splice 1-Feb; nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; pyruvate kinase, muscle; telomeric repeat binding factor 2; cell division cycle 2, G1 to S and G2 to M; ADP-ribosylation factor 3; NRF1 Protein; H factor (complement)-like 3; serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 6; mRNA of muscle specific gene M9; solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3; ribosomal protein L36a; suppressor of Ty (S. cerevisiae) 4 homolog 1; amino-terminal enhancer of split; ubiquitin A-52 residue ribosomal protein fusion product 1; hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase; chaperonin containing TCP1, subunit 2 (beta); tyrosine kinase with immunoglobulin and epidermal growth factor homology; domains; Fc fragment of IgG, receptor, transporter, alpha; NRD1 convertase; ADP-ribosylation factor 5; transcription elongation factor A (SII), 1; like mouse brain protein E46; titin; fibromodulin; Abl-interactor 2 (Abi-2); and other differentially expressed genes disclosed herein. In one embodiment, the differentially expressed gene includes one or more of the genes listed in Table 9. The number of differentially expressed genes analyzed in the sample can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more.

In another embodiment, the differentially expressed gene is at least one of CDKN1B, CDKN1C, CTGF, fibromodulin, and Abl-interactor 2 (Abi-2).

For modulating the expression of a gene, a method can comprise, in any effective order, one or more of the following steps, *e.g.*, contacting a gene (*e.g.*, in a cell population) with a test agent under conditions effective for the test agent to modulate the expression of the gene, and determining whether the test agent modulates the gene. An agent can modulate expression of a gene at any level, including transcription (*e.g.*, by modulating the promoter), translation, and/or perdurance of the nucleic acid (*e.g.*, degradation, stability, *etc.*) in the cell.

For modulating the biological activity of polypeptides, a method can comprise, in any effective order, one or more of the following steps, *e.g.*, contacting a polypeptide

(*e.g.*, in a cell, lysate, or isolated) with a test agent under conditions effective for the test agent to modulate the biological activity of the polypeptide, and determining whether the test agent modulates the biological activity.

Contacting the gene or polypeptide with the test agent can be accomplished by any suitable method and/or means that places the agent in a position to functionally control expression or biological activity of the gene or its product in the sample. Functional control indicates that the agent can exert its physiological effect through whatever mechanism it works. The choice of the method and/or means can depend upon the nature of the agent and the condition and type of environment in which the gene or its product is presented, *e.g.*, lysate, isolated, or in a cell population (such as, *in vivo*, *in vitro*, organ explants, *etc.*). For instance, if the cell population is an *in vitro* cell culture, the agent can be contacted with the cells by adding it directly into the culture medium. If the agent cannot dissolve readily in an aqueous medium, it can be incorporated into liposomes, or another lipophilic carrier, and then administered to the cell culture. Contact can also be facilitated by incorporation of agent with carriers and delivery molecules and complexes, by injection, by infusion, *etc.*

Agents can be directed to, or targeted to, any part of the polypeptide that is effective for modulating it. For example, agents, such as antibodies and small molecules, can be targeted to cell-surface, exposed, extracellular, ligand binding, functional, *etc.*, domains of the polypeptide. Agents can also be directed to intracellular regions and domains, *e.g.*, regions where the polypeptide couples or interacts with intracellular or intramembrane binding partners.

After the agent has been administered in such a way that it can gain access to the gene or gene product (including DNA, mRNA, and polypeptides), it can be determined whether the test agent modulates its expression or biological activity. Modulation can be of any type, quality, or quantity, *e.g.*, increase, facilitate, enhance, up-regulate, stimulate, activate, amplify, augment, induce, decrease, down-regulate, diminish, lessen, reduce, *etc.* The modulatory quantity can also encompass any value, *e.g.*, 1%, 5%, 10%, 50%, 75%, 1-fold, 2-fold, 5-fold, 10-fold, 100-fold, *etc.* To modulate gene expression means, *e.g.*, that the test agent has an effect on its expression, *e.g.*, to effect the amount of transcription, to effect RNA splicing, to effect translation of the RNA into polypeptide, to effect RNA or polypeptide stability, to effect polyadenylation or other processing of the RNA, to effect post-transcriptional or post-translational processing, *etc.* To modulate

biological activity means, *e.g.*, that a functional activity of the polypeptide is changed in comparison to its normal activity in the absence of the agent. This effect includes, increase, decrease, block, inhibit, enhance, *etc.*

A test agent can be of any molecular composition, *e.g.*, chemical compounds, biomolecules, such as polypeptides, lipids, nucleic acids (*e.g.*, antisense, siRNA, or ribozyme targeted to a polynucleotide), carbohydrates, antibodies, ribozymes, double-stranded RNA, aptamers, *etc.* For example, if a polypeptide to be modulated is a cell-surface molecule, a test agent can be an antibody that specifically recognizes it and, *e.g.*, causes the polypeptide to be internalized, leading to its down regulation on the surface of the cell. Such an effect does not have to be permanent, but can require the presence of the antibody to continue the down-regulatory effect. Antibodies can also be used to modulate the biological activity of a polypeptide in a lysate or other cell-free form.

The present invention also relates to methods of identifying modulators of a gene, differentially-expressed in fibrotic tissue or during fibrogenesis, in a cell population capable of forming fibrotic tissue, comprising, one or more of the following steps in any effective order, *e.g.*, contacting the cell population with a test agent under conditions effective for the test agent to modulate a differentially-expressed gene disclosed herein, or a polypeptide thereof. These methods are useful, *e.g.*, for drug discovery in identifying and confirming the pro-fibrotic or anti-fibrotic activity of agents, for identifying molecules in the normal pathway of fibrogenesis, *etc.*

Any cell population capable of forming (contributing to) fibrotic tissue can be utilized. Cells can include, *e.g.*, endothelial, epithelial, muscle, embryonic and adult stem cells, ectodermal, mesenchymal, endodermal, neoplastic, *etc.* The phrase "capable of forming fibrotic tissue" does not indicate a particular cell-type, but simply that the cells in the population are able under appropriate conditions to form or contribute to fibrotic tissue structure. In some circumstances, the population may be heterogeneous, comprising more than one cell-type, only some which actually form fibrotic tissue, but others which are necessary to initiate, maintain, *etc.*, the process of fibrogenesis.

The cell population can be contacted with the test agent in any manner and under any conditions suitable for it to exert an effect on the cells, and to modulate the differentially-expressed gene or polypeptide. The means by which the test agent is delivered to the cells may depend upon the type of test agent, *e.g.*, its chemical nature, and the nature of the cell population. Generally, a test agent must have access to the cell

population, so it must be delivered in a form (or pro-form) that the population can experience physiologically, *i.e.*, to put in contact with the cells. For instance, if the intent is for the agent to enter the cell, if necessary, it can be associated with any means that facilitate or enhance cell penetrance, *e.g.*, associated with antibodies or other reagents specific for cell-surface antigens, liposomes, lipids, chelating agents, targeting moieties, *etc.* Cells can also be treated, manipulated, *etc.*, to enhance delivery, *e.g.*, by electroporation, pressure variation, *etc.*

A purpose of administering or delivering the test agents to cells capable of forming blood vessels is to determine whether they modulate a gene that is differentially expressed in fibrotic tissue, such as those disclosed herein. By the phrase "modulate," it is meant that the gene or polypeptide affects the polypeptide or gene in some way. Modulation includes effects on transcription, RNA splicing, RNA editing, transcript stability and turnover, translation, polypeptide activity, and, in general, any process involved in the expression and production of the gene and gene product. The modulatory activity can be in any direction, and in any amount, including, up, down, enhance, increase, stimulate, activate, induce, turn on, turn off, decrease, block, inhibit, suppress, prevent, *etc.*

Any type of test agent can be used, comprising any material, such as chemical compounds, biomolecules, such as polypeptides (including polypeptide fragments and mimics), lipids, nucleic acids (such as short interfering RNA (siRNA), antisense, or ribozymes), carbohydrates, antibodics, small molecules, fusion proteins, etc. Test agents can include, *e.g.*, protamine, heparins, steroids, angiostatins, triazines, endostatins, cytokines, chemokines, FGFs, *etc.* The agent can be one based on a pyrazolopyridine scaffold (Beight, D.W. *et al.*, WO 2004/026871), a pyrazole scaffold (Gellibert, F. *et al.*, *J. Med. Chem.*, 2004, 47:4494-4506), an imidazopyridine scaffold (Lee, W.C. *et al.*, Wo 2004/021989), triazole scaffold (Blumberg, L.C. *et al.*, WO 2004/026307), a pyridopyrimidine scaffold (Chakravarty, S. *et al.*, WO 2000/012497), or an isothiazole scaffold (Munchhof, M.J., WO 2004/147574), for example.

Whether the test agent modulates a differentially expressed gene or polypeptide encoded by a differentially expressed gene can be determined by any suitable method. These methods include, detecting gene transcription, detecting mRNA, detecting polypeptide and activity thereof. The detection methods include those mentioned herein, *e.g.*, PCR, RT-PCR, Northern blot, ELISA, Western, RIA, *etc.* In addition to detecting

nucleic acid and polypeptide, further downstream targets can be used to assess the effects of modulators, including, the presence or absence of TGF-beta receptor signal transduction (*e.g.*, TGF-beta II receptor signal transduction) as modulated by a test agent.

The method for identifying modulators of differentially expressed genes or polypeptides encoded by differentially expressed genes can include the additional step of evaluating the effects of the test agent on an animal model of fibrosis. The use of an animal model can be used before, during, or after a test agent has been identified as a modulator of a differentially expressed gene or polypeptide encoded by a differentially expressed gene in accordance with the present invention. Animal models that are genetically susceptible to the development of tumors may be used. For example, the Eker rat carries a mutation in the tuberous sclerosis 2 (Tsc-2) tumor suppressor gene and is predisposed to the development of tumors of the digestive tract (renal cell carcinomas) and reproductive tract (uterine leiomyomas) (Everitt J.I. *et al.*, *American Journal of Pathology*, 1995, 146:1556-1567; Hunter D.S. *et al.*, *Cancer Research*, 59:3090-3099; Walker C.L. *et al.*, *Genes Chromosomes Cancers*, 2003, 38(4):349-356; Everitt J.I. *et al.*, *Toxicol. Lett.*, 1995, 82-83:621-625; Yoon H. *et al.*, *Am. J. Physiol. Renal. Physiol.*, 2002, 283:F262-F270; Everitt J.I. *et al.*, *American Journal of Pathology*, 1995, 146:1556-1567; each of which is incorporated herein by reference in its entirety). Because of their inherited susceptibility to tumor development, Eker rats are an excellent model system for studying the effects of chemical carcinogens on predisposed individuals and for identifying the mechanisms by which chemical carcinogens interact with tumor susceptibility genes. In addition to being useful for studying the effects of carcinogens on tumor susceptibility genes, animal models in which spontaneous tumors occur at a high frequency are also useful in preclinical studies conducted to identify agents that may be used to prevent or treat fibrosis. Thus, test agents may be administered to rats carrying the Eker mutation or other animal model to determine if the test agent is capable of preventing or reducing the growth of fibrotic tissue, such as fibrotic tissue of the uterus.

In another aspect, the invention concerns an array, such as a gene array, including a substrate (such as a solid support) having a plurality of addresses (such as wells), wherein each address disposed thereon has a capture probe that can specifically bind at least one polynucleotide that is differentially expressed in fibrotic disorders, or a complement thereof. In one embodiment, the at least one polynucleotide is selected from the group consisting of docking protein 1, 62 kD (downstream of tyrosine kinase 1);

centromere protein A (17 kD); catenin (cadherin-associated protein), beta 1 (88 kD); nuclear receptor subfamily 1, group I, member 2; v-rel avian reticuloendotheliosis viral oncogene homolog A; LGN Protein; CDC28 protein kinase 1; hypothetical protein; solute carrier family 17 (sodium phosphate), member 1; FOS-like antigen-1; nuclear matrix protein p84; LERK-6 (EPLG6); visinin-like 1; phosphodiesterase 10A; KH-type splicing regulatory protein (FUSE binding protein 2); Polyposis locus (DP1 gene) mRNA; microtubule-associated protein 2; CDC5 (cell division cycle 5, S pombe, homolog)-like; Centromere autoantigen C (CENPC) mRNA; RNA guanylyltransferase and 5'-phosphatase; Nijmegen breakage syndrome 1 (nibrin); ribonuclease, RNase A family, 4; keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris); basic helix-loop-helix domain containing, class B, 2; dual specificity phosphatase 1; annexin A11; putative receptor protein; Human endogenous retrovirus HERV-K(HML6); mitogen-activated protein kinase kinase kinase 12; TXK tyrosine kinase; kynureninase (L-kynurenine hydrolase); ubiquitin specific protease 4 (proto-oncogene); peroxisome biogenesis factor 13; olfactory receptor, family 2, subfamily F, member 1; membrane protein, palmitoylated 3 (MAGUK p55 subfamily member 3); origin recognition complex, subunit 1 (yeast homolog)-like; dTDP-D-glucose 4,6-dehydratase; cytochrome c oxidase subunit VIa polypeptide 2; gamma-tubulin complex protein 2; Monocyte chemotactic protein-3; myelin transcription factor 1; inhibitor of growth family, member 1-like; thyroid hormone receptor, alpha myosin-binding protein C, slow-type; fragile X mental retardation 2; sonic hedgehog (Drosophila) homolog; 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2; SFRS protein kinase 2; excision repair cross-complementing rodent repair deficiency; cyclin-dependent kinase 5, regulatory subunit 1 (p35); poly(A)-specific ribonuclease (deadenylation nuclease); solute carrier family 12 (potassium/chloride transporters), member 4; Pseudogene for metallothionein; natriuretic peptide precursor A; intercellular adhesion molecule 2; apoptosis antagonizing transcription factor; similar to rat HREV107; major histocompatibility complex, class II, DP beta 1; MpV17 transgene, murine homolog, glomerulosclerosis; uroporphyrinogen decarboxylase; proteasome (prosome, macropain) 26S subunit, ATPase, 1; fms-related tyrosine kinase 3 ligand; actin, gamma 1; Protein Kinase Pitslre, Alpha, Alt. Splice 1-Feb; nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; pyruvate kinase, muscle; telomeric repeat binding factor 2; cell division cycle 2, G1 to S and G2 to M; ADP-ribosylation factor 3; NRF1 Protein; H factor (complement)-like 3;

serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 6; mRNA of muscle specific gene M9; solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3; ribosomal protein L36a; suppressor of Ty (S. cerevisiae) 4 homolog 1; amino-terminal enhancer of split; ubiquitin A-52 residue ribosomal protein fusion product 1; hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase; chaperonin containing TCP1, subunit 2 (beta); tyrosine kinase with immunoglobulin and epidermal growth factor homology; domains; Fc fragment of IgG, receptor, transporter, alpha; NRD1 convertase; ADP-ribosylation factor 5; transcription elongation factor A (SII), 1; like mouse brain protein E46; titin; fibromodulin; and Abi-interactor 2 (Abi-2).

In another embodiment of the array, the at least one polynucleotide includes at least one gene selected from the group consisting of CDKN1B, CDKN1C, CTGF, fibromodulin, and Abi-2.

In another embodiment of the array, the at least one polynucleotide includes at least one gene selected from the group consisting of IL-11, IL-13, EGR1, EGR2, EGR3, CITED2, P300, E2F1, E2F2, E2F3, E2F4, E2F5, MCP3, CXCL5, CCL7, SMAD3, TYMS, GT198, SMAD7, NCOR2, TIMP-1, and ADAM17.

In another embodiment of the array, the at least one polynucleotide includes at least one of those genes listed in Table 9.

In another embodiment of the array, the at least one polynucleotide includes at least one gene selected from the group consisting of stanniocalcin 2, interleukin 11, disintegrin and metalloproteinase domain 17, early growth response 3, fibromodulin, collagen type XVIII alpha 1, and interleukin 13.

In another embodiment of the array, the at least one polynucleotide includes a plurality of genes comprising stanniocalcin 2, interleukin 11, disintegrin and metalloproteinase domain 17, early growth response 3, fibromodulin, collagen type XVIII alpha 1, and interleukin 13.

In another embodiment of the array, the array further comprises a capture probe that can specifically bind at least one polynucleotide encoding a house-keeping gene as a control.

In another embodiment of the array, each of the addresses comprises a well, and each of the capture probes comprises a primer for amplifying RNA in a biological sample that is deposited in the well

In one embodiment, the capture probes are polynucleotides that hybridize to the differentially expressed polynucleotides under stringent conditions or mild conditions. In another embodiment of the array, each of the capture probes binds the polynucleotides (*e.g.*, hybridizes with the polynucleotide along the full length of the polynucleotide or along substantially the full length of the polynucleotide) under stringent conditions. As used herein "stringent" conditions for hybridization refers to conditions which achieve the same, or about the same, degree of specificity of hybridization as the conditions employed by the current applicants. Specifically, hybridization of immobilized DNA on Southern blots with 32P-labeled gene-specific probes was performed by standard methods (Maniatis, T., E. F. Fritsch, J. Sambrook [1982] Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). In general, hybridization and subsequent washes are carried out under stringent conditions that allow for hybridization of target sequences with homology to the capture probes. For double-stranded DNA gene probes, hybridization was carried out overnight at 20-25 °C. below the melting temperature (Tm) of the DNA hybrid in 6 x SSPE, 5X Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. The melting temperature is described by the following formula (Beltz, G. A., K. A. Jacobs, T. H. Eickbush, P. T. Cherbas, and F. C. Kafatos, Methods of Enzymology, 1983, R. Wu, L. Grossman and K. Moldave [eds.] Academic Press, New York 100:266-285).

Tm=81.5 °C+16.6 Log[Na+]+0.41(% G+C)-0.61(% formamide)-600/length of duplex in base pairs.

Washes are typically carried out as follows:

(1) Twice at room temperature for 15 minutes in 1 x SSPE, 0.1% SDS (low stringency wash).

(2) Once at Tm-20 °C. for 15 minutes in 0.2 x SSPE, 0.1% SDS (moderate stringency wash).

For oligonucleotide probes, hybridization was carried out overnight at 10-20 °C. below the melting temperature (Tm) of the hybrid in 6 x SSPE, 5 x Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. Tm for oligonucleotide probes can be determined by the "nearest-neighbor" method. See Breslauer *et al.*, "Predicting DNA duplex stability from the base sequence," *Proc. Natl. Acad. Sci. USA*, 83 (11): 3746-3750 (June 1986); Rychlik and Rhoads, "A computer program for choosing optimal oligonucleotides for filter hybridization, sequencing and *in vitro* amplification of DNA," *Nucleic Acids Res.*,

17 (21): 8543-8551 (Nov. 11, 1989); Santa Lucia *et al.*, "Improved nearest-neighbor parameters for predicting DNA duplex stability," *Biochemistry* 35 (11): 3555-3562 (Mar. 19, 1996); Doktycz *et al.*, "Optical melting of 128 octamer DNA duplexes. Effects of base pair location and nearest neighbors on thermal stability," *J. Biol. Chem.*, 270 (15): 8439-8445 (Apr. 14, 1995). Alternatively, the Tm can be determined by the following formula:

Tm (°C)=2(number T/A base pairs)+4(number G/C base pairs) (Suggs, S. V., T. Miyake, E. H. Kawashime, M. J. Johnson, K. Itakura, and R. B. Wallace [1981] ICN-UCLA *Symp. Dev. Biol. Using Purified Genes*, D. D. Brown [ed.], Academic Press, New York, 23:683-693).

Washes are typically carried out as follows:

(1) Twice at room temperature for 15 minutes 1 x SSPE, 0.1% SDS (low stringency wash).

(2) Once at the hybridization temperature for 15 minutes in 1 x SSPE, 0.1% SDS (moderate stringency wash).

In another embodiment of the array, each polynucleotide bound by the capture probe of each address is unique among the plurality of addresses.

In another embodiment of the array, the substrate has no more than 500 addresses. In another embodiment of the array, the substrate has 200 to 500 addresses.

The substrate of the array of the invention can be any solid support suitable for disposing the capture probes, such as those materials known in the art used for fabrication of gene arrays and/or microfluidics. "Arraying" refers to the act of organizing or arranging members of a library, or other collection, into a logical or physical array. Thus, an "array" refers to a physical or logical arrangement of, *e.g.*, library members (candidate agent libraries). A physical array can be any "spatial format" or physically gridded format" in which physical manifestations of corresponding library members are arranged in an ordered manner, lending itself to combinatorial screening. For example, samples corresponding to individual or pooled members of a candidate agent library or patient library can be arranged in a series of numbered rows and columns, *e.g.*, on a multiwell plate. Similarly, capture probes can be plated or immobilized (in a lyophilized or other state) or otherwise deposited in microtitered, *e.g.*, 96-well, 384-well, or-1536 well, plates (or trays).

A "solid support" (also referred to herein as a "solid substrate") has a fixed organizational support matrix that preferably functions as an organization matrix, such as a microtiter tray. Solid support materials include, but are not limited to, glass, polacryloylmorpholide, silica, controlled pore glass (CPG), polystyrene, polystyrene/latex, polyethylene, polyamide, carboxyl modified teflon, nylon and nitrocellulose and metals and alloys such as gold, platinum and palladium. The solid support can be biological, non-biological, organic, inorganic, or a combination of any of these, existing as particles, strands, precipitates, gels, sheets, tubing, spheres, containers, capillaries, pads, slices, films, plates, slides, *etc.*, depending upon the particular application. Other suitable solid substrate materials will be readily apparent to those of skill in the art. The surface of the solid substrate may contain reactive groups, such as carboxyl, amino, hydroxyl, thiol, or the like for the attachement of nucleic acids, proteins, *etc.* Surfaces on the solid substrate will sometimes, though not always, be composed of the same material as the substrate. Thus, the surface can be composed of any of a wide variety of materials, for example, polymers, plastics, resins, polysaccharides, silica or silica-based materials, carbon, metals, inorganic glasses, membranes, or any of the above-listed substrate materials.

In addition to standard gene arrays, such as the commercially available gene arrays described herein, micro fluidic cards (*e.g.*, 7900 HT Micro Fluidic Card, APPLIED BIOSYSTEMS) may be used to profile gene expression using the comparative C_T method of relative quantification. Such cards are also contemplated in the arrays of the present invention. Microfluidic card experiments use a two-step RT-PCR process. In the reverse-transcription (RT) step, cDNA is reverse transcribed from total RNA samples using random primers from the high capacity cDNA archive kit. Additional details about the RT-PCR process are contained in the high capacity cDNA archive kit protocol (PN 4322169). In the PCR step, PCR products are synthesized from cDNA samples using the TAQMAN universal PCR master mix. The PCR step employs the 5' nuclease assay, which is described in Appendix C of the user's guide for the 7900HT system. Relative gene expression values can be obtained from 7900HT system data using the comparative C_T method for relative quantification. In the comparative C_T method, quantity is expressed relative to a calibrator sample that is used as the basis for comparative results (see Applied Biosystems 7900HT Micro Fluidic Card Getting Started Guide, APPLIED BIOSYSTEMS, which is incorporated herein by reference in its entirety). Real-time

quantitative gene expression results are available as soon as the thermal cycling process is complete.

All wells on the card are connected by a series of channels, and assays are loaded at the factory before shipping. The biological sample is combined with TAQMAN Universal PCR Master Mix and loaded into the card ports. The card may contain any number of wells, such as 96, 192, 384, 500, 1000, *etc.* Real-time performance can be obtained by using a micro fluidic card in a high throughput 384-well format, 2 microliter reaction volume, and eight loading ports. Briefly, sample (*e.g.*, isolated RNA) is loaded into the micro fluidic card, the card is centrifuged to transfer mixes into the individual wells, and the card is sealed using a sealing device which individually seals each well to avoid diffusion and cross-talk. The sealed card is then ready for real-time PCR. The fill reservoirs are trimmed and the card is loaded on the 7900HT system for real-time PCR. The 384 well format provides configuration flexibility. For example, using one sample per micro fluidic card, 384 genes with single data points, or 96 genes with 4 replicates may be assayed. Using eight samples per micro fluidic card, 48 genes with single data points, 24 genes with 2 replicates, or 12 genes with 4 replicates may be assayed. Isolated RNA from tumor tissues, normal tissues, or cells can be injected into the card. The card can be divided into normal tissue and tumor tissue, for example. Using a 384 well format, 48 genes of four individuals (human or non-human animal subjects) with normal tissue and tumor tissue can be assayed.

The effects of test agents, such as TGF-beta receptor inhibitors (*e.g.*, SB505124/SB431542), TGF-beta signaling inhibitors (halofuginone), and potential environment carcinogens or gene express can be determined using the method of the invention.

For differential expression analysis, it is preferable to include at least one house-keeping gene (as a control gene) whose expression should not change, such as GAPD (GenBank accession number NM_002046), or other house-keeping genes described herein.

Table 9 lists genes that may be used on a micro fluidic card in accordance with the subject invention. For example, one or more genes from each category listed in Table 9 can be assayed for differential expression (*e.g.*, cell adhesion molecule, extracellular matrix, kinase, oxidoreductase, protease, signaling molecule, transcription factor).

Optionally, once a test agent is identified as a modulator, the method of the invention may further include the step of manufacturing the identified modulator. The manufacturing step may involve synthesis of the modulator (*e.g.*, if a small molecule) or genetic engineering, for example. Optionally, the manufacturing step may further comprise combining the manufactured modulator with another active substance and/or a pharmaceutically acceptable carrier or excipient, as a formulated composition.

As used herein, the terms "bind," "binds," or "interacts with" mean that one molecule recognizes and adheres to a particular second molecule in a sample, but does not substantially recognize or adhere to other structurally unrelated molecules in the sample. Generally, a first molecule that "specifically binds" a second molecule has a binding affinity greater than about 10^5 to 10^6 moles/liter for that second molecule.

By reference to an "antibody that specifically binds" another molecule is meant an antibody that binds the other molecule, and displays no substantial binding to other naturally occurring proteins other than those sharing the same antigenic determinants as other molecule. The term "antibody" includes polyclonal and monoclonal antibodies as well as antibody fragments or portions of immunolglobulin molecules that can specifically bind the same antigen as the intact antibody molecule.

As used herein, a "nucleic acid," "nucleic acid molecule," "oligonucleotide," or "polynucleotide" means a chain of two or more nucleotides such as RNA (ribonucleic acid) and DNA (deoxyribonucleic acid).

The term "subject," as used herein, means a human or non-human animal, including but not limited to mammals, such as a dog, cat, horse, cow, pig, sheep, goat, chicken, primate, rat, and mouse. In a preferred embodiment, the subject is female, such as a human female.

The term "differentially expressed gene", as used herein, means a gene that is either over-expressed or underexpressed in fibrotic tissue (such as leiomyoma), compared to normal, non-fibrotic tissue. Accordingly, the method of treatment of the present invention is directed to upregulating the expression of one or more genes that are underexpressed in fibrotic tissue, or increasing the activity of the polypeptide encoded by the gene; and downregulating the expression of one or more genes that are overexpressed in fibrotic tissue, or decreasing the activity of the polypeptide encoded by the gene.

When referring to a differentially expressed gene, the phrase "modulates the expression of" means upregulates or downregulates the amount or functional activity of

the gene, or otherwise modifies the activity of the gene product, *e.g.*, the availability of the gene product to interact with a receptor.

The terms, "treat", "treatment", and "treating", as used herein, are intended to include the prevention of a fibrotic disorder and partial or full alleviation of an existing fibrotic disorder within a human or non-human animal subject (*e.g.*, a reduction in the severity of one or more symptoms associated with the fibrotic disorder). For example, treating a fibroid, such as a uterine fibroid, can include a reduction in the size of the fibroid and/or a reduction in the rate of the fibroid's growth.

Materials and Methods

The following materials and methods describe those utilized in Examples 1-8.

Tissues. Portions of leiomyoma and matched myometrium were collected from premenopausal women (N=6) who were scheduled to undergo hysterectomy for indications related to symptomatic leiomyomas. Three of the patients received GnRHa therapy for three months prior to surgery. The untreated patients did not receive any medications (including hormonal therapy) during the previous 3 months prior to surgery, and based on endometrial histology and the patient's last menstrual period they were from early-mid secretory phase of the menstrual cycle. To maintain a standard, all leiomyomas selected for this study were between 2 to 3 cm in diameter. Following collection, the tissues were divided into several pieces and either immediately snap frozen and stored in liquid nitrogen for further processing, fixed and paraffin embedded for histological evaluation and immunohistochemistry, or used for isolation of leiomyoma and myometrial smooth muscle cells and culturing (Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557; Xu, J *et al. J Clin Endocrinol Metab*, 2003, 88:1350-61). The tissues were collected at the University of Florida affiliated Shands Hospital with prior approval obtained from the Institutional Review Board.

Isolation and Culture of Leiomyoma and Myometrial Smooth Muscle Cells. To determine the direct action of GnRHa on global gene expression in leiomyoma and myometrial smooth muscle cells (LSMC and MSMC), the cells were isolated and cultured as previously described (Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557; Chegini, N *et al. Mol Hum Reprod*, 2002, 8:1071-8). Only untreated tissues were used for isolation of LSMC and MSMC. Prior to use in these experiments, the primary cell cultures were seeded in 8-well culture slides (Nalge Nunc, Naperville, IL) and after

24 hours of culturing they were characterized using immunofluroscence microscopy and antibodies to α smooth muscle actin, desmin and vimentin (Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557; Xu, J *et al. J Clin Endocrinol Metab*, 2003, 88:1350-61). LSMC and MSMC were cultured in 6-well plates at an approximate density of 10^6 cells/well in DMEM-supplemented media containing 10% FBS. After reaching visual confluence, the cells were washed in serum-free media and incubated for 24hrs under serum-free, phenol red-free condition (Chegini, N *et al. Mol Hum Reprod*, 2002, 8:1071-8). The cells were then treated with 0.1 μM of GnRHa (leuprolide acetate, Sigma Chemical, St Louis, MO) for a period of 2, 6 and 12 hours (Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557).

cDNA Microarray and Gene Expression Profiling. Total cellular RNA was isolated from the tissues and cells using Trizol (INVITROGEN, Carlsbad, CA). The isolated RNA was treated with DNase I (Roche, Molecular Biochemicals, Indianaplis, IN) at 1 unit/10 μg of RNA for 20 min at 25°C, heat-inactivated at 75°C and subjected to further purification using RNeasy Kit (QIAGEN, Valencia, CA). The RNA was then subject to amplification by reverse transcription using SuperScript Choice system (Invitrogen), with final concentrations in 20 μl first-strand reaction of 100 pmol of high performance liquid chromatography-purified T7-(dT)24 primer (Genset Corp, La Jolla, CA.), 8 μg of RNA, 1× first-strand buffer, 10 mM dithiothreitol, 500μM of each dNTP, and 400 units of Superscript II reverse transcriptase (T7 Megascript kit; Ambion, Austin, TX). The second-strand cDNA synthesis was performed in a 150 μl reaction consisting of, at the final concentrations, 1× second-strand reaction buffer, 200 μM each dNTP, 10 units of DNA ligase, 40 units of DNA polymerase I, and 2 units of RNase H (INVITROGEN), and double-stranded cDNA was purified by phenol:chloroform extraction using phase lock gels (Eppendorf-5 Prime, Inc. Westbury, NY) and an ethanol precipitation (Chegini, N *et al. J Soc Gynecol Investig*, 2003, 10:161-71).

Five micrograms of purified cDNA was reverse transcribed using Enzo BioArray high yield RNA transcript labeling kit (AFFYMETRIX, Santa Clara, CA) and the product was purified in RNeasy spin columns (QIAGEN) according to manufacture's instructions. Following an overnight ethanol precipitation, cRNA was re-suspended in 15 μl of diethyl pyrocarbonate-treated water (AMBION) and quantified using a Beckman DU530 Life Science UV-visible spectrophotometer. Following quantification of cRNA to reflect any carryover of unlabeled total RNA according to an equation given by Affymetrix (adjusted

cRNA yield = cRNA (μg) measured after in vitro transcription (starting total RNA) (fraction of cDNA reaction used in in vitro transcription), 20μg of cRNA was fragmented (0.5μg/μl) according to Affymetrix instructions using the 5× fragmentation buffer containing 200 mM Tris acetate, pH 8.1, 500 mM potassium acetate and 150 mM magnesium acetate (SIGMA Chemical, St. Louis, MO). 20 μg of the adjusted fragmented cRNA was added to a 300 μl of hybridization mixture containing at final concentrations 0.1 mg/ml herring sperm DNA (Promega/Fisher, Madison, WI), 0.5 mg/ml acetylated bovine serum albumin (INVITROGEN), and 2× MES hybridization buffer (Sigma). 200 μl of the mixture was hybridized to the human U95A Affymetrix GeneChip arrays, purchased at the same time from the same lot number and used within two weeks of purchase in order to maintain standard. In addition, an aliquot of random samples were first hybridized to an Affymetrix Test 2 Array to determine sample quality according to manufacturer's criteria. After meeting recommended criteria for use of the expression arrays, the hybridization was performed for 16 hrs at 45°C, followed by washing, staining, signal amplification with biotinylated anti-strepavidin antibody, and the final staining step according to manufactures protocol.

Microarray Data Analysis. The Chips were scanned to obtain the raw hybridization values using Affymetrix Genepix 5000A scanner. Difference in the levels of fluorescence spot intensities representing the rate of hybridization between the 25 basepair oligonucleotides and their mismatches were analyzed by multiple decision matrices to determine the presence or absence of gene expression, and to derive an average difference score representing the relative level of gene expression. The fluorescence spot intensities, qualities and local background were assessed automatically by Genepix software with a manual supervision to detect any inaccuracies in automated spot detection. Background and noise corrections were made to account for nonspecific hybridization and minor variations in hybridization conditions. The net hybridization values for each array were normalized using a global normalization method as previously described (Chegini, N *et al. J Soc Gynecol Investig*, 2003, 10:161-71). To identify the changes in pattern of gene expression, the average and standard deviation (SD) of the globally normalized values were calculated followed by subtraction of the mean value from each observation and division by the SD. The mean transformed expression value of each gene in the transformed data set was set at 0 and the SD at 1 (Chegini, N *et al. J Soc Gynecol Investig*, 2003, 10:161-71).

The transformed gene expression values were subjected to Affymetrix Analysis Suite V 5.0. Briefly, probe sets that were flagged as absent on all arrays using default settings were removed from the datasets. After application of this filtering, the dataset was reduced from 12,625 probe sets to 8580 probe sets. The gene expression value of the remaining probe sets was then subjected to unsupervised and supervised learning, discrimination analysis, and cross validation (Eisen, MB *et al. Proc Natl Acad Sci USA*, 1998, 95:14863-14868; Varela, JC *et al. Invest Ophthalmol Vis Sci*, 2002, 43:1772-1782; Tusher, VG *et al. Proc Natl Acad Sci USA*, 2001, 98:5116-5121; Pavlidis, P *Methods*, 2003, 31:282-289; Peterson, LE *Comput Methods Programs Biomed*, 2003, 70:107-19; Butte, A *Nat Rev Drug Discov*, 2002, 1:951-960). After variation filtering, the coefficient of variation was calculated for each probe set across all chips and the probe sets were ranked by the coefficient of variation of the observed single intensities. The expression values of the selected genes were then subjected to R programming analysis that assesses multiple test correction to identify statistically significant gene expression values (Pavlidis, P *Methods*, 2003, 31:282-289; Peterson, LE *Comput Methods Programs Biomed*, 2003, 70:107-19; Butte, A *Nat Rev Drug Discov*, 2002, 1:951-960). The gene expression values having a statistical significance of $p \leq 0.02$ (ANOVA, Tukey test) between leiomyoma and myometrium from GnRH-treated and untreated cohorts, and $p \leq 0.005$ between GnRHa-treated and untreated cells (control) were selected. The validity of gene sets identified at these p values in predicting treatment class was established using "leave-one-out" cross validation where the data from one array was left out of the training set and probe sets with differential hybridization signal intensities were identified from the remaining arrays (Varela, JC *et al. Invest Ophthalmol Vis Sci*, 2002, 43:1772-1782; Butte, A *Nat Rev Drug Discov*, 2002, 1:951-960). Hierarchical clustering and K-means analysis was performed and viewed with the algorithms in the software packages Cluster and TreeView (Eisen, MB *et al. Proc Natl Acad Sci USA*, 1998, 95:14863-14868).

Gene Classification and Ontology Assessment. The selected differentially expressed and regulated genes in the above cohorts were subjected to functional annotation and visualization using Database for Annotation, Visualization, and Integrated Discovery (DAVID) software (Dennis G Jr. *et al.*, DAVID: Database for Annotation, Visualization, and Integrated Discovery, Genome Biology, 2003; 4(5):P3; Hosack D.A. *et al.*, Glynn Dennis Jr, Brad T Sherman, HClifford Lane, Richard A Lempicki. Identifying

Biological Themes within Lists of Genes with EASE, Genome Biology, 2003, 4(6):P4). The integrated GoCharts assigns genes to specific ontology functional categories based on selected classifications, KeggCharts assigns genes to KEGG metabolic processes and context of biochemical pathway maps, and DomainCharts assigning genes according to PFAM conserved protein domains.

Quantitative RealTime PCR. Realtime PCR was utilized for verification of 10 differentially expressed and regulated genes identified in leiomyoma and myometrium as well as LSMC and MSMC from untreated and GnRHa-treated cohorts. The selection of these genes was based not only on their expression values (up or downregulation), but classification and biological functions important to leiomyoma growth and regression, regulation by ovarian steroids, GnRHa and TGF-β. They are IL-11, CITED2, Nur77, EGR3, TGIF, TIEG, p27, p57, GAS-1 and GPRK5 representing cytokines, transcription factors, cell cycle regulators and signal transduction. Realtime PCR was carried out as previously described using Taqman and ABI-Prism 7700 Sequence System and Sequence Detection System 1.6 software (Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557). Results were analyzed using the comparative method and following normalization of expression values to the 18S rRNA expression according to the manufacturer's guidelines (Applied Biosystems) as previously described (Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557).

Western Blot Analysis and Immunohistochemical Localization. For immunoblotting, total protein was isolated from small portions of GnRHa-treated and untreated leiomyoma and myometrium as well as the GnRHa-treated and untreated cells as previously described (Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557; Chegini, N *et al. Mol Cell Endocrinol*, 2003, 209:9-16). Following determination of the tissue homogenates and cell lysates protein content an equal amount of sample proteins were subjected to SDS-PAGE and transferred to polyvinyldiene difluoride (PVDF) membrane. The blots were incubated with anti-TIEG antibody, kindly provided by Dr. Thomas Spelsberg, Department of Biochemistry, Mayo Clinic, Rochester, MN (Johnsen, SA *et al. Oncogene*, 2002, 21:5783-90), TGIF, EGR3, p27, p57, Nur77 and Gas1 antibodies purchased from Santa Cruz Biochemical (Santa Cruz, CA), IL-11 antibodies purchased from R & D system (Minneapolis, MN) for 1 hr at room temperature. The membranes were washed, exposed to corresponding HRP-conjugated IgG for 1 hr and immunostained proteins were visualized using enhanced chemiluminesence reagents

(Amersham-Pharmacia Biotech, Piscataway, NJ) as previously described (Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557; Chegini, N *et al. Mol Cell Endocrinol*, 2003, 209:9-16; Xu, J *et al. J Clin Endocrinol Metab*, 2003, 88:1350-61).

For immunohistochemical localization, tissue sections were prepared from formalin-fixed and paraffin embedded leiomyoma and myometrium. Tissue sections were microwave prior to immunostaining using antibodies to IL-11, TGIF, TIEG, EGR3, Nur77, p27, p57 and Gas1. The antibodies were used at concentrations of 5 μg of IgG/ml for 2-3 hours at room temperature. Following further processing including incubation with biotinylated secondary antibodies and avidin-conjugated HRP (ABC ELITE kit, VECTOR Laboratories, Burlingame, CA), the chromogenic reaction was detected with 3,3'-diaminobenzidine tetrahydrochloride solution. In some instances the slides were counter stained with hematoxylin. Omission of primary antibodies or incubation of tissue sections with non-immune mouse IgG instead of primary antibodies at the same concentration during immunostaining served as controls (Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557; Chegini, N *et al. Mol Cell Endocrinol*, 2003, 209:9-16; Xu, J *et al. J Clin Endocrinol Metab*, 2003, 88:1350-61).

Determination of TGF-β1 on global gene expression in LSMC and MSMC. All the materials utilized for this study including isolation of leiomyoma and myometrial cells are identical to those described in detail above. To determine the effect of TGF-β1 on global gene expression in LSMC and MSMC, the cells were cultured in 6-well plates at approximate density of 10^6 cells/well in DMEM-supplemented media containing 10% FBS. After reaching visual confluence the cells were washed in serum-free media and incubated for 24 hrs under serum-free, phenol red-free condition (Xu, J *et al. J Clin Endocrinol Metab*, 2003, 88:1350-1361; Ding, L. *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557). The cells were then treated with 2.5 ng/ml of TGF-β1 (R & D System, Minneapolis, MI) for 2, 6 and 12 hours. To further profile the autocrine/paracrine action of TGF-β1 on gene expression in LSMC and MSMC, the cells were cultured as above and treated with 1 μM of TGF-β type II receptor antisense or sense oligonucleotides for 24 hours as previously described (Xu, J *et al. J Clin Endocrinol Metab*, 2003, 88:1350-1361; Ding, L. *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557). The cells were washed and then treated with TGF-β1 (2.5 ng/ml) for 2 hours. Parallel experiments using untreated cells were used as controls including an additional control for TGF-β type II receptor antisense and sense experiments.

Total cellular RNA was isolated from LSMC- and MSMC-treated and untreated controls and subjected to microarray analysis. To maintain standard and allow for comparative analysis, the GeneChips used in this study were utilized, simultaneously processed and their gene expression values were subjected to global normalization and transformation. Following these unsupervised assessments the coefficient of variation was calculated for each probe set across all the chips used in this study, and the selected gene expression values of this study were independently subjected to supervised learning including R programming analysis and ANOVA with false discovery rate selected at $p \leq 0.001$ (Moustakas, A. *Immunol Lett*, 2002, 82:85-91; Verrecchia, F. *et al. J Biol Chem*, 2001, 276:17058-17062). The genes identified in these cohorts were analyzed for functional annotation and visualized using Database for Annotation, Visualization, and Integrated Discovery (DAVID) software with integrated GoCharts. Following the analysis, we selected 12 of the differentially expressed and regulated genes, including 10 identified and validated in leiomyoma and myometrium from untreated and GnRHa-treated cohorts, as well as LSMC and MSMC treated *in vitro* with GnRHa, for validation in response to TGF-β-time dependent action using Realtime PCR. They include IL-11, EGR3, CITED2, TIEG, TGIF, Nur77, p27, p57, GAS-1 and GPRK5. In addition, the expression of Runx1 and Runx2, transcription factors that interact with TGF-β receptor signaling pathways (Zavadil, J. *et al. Proc Natl Acad Sci USA*, 2001, 98:6686-6691), was validated in LSMC and MSMC as well as in leiomyoma and myometrium from GnRHa-treated and untreated cohorts. Detail description of the materials and methods for Realtime PCR as well as data analysis is provided in Chegini, N. *et al. J Soc Gynecol Investig*, 2003, 10:161-71.

Example 1—Gene Expresssion Profiles in Leiomyoma and Normal Myometrium

Global gene expression profiling has been instrumental in identifying the molecular environment of tissues with respect to fingerprints of their physiological and pathological behavior, and *in vitro* cellular responses to various regulatory molecules. The present inventors used this approach and characterized the gene expression profile of leiomyoma and matched myometrium, and their transcriptional changes in response to hormonal transition induced by GnRHa therapy. The initial assessment of the gene expression values in leiomyoma, myometrium and their isolated smooth muscle cells from untreated as well as

GnRHa- and TGF-β-treated cohorts revealed a uniform expression of transcripts for the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase, α-actin and a large number of ribosomal proteins, indicating that the expression profile is consistent with established standards for gene expression analysis. Following global normalization and transformation of the gene expression values, supervised learning, discrimination analysis, cross validation and variation filtering, the gene expression values were subjected to R programming analysis and ANOVA with false discovery rate selected at $p \leq 0.02$.

Using the above analysis, the present inventors identified a total of 153 genes, including 19 EST, or 1.23% of the genes, and 122 genes including 21 EST or 0.98% of the genes on the array, as differentially expressed in leiomyoma compared to matched myometrium from untreated and GnRHa-treated tissues, respectively. Hierarchical clustering and Tree-View analysis separated the genes in each cohort into distinctive clusters with sufficient variability allowing division into their respective subgroups. Of the 153 (excluding 19 EST) differentially expressed genes in untreated cohorts, 82 were upregulated and 52 downregulated in leiomyoma compared to myometrium (Table 1). Of the 122 genes (excluding 21 EST) in leiomyoma and myometrium from patients who received GnRHa therapy, 34 transcripts were upregulated and 67 downregulated, in leiomyoma compared to myometrium, respectively (Table 2). Analysis of the variance-normalized mean (K-means) separated the differentially expressed and regulated genes in these cohorts into 4 distinctive clusters, with genes in clusters A and D displaying a tissue-specific response, while genes in cluster B and C showing regulatory response to GnRHa therapy. To further characterize the molecular environment of leiomyoma from myometrium and their response to GnRHa therapy, we compared the gene expression profiles in GnRHa-treated with corresponding untreated tissues. The analysis indicated that the expression of 170 (excluding 26 EST) and 167 (excluding 31 EST) genes are targeted by GnRHa therapy in leiomyoma and myometrium, compared to their respective untreated cohorts (Tables 3 and 4). Of these genes, 96 and 89 transcripts were downregulated in leiomyoma and myometrium, respectively, due to GnRHa therapy, compared to their respective untreated tissues, with 3 transcripts were commonly found among the tissues in these cohorts, with different regulatory pattern of expression (compare Tables 3 and 4).

Table 1 is a categorical list of differentially expressed genes identified in leiomyoma compared to matched myometrium. The genes were identified following unsupervised and supervised analysis of their expression values and subjected to R programming environment and ANOVA with a false-discovery rate of rate of $p \leq 0.02$ as described in materials and methods. Of the 153 genes identified as differentially expressed, 82 genes were up (+) and 52 genes were downregulated (-) in leiomyoma compared to myometrium excluding 19 EST.

Table 2 is a categorical list of differentially expressed genes identified in leiomyoma compared to myometrium in response to GnRHa therapy. The genes were identified following unsupervised and supervised analysis of their expression values and statistical analysis in R programming environment and ANOVA with a false-discovery rate selected at $p \leq 0.02$. Of the 122 genes identified, the expression of 34 genes was up (+) and 67 gene downregulated (-) in GnRH-treated leiomyoma (LYM) compared to myometrium (MYM) excluding 21 EST).

Table 3 is a categorical list of differentially expressed genes identified in leiomyoma from GnRHa-treated compared to untreated leiomyoma. The genes were identified following unsupervised and supervised analysis of their expression values and statistical analysis in R programming environment and ANOVA with a false-discovery rate selected at $p \leq 0.02$. Of the 170 genes identified, the expression of 74 genes was up (+) and 96 genes downregulated (-) in GnRH-treated compared to untreated leiomyoma (LMY) excluding 26 EST.

Table 4 is a categorical list of differentially expressed genes identified in myometrium from GnRHa-treated compared to untreated myometrium. The genes were identified following unsupervised and supervised analysis of their expression values and statistical analysis in R programming environment and ANOVA with a false-discovery rate selected at $p \leq 0.02$. Of the 167 genes identified, the expression of 47 genes was up (+) and 89 genes downregulated (-) in GnRH-treated compared to untreated myometrium (MYM) excluding 31 EST.

A few microarray studies have reported the gene expression profile of leiomyoma and myometrium (Tsibris, JCM *et al. Fertil Steril*, 2002, 78:114-121; Chegini, N *et al. J Soc Gynecol Investig*, 2003, 10:161-71; Wang, H *et al. Fertil Steril*, 2003, 80:266-76; Weston, G *et al. Mol Hum Reprod*, 2003, 9:541-9; Ahn, WS *et al. Int J Exp Pathol*, 2003, 84:267-79; Quade, BJ *et al. Genes Chromosomes Cancer*, 2004, 40:97-108). The present

inventors performed a comparative analysis using the differentially expressed genes identified in the untreated leiomyoma and matched myometrium of this study, with the list of genes reported in four of the other studies, searching for a set of commonly expressed genes. The comparison identified 2 genes in this study in common with at least one of the other studies. However, lowering the false discover rate to $p \leq 0.05$ enabled the identification of a larger number of genes (422 including 49 EST), of which 11 transcripts were found in common with other studies (Table 5).

Table 5 is a list of the common genes found in this study of leiomyoma and matched myometrium from early-med secretory phase of the menstrual cycle following unsupervised and supervised analysis of their expression values and statistical analysis in R programming environment and ANOVA with a false-discovery rate selected at $p \leq 0.05$ to allow comparison with the results of four other microarray studies utilizing leiomyoma and myometrium from proliferative and secretory phases of the menstrual cycle.

Gene ontology assessment and division of differentially expressed genes into similar functional categories indicated that the products of a large percentage of these genes (40% to 67%), in leiomyoma and myometrium from both GnRHa treated and untreated cohorts, are involved in metabolic processes, catalytic activities, binding, signal transduction, transcriptional and translational activities, cell cycle regulation, cell and tissue structure, *etc.* (Tables 1-4). In addition, 15% to 23% of the genes were either functionally unclassified, or their roles in biological process are still unknown.

Example 2—Time-Dependent action of GnRHa on Gene Expression Profile of Leiomyoma and Myometrial Smooth Muscle Cells (LSMC and MSMC)

Leiomyoma and myometrium and their smooth muscle cells (LSMC and MSMC) express GnRH and GnRH receptors, and GnRH through the activation of specific signal transduction pathways results in transcriptional regulation of several genes downstream from these signals in LSMC and MSMC (Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557; Chegini, N *et al. Mol Cell Endocrinol*, 2003, 209:9-16; Xu, J *et al. J Clin Endocrinol Metab*, 2003, 88:1350-61. To obtain a comprehensive picture of transcriptional changes induced by GnRHa direct action in leiomyoma and myometrium, we isolated LSMC and MSMC from the untreated cohorts. The serum starved LSMC and MSMC were treated with GnRHa (0.1 μM) for 2, 6 and 12 hours and their isolated RNA

was subjected to microarray analysis. Based on the same data analysis criteria described above with a false discovery rate of $p \leq 0.005$, we identified 281 genes including 36 EST or 2.2% of the genes on the array displaying differential expression and regulation in LSMC and MSMC in response to GnRHa treatment in a time-dependent manner compared to untreated controls. Hierarchical clustering analysis also separated these genes into different clusters in response to time-dependent action of GnRHa in LSMC and MSMC, with expression patterns sufficiently different to cluster into their respective subgroups. Analysis of the variance-normalized mean (K-means) separated the differentially expressed and regulated genes in these cohorts into 4 distinctive clusters, with genes in clusters A and D displaying a cell-specific response, while genes in cluster B and C showing regulatory behaviors to GnRHa time-dependent action. Among the differentially expressed and regulated genes, the transcripts of 48 genes were identified as commonly expressed in LSMC and the original tissues (leiomyoma) from the untreated cohort used (Table 6).

Table 6 is a categorical list of differentially expressed genes in leiomyoma from GnRHa treated and LSMC treated with GnRHa for 2, 6 and 12 hours. The genes were identified following unsupervised and supervised analysis of their expression values and statistical analysis in R programming environment and ANOVA with a false-discovery rate selected at $p \leq 0.005$. Of the 130 genes identified, the expression of 34 genes was up-(+) and 96 genes downregulated (-) excluding 26 EST.

Gene ontology and functional annotation of the differentially expressed and regulated genes into similar functional categories indicated that in LSMC and MSMC, similar to their original tissues, the majority of the gene products are involved in cellular processes, catalytic activities, binding, signal transduction, transcriptional and translational activities, metabolism, cell cycle regulation and cellular structure. The time-dependent action of GnRHa on the expression of a selective group of genes representing growth factors/cytokines/chemokines/receptors, intracellular signal transduction pathways, transcription factors, cell cycle, cell adhesion/receptor/ECM/cytoskeleton in LSMC and MSMC are shown in Figures 1A-1J.

Example 3—Verification of Gene Transcripts in Leiomyoma, Myometrium and LSMC and MSMC

Among the differentially expressed and regulated genes identified in these tissues and cells, we selected 10 genes for verification using Realtime PCR, western blotting and immunohistochemistry. The selection of these genes was based not only on their expression values (up or downregulated), but also on gene classification, biological functions important to leiomyoma growth and regression, and regulation by ovarian steroids, GnRH and TGF-β. The genes selected for validation were IL-11, CITED2, Nur77, EGR3, TGIF, TIEG, CDKN1B (p27), CDKN1C (p57), GAS-1 and GPRK5, representing cytokines, transcription factors, cell cycle regulators, and signal transduction. The pattern of expression of these genes in leiomyoma and myometrium from untreated and GnRHa-treated cohorts (Figures 2A-2J), as well as in LSMC and MSMC treated with GnRHa for 2, 6 and 12 hours (Figures 3A-3T) as determined by Realtime PCR, closely overlapped with their expression profiles identified by the microarray analysis.

Western blotting also indicated that leiomyoma and myometrium, as well as LSMC and MSMC locally produce IL-11, TGIF, TIEG, Nur77, EGR3, CITED2, p27, p57 and Gas1 proteins. Immunohistochemically, IL-11, TGIF, TIEG, Nur77, EGR3, CITED2, p27, p57 and Gas1 were localized in various cell types in leiomyoma and myometrium, including LSMC and MSMC (Figures 4A-4E). The present inventors did not have access to antibody to GPRK5 and have not yet attempted to quantitate the level of IL-11, TGIF, TIEG, Nur77, EGR3, CITED2, p27, p57 and Gas1 production in leiomyoma and myometrium as well as in LSMC and MSMC in response to GnRHa treatment. However, these results provided further support for the microarray and Realtime PCR data, indicating that various cells types contribute to overall expression of these genes in leiomyoma and myometrium. In addition to these genes, the expression of 15 more genes was validated with Realtime PCR including CTGF, Abl-interactor 2 (Abi2), fibromodulin, Runx1 and Runx2 (Levens, E *et al.* "Differential Expression of fibromodulin and Abl-interactor 2 in leiomyoma and myometrium and regulation by gonadotropin releasing hormone analogue (GnRHa) therapy" *Fertil Steril*, 2004, (In press)).

Uterine leiomyoma affect 30 to 35% of women during their reproductive years and up to 70 to 80% before menopause (Baird, DD *et al. Am. J Obstet Gynecol*, 2003,

188: 100-107). The etiology of leiomyoma remains unknown, however they are thought to derive from the transformation of MSMC and/or connective tissue fibroblasts, and display high sensitivity to ovarian steroids for their growth. For this reason, GnRHa therapy is often used for medical management of symptomatic leiomyomas. In addition to GnRHa therapy, clinical and preclinical assessments of selective estrogen and progesterone receptor modulators, either alone, or in combination with GnRHa therapy, have shown efficacy in leiomyoma regression (Steinauer, J *et al. Obstet Gynecol*, 2004, 103:1331-6; Palomba, S *et al. Hum Reprod*, 2002, 17:3213-3219; DeManno, D *et al. Steroids*, 2003, 68:1019-32). Despite their prevalence and the efficacy of these therapies for their medical management, the molecular environment differentiating leiomyoma from adjacent myometrium, and their response to the above therapies is unknown. In the present study, the present inventors characterized gene expression fingerprints of leiomyoma and matched myometrium from the early-mid secretory phase of the menstrual cycle, a period associated with their rapid growth, their response to hormonal transition induced by GnRHa therapy, and to direct action of GnRHa in isolated LSMC and MSMC prepared from the untreated tissues.

Combining global normalization and unsupervised assessment of the gene expression values derived from all the cohorts enabled us to sort potential candidate genes prior to their putative identification in each cohort. Transcripts of many of the genes on the array were found in leiomyoma and myometrium as well as in LSMC and MSMC. However, leiomyoma/LSMC were not distinguished as a single class from myometrium/MSMC based on single gene markers uniformly expressed only in leiomyoma and/or myometrium. This is not unique to leiomyoma/myometrium since many large-scale gene expression profiling studies have shown the existence of a significant degree of shared gene expression between various tumors and their normal tissue counterparts. However, supervised assessment and multiple test correction in R programming environment (Tusher, VG *et al. Proc Natl Acad Sci USA*, 2001, 98:5116-5121; Pavlidis, P *Methods*, 2003, 31:282-289; Peterson, LE *Comput Methods Programs Biomed*, 2003, 70:107-19; Butte, A *Nat Rev Drug Discov*, 2002, 1:951-960) with reduced false discovery rate, allowed the identification of a specific set of differentially expressed and regulated genes in descending order of significance in each cohort. The analysis separated these genes into several clusters with a sufficient difference allowing their subdivision into their respective subgroup in leiomyoma, myometrium, their isolated cells, as well as due to

GnRHa therapy at the tissue and cellular levels. We identified 153 genes (excluding 19 EST) in these cultures as differentially expressed in leiomyoma compared to myometrium, of which 82 genes were upregulated and 52 downregulated in leiomyoma. GnRHa therapy affected the expression of 122 genes (excluding 21 EST), with 34 upregulated and 67 downregulated genes in leiomyoma compared to myometrium. However, their gene profiles in untreated and GnRHa treated leiomyoma/myometrium differed substantially, pointing out a unique molecular environment that is targeted by GnRHa therapy. Analysis of the variance-normalized mean gene expression values divided these genes into 4 clusters with two clusters showing treatment-specific, while other clusters displayed a tissue-specific response to GnRHa therapy. A similar behavior was also observed with gene clusters identified in LSMC and MSMC in response to GnRHa action in vitro. The significance of these findings are related to clinical observations indicating that GnRHa therapy affects both leiomyoma and myometrium, with non-myoma tissue regressing more in response to therapy (Carr, BR *et al. J Clin Endocrinol Metab*, 1993, 76:1217-1223). The gene expression profiling disclosed herein supports the clinical observations, and further points out that GnRHa therapy targets different genes in leiomyoma and myometrium although they may group in a similar functional category. The recent microarray study using a small-scale array containing probe sets of 1200 known genes (Chegini, N *et al. J Soc Gynecol Investig*, 2003, 10:161-71) provides support for the current study; however, the present inventors are not aware of any other study using a large-scale gene expression profiling in leiomyoma and myometrium from women who received GnRHa therapy for further comparison.

Since this study was completed, a few other microarray studies have reported the gene expression profiles of leiomyoma and myometrium from the proliferative and secretory phases of the menstrual cycle (Tsibris, JCM *et al. Fertil Steril*, 2002, 78:114-121; Wang, H *et al. Fertil Steril*, 2003, 80:266-76; Weston, G *et al. Mol Hum Reprod*, 2003, 9:541-9; Quade, BJ *et al. Genes Chromosomes Cancer*, 2004, 40:97-108). To broaden the scope of this study, the present inventors compared the genes list identified in untreated leiomyoma and matched myometrium of the present study, with the data sets reported in four of these otherstudies (Tsibris, JCM *et al. Fertil Steril*, 2002, 78:114-121; Wang, H *et al. Fertil Steril*, 2003, 80:266-76; Weston, G *et al. Mol Hum Reprod*, 2003, 9:541-9; Quade, BJ *et al. Genes Chromosomes Cancer*, 2004, 40:97-108). This comparison resulted in identification of only a few genes in common among these studies. Although intrinsic

individual tissue variation may contribute toward differences among these studies, standard of experimental process, utilization of different microarry platforms, utilization of tissues from different phases of the menstrual cycle, differences of leiomyoma size, and most importantly the method of data acquisition and analysis (Tsibris, JCM *et al. Fertil Steril*, 2002, 78:114-121; Wang, H *et al. Fertil Steril*, 2003, 80:266-76; Weston, G *et al. Mol Hum Reprod*, 2003, 9:541-9; Quade, BJ *et al. Genes Chromosomes Cancer*, 2004, 40:97-108) are among other key contributing factors accounting for different study results (Pavlidis, P *Methods*, 2003, 31:282-289; Peterson, LE *Comput Methods Programs Biomed*, 2003, 70:107-19; Butte, A *Nat Rev Drug Discov*, 2002 1:951-960). To maintain a standard, the present inventors used leiomyoma of uniform sizes (2-3 cm in diameters) and matched myometrium, and the untreated cohorts were collected from the early-mid secretory phase of the menstrual cycle, a period associated with leiomyoma maximum growth. However, lowering the false discovery rate of the present study allowed the identification of more transcripts and the appearance of additional common genes with other studies (see Table 5; Refs. Tsibris, JCM *et al. Fertil Steril*, 2002, 78:114-121; Wang, H *et al. Fertil Steril*, 2003, 80:266-76; Weston, G *et al. Mol Hum Reprod*, 2003, 9:541-9; Quade, BJ *et al. Genes Chromosomes Cancer*, 2004, 40:97-108). Considering the presence of a large number of probe sets on these arrays (i.e. 6800-12,500), selection of genes based only on fold change (Tsibris, JCM *et al. Fertil Steril*, 2002), or higher statistical levels (Wang, H *et al. Fertil Steril*, 2003, 80:266-76; Weston, G *et al. Mol Hum Reprod*, 2003, 9:541-9; Ahn, WS *et al. Int J Exp Pathol*, 2003, 84:267-79; Quade, BJ *et al. Genes Chromosomes Cancer*, 2004, 40:97-108) is no better than what one would expect by chance alone (Pavlidis, P *Methods*, 2003, 31:282-289; Peterson, LE *Comput Methods Programs Biomed*, 2003, 70:107-19; Butte, A *Nat Rev Drug Discov*, 2002 1:951-960). Since the present inventors employed a similar data analysis, a larger number of genes was found in common with our previous microarray study which used only a small-scale array containing about 1200 known genes (Chegini, N *et al. et al. J Soc Gynecol Investig*, 2003, 10:161-71). The present inventors recognize that exclusion of moderately regulated genes during microarray data analysis does not reflect lack of functional importance, since a number of genes previously identified in leiomyoma and myometrium by conventional methods are not included among the differentially expressed genes in our study and other reports (Chegini, N Implication of growth factor and cytokine networks in leiomyomas. In; Cytokines in human reproduction. J. Hill ed.

New York, Wiley & Sons Publisher, 2000, 133-162; Maruo, T *et al. Hum Reprod Update*, 2004, 10:207-20; Tsibris, JCM *et al. Fertil Steril*, 2002, 78:114-121; Chegini, N *et al. J Soc Gynecol Investig*, 2003, 10:161-71; Wang, H *et al. Fertil Steril*, 2003, 80:266-76; Weston, G *et al. Mol Hum Reprod*, 2003, 9:541-9; Ahn, WS *et al. Int J Exp Pathol*, 2003, 84:267-79; Quade, BJ *et al. Genes Chromosomes Cancer*, 2004, 40:97-108). However, the expression of newly identified genes requires verification, and their regulation would allow linking their potential biological functions in leiomyoma growth and regression.

GnRHa therapy and most recently SERM and SPRM have been utilized for medical management of leiomyoma (Takeuchi, H *et al. J Obstet Gynaecol Res*, 2000, 26:325-331; Steinauer, J *et al. Obstet Gynecol*, 2004, 103:1331-6; Palomba, S *et al. Hum Reprod*, 2002, 17:3213-3219; DeManno, D *et al. Steroids*, 2003, 68:1019-32; Carr, BR *et al. J Clin Endocrinol Metab*, 1993, 76:1217-1223). Unlike SERM and SPRM that act directly on estrogen/progesterone sensitive tissues such as the uterus (Palomba, S *et al. Hum Reprod*, 2002, 17:3213-3219; DeManno, D *et al. Steroids*, 2003, 68:1019-32), GnRHa is traditionally believed to act primarily at the level of the pituitary-gonadal axis to implement its therapeutic benefits (Klausen, C *et al. Prog Brain Res*, 2002, 141:111-128). However, identification of GnRH and GnRH receptors in several peripheral tissues, including leiomyoma, has led the present inventors to propose an autocrine/paracrine role for GnRH, and an additional site of action for GnRHa therapy (Chegini, N *et al. J Clin Endocrinol Metab*, 1996, 81:3215-3221; Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557; Chegini, N *et al. Mol Cell Endocrinol*, 2003, 209:9-16; Xu, J *et al. J Clin Endocrinol Metab*, 2003, 88:1350-61). *In vitro* studies have provided evidence for direct action of GnRHa on several cell types derived from these tissues resulting in alterations of a wide range of cellular activities, including cell growth, apoptosis and gene expression (Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557; Chegini, N *et al. Mol Cell Endocrinol*, 2003, 209:9-16; Xu, J *et al. J Clin Endocrinol Metab*, 2003, 88:1350-61; Chegini, N and Kornberg, L *J Soc Gynecol Investig*, 2003, 10:21-6; Chegini, N *et al. Mol Hum Reprod*, 2002, 8:1071-8; Klausen, C *et al. Prog Brain Res*, 2002, 141:111-128; Mizutani, T *et al. J Clin Endocrinol Metab*, 1998, 83:1253-1255). Using isolated LSMC and MSMC prepared from the untreated tissues allowed the present inventors to identify novel regulatory functions for GnRHa in leiomyoma and myometrium, and discover a wide range of genes whose expression has not previously been recognized to be the target of GnRHa direct action. Similar to their distinct clustering at tissue levels, the

differentially expressed and regulated genes identified in LSMC and MSMC were also divided into clusters according to time-dependent response to GnRHa action. The genes in these clusters were either rapidly induced by GnRHa treatment, or required prolong exposure, while others displayed biphasic patterns of temporal regulation in both treatment- and cell- specific fashions. Despite differences in their profiles, substantial similarity existed in functional grouping of the genes affected by GnRHa therapy in leiomyoma/myometrium, and GnRHa direct action on LSMC/MSMC (*in vitro*), with the expression of 48 genes commonly identified in tissues and cells. The present inventors propose that the hypoestrogenic condition created by GnRHa therapy and contributions of other cell types to overall gene expression at the tissue level may account for the difference in profiles of gene expression between tissues and cell cultures. Gene ontology and division into similar functional categories indicated that the products of the majority of the genes in these clusters are involved in transcriptional and signal transduction activities, cell cycle regulation, extracellular matrix turnover, cell-cell communication, transport and enzyme regulatory activities.

Among the genes in these functional categories are several growth factors, cytokines and chemokines, and polypeptide hormones, identified as differentially expressed in leiomyoma, myometrium and their isolated smooth muscle cells, and were the target of GnRHa action *in vivo* and *in vitro*. Using several conventional methods, previous reports have documented the expression of PDGF, EGF, IGFs, VEGF, FGF, TGF-βs, CTGF, TNF-α, IFN-γ, MCP-1 and IL-8 as well as some of their receptors in leiomyoma and myometrium (Chegini, N "Implication of growth factor and cytokine networks in leiomyomas" In Cytokines in human reproduction. J. Hill ed. New York, Wiley & Sons Publisher, 2000; Maruo, T *et al. Hum Reprod Update*, 2004, 10:207-20; Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557; Chegini, N *et al. Mol Cell Endocrinol*, 2003, 209:9-16; Chegini, N *et al. Mol Hum Reprod*, 2002, 8:1071-8; Wu, X *et al. Acta Obstet Gynecol Scand*, 2001, 80:497-504; Senturk, LM *et al. Am J Obstet Gynecol*, 2001, 184:559-566; Sozen, I *et al. Fertil Steril*, 1998, 69:1095-1102). However, the expression of some of these and other genes in this category did not meet the selection criteria of this study, a common discrepancy often observed in microarray analysis, particularly in identifying moderately expressed and regulated genes (Varela, JC *et al. Invest Ophthalmol Vis Sci*, 2002, 43:1772-1782; Tusher, VG *et al. Proc Natl Acad Sci USA*, 2001, 98:5116-5121; Pavlidis, P *Methods*, 2003, 31:282-289; Peterson, LE *Comput*

Methods Programs Biomed, 2003, 70:107-19; Butte, A *Nat Rev Drug Discov*, 2002, 1:951-960). For example, the expression of TGF-β isoforms, TGF-β receptors and components of their signaling pathway that are well documented in leiomyoma and myometrium, as well as in their isolated smooth muscles cells (Chegini, N *et al. Mol Cell Endocrinol*, 2003, 209:9-16; Xu, J *et al. J Clin Endocrinol Metab*, 2003, 88:1350-61; Chegini, N and Kornberg, L *J Soc Gynecol Investig*, 2003, 10:21-6; Chegini, N *et al. Mol Hum Reprod*, 2002, 8:1071-8; Dou, Q *et al. J Clin Endocrinol Metab*, 1996, 81:3222-3230; Arici, A and Sozen, I *Fertil Steril*, 2000, 73:1006-1011; Lee, BS and Nowak, RA *J Clin Endocrinol Metab*, 2001, 86:913-920), are not consistently identified in microarray studies (Tsibris, JCM *et al. Fertil Steril*, 2002, 78:114-121; Chegini, N *et al. J Soc Gynecol Investig*, 2003, 10:161-71; Wang, H *et al. Fertil Steril*, 2003, 80:266-76; Weston, G *et al. Mol Hum Reprod*, 2003, 9:541-9; Ahn, WS *et al. Int J Exp Pathol*, 2003, 84:267-79; Quade, BJ *et al. Genes Chromosomes Cancer*, 2004, 40:97-108), although in the current and previous (Chegini, N *et al. J Soc Gynecol Investig*, 2003, 10:161-71) studies we identified most of the members of TGF-β system. Among the cytokines whose expression was identified and validated in the present study is IL-11. IL-11 is recognized to play key regulatory functions in inflammation, angiogenesis and tissue remodeling (Leng, SX and Elias, JA *Int J Biochem Cell Biol*, 1997, 29:1059-62; Tang, W *et al. J Clin Invest*, 1996, 98:2845-53; Zhu, Z *et al. Am J Respir Crit Care Med*, 2001, 164:S67-70; Zimmerman, MA *et al. Am J Physiol Heart Circ Physiol*, 2002, 283:H175-80; Bamba, S *et al. Am J Physiol Gastrointest Liver Physiol*, 2003, 285:G529-38), events that are central to leiomyoma pathophysiology. IL-11 is a member of the IL-6 family and produced by various cell types, including the uterus, and its overexpression is reported to cause sub-epithelial airway fibrosis particularly through interaction with IL-13 and TGF-β (Leng, SX and Elias, JA *Int J Biochem Cell Biol*, 1997, 29:1059-62; Tang, W *et al. J Clin Invest*, 1996, 98:2845-53; Zhu, Z *et al. Am J Respir Crit Care Med*, 2001, 164:S67-70; Zimmerman, MA *et al. Am J Physiol Heart Circ Physiol*, 2002, 283:H175-80; Bamba, S *et al. Am J Physiol Gastrointest Liver Physiol*, 2003, 285:G529-38; Karpovich, N *et al. Mol Hum Reprod*, 2003, 9:75-80). Evidence has been provided that IL-11, similar to TGF-β and IL-13, is overexpressed in leiomyoma compared to myometrium and GnRHa therapy suppressed their expression in these tissues (Chegini, N *et al. Mol Cell Endocrinol*, 2003, 209:9-16; Chegini, N *et al. Mol Hum Reprod*, 2002, 8:1071-8; Dou, Q *et al. J Clin Endocrinol Metab*, 1996, 81:3222-3230; Ding, L *et al. J Soc Gyncol Invest*,

2004, 00, 00). At the cellular level, unlike the expression of TGF-β and IL-13, GnRHa increased IL-11 expression in LSMC and MSMC within 2 to 6 hours of treatment, which sharply declined to control levels after 12 hours. Although the nature of differential regulation of IL-11 at the tissue and cellular levels requires detailed investigation, prolonged treatment with GnRHa, the contribution of other cell types and the influence of other autocrine/paracrine regulators, may account for the difference in IL-11 expression between *in vivo* and *in vitro* conditions.

Other differentially expressed and regulated genes identified in the present study functionally belong to signal transduction pathways that are recruited and activated by various growth factors/cytokines/chemokines, polypeptide hormones, extracellular matrix and adhesion molecules. However the expression of few of these components and other signal transduction pathways has been documented in leiomyoma and myometrium (Chegini, N "Implication of growth factor and cytokine networks in leiomyomas" In; Cytokines in human reproduction. J. Hill ed. New York, Wiley & Sons Publisher, 2000, 133-162; Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557; Chegini, N and Kornberg, L *J Soc Gynecol Investig*, 2003, 10:21-6; Orii, A *et al. J Clin Endocrinol Metab*, 2002, 87:3754-9), and little is known about their recruitment and activation in LSMC and MSMC. The expression of Smads, MAPK and FAK has been identified in leiomyomas and myometrium and evidence has been provided for their regulation and activation by GnRHa in LSMC and MSMC (Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557; Xu, J *et al. J Clin Endocrinol Metab*, 2003, 88:1350-61; Chegini, N and Kornberg, L *J Soc Gynecol Investig*, 2003, 10:21-6). Here, the present inventors validated the expression of GPRK5 identified as one of the differentially expressed and regulated genes in leiomyoma and myometrium and demonstrated that GnRHa therapy, and *in vitro* treatment of LSMC and MSMC with GnRHa inhibits GPRK5 expression. G-protein-coupled receptor kinases (GPRKs), consisting of six members GPRK1 to GPRK6, act as key regulators of signaling via GPRKs, and are widely expressed in various tissues and cells (Mak, JC *et al. Eur J Pharmacol*, 2002, 436:165-72; Simon, V *et al. Endocrinology*, 2001, 142:1899-905; Simon, V *et al. Endocrinology*, 2003, 144:3058-66; Krasel, C *et al. J Biol Chem*, 2001, 276:1911-1915). Previous studies have demonstrated that pregnant and non-pregnant human myometrium as well as cultured myometrial cells express GPRK2, GPRK4γ and GPRK5, however GPRK3 and GPRK4α, β, and δ were not detected in myometrium (Simon, V *et al. Endocrinology*, 2001, 142:1899-905; Simon, V

et al. Endocrinology, 2003, 144:3058-66). GPRK5 has been shown to serve as a substrate for PKC, although PKC-mediated phosphorylation inhibits GPRK5 (Klausen, C *et al. Prog Brain Res*, 2002, 141:111-128; Krasel, C *et al. J Biol Chem*, 2001, 276: 1911-1915). In addition, the extreme N terminus of GPRK5 contains a binding site for Ca2+/calmodulin, where upon binding it inhibits GPRK activity, a mechanism suggested to regulate GPRKs activity (Krasel, C *et al. J Biol Chem*, 2001, 276: 1911-1915). Since GnRH receptors are a member of the G-protein coupled receptor (GPCR) family and recruit and activate the components of several signaling pathways, including PKC and Ca2+/calmodulin, their regulatory interaction with GPRKs may serve in regulating various events downstream from these signals in LSMC and MSMC.

Nuclear translocation of many activated intracellular signaling molecules results in phosphorylation and activation of transcription factors, major elements in these signaling networks that regulate specific gene expression. In previous studies (Chegini, N *et al. J Soc Gynecol Investig*, 2003, 10:161-71) and the present study, several transcription factors were identified as differentially expressed and regulated in leiomyoma and myometrium and targeted by GnRHa direct action in LSMC and MSMC (see Tables 1-4). Many of these transcription factors are involved in ovarian steroids, polypeptide hormones, inflammatory cytokines, growth factors and ECM receptor mediated-actions, by regulating the promoter of their target genes in various normal and cancer cells. However, little is known regarding the expression and regulation of these and other transcription factors in leiomyoma and myometrium. For this reason, the present inventors placed a greater emphasis on verification of the expression of transcription factors such as Nur77, CITED2, EGR3, TIEG and TGIF in leiomyoma, myometrium and their temporal regulation by GnRHa in LSMC and MSMC.

Nur77 (also known as NR4A1, TR3, NGFI-B, NAK-1) is a member of the orphan nuclear receptor superfamily originally identified as an immediate-early gene in serum-treated fibroblasts (Maira, M *et al. Mol and Cell Biol*, 2003, 23;763-776; Drouin, J *et al. J. Steroid Biochem Mol Biol*, 1998, 65:59-63; Fernandez, P *et al. Endocrinology*, 2000, 141:2392-2400; Gelman, L *et al. J Biol Chem*, 1999, 274:7681-7688; Sadie, H *et al. Endocrinology*, 2003, 144:1958-71; Wilson, TE *et al. Mol Cell Biol*, 1993, 13:861-868; Song, KH *et al. Endocrinology*, 2001, 142:5116-23; Zhang, P and Mellon, SH *Mol Endocrinol*, 1997, 11:891-904). It is also identified as NGF-inducible gene, which is constitutively expressed in various tissues and is strongly induced by several stimuli,

resulting in regulation of gene expression related to inflammation, angiogenesis, apoptosis and steriodogenesis, including steroid-21 and 17α-hydroxylases and 20α hydroxysteroid dehydrogenase in the hypothalamic-pituitary-adrenal axis (Maira, M *et al. Mol and Cell Biol*, 2003, 23;763-776; Drouin, J *et al. J. Steroid Biochem Mol Biol*, 1998, 65:59-63; Fernandez, P *et al. Endocrinology*, 2000, 141:2392-2400; Gelman, L *et al. J Biol Chem*, 1999, 274:7681-7688; Sadie, H *et al. Endocrinology*, 2003, 144:1958-71; Wilson, TE *et al. Mol Cell Biol*, 1993, 13:861-868; Song, KH *et al. Endocrinology*, 2001, 142:5116-23; Zhang, P and Mellon, SH *Mol Endocrinol*, 1997, 11:891-904). In the anterior pituitary, Nur77 is reported to mediate the stimulatory effect of CRH and the negative-feedback regulation of POMC transcription by glucocorticoids, as well as GnRH-induced GnRH receptor expression (Drouin, J *et al. J. Steroid Biochem Mol Biol*, 1998, 65:59-63; Sadie, H *et al. Endocrinology*, 2003, 144:1958-71). LH-induced Nur77 is also reported to regulate cytochorome p450 expression in granulosa and leydig cells (Sadie, H *et al. Endocrinology*, 2003, 144:1958-71; Wilson, TE *et al. Mol Cell Biol*, 1993, 13:861-868; Song, KH *et al. Endocrinology*, 2001, 142:5116-23). More importantly, overexpression of Nur77 is implicated as an important regulator of apoptosis in different cells. In response to apoptotic stimuli, Nur77 translocation from the nucleus to mitochondria results in cytochrome C release and apoptosis of LNCaP human prostate cancer cells (Rajpal, A *et al. EMBO J*, 2003, 22:6526-36; Castro-Obregon, S *et al. J Biol Chem*, 2004, 279:17543-53; Li, H *et al. Science*, 2000, 289:1159-1164). The present inventors found a relatively similar expression of Nur77 in myometrium and leiomyoma; however, GnRHa therapy resulted in a significant elevation of Nur77 in both tissues. GnRHa treatment also resulted in a rapid induction of Nur77 in MSMC and LSMC, which subsequently declined to control levels, and in LSMC fell to below the levels detected in untreated cells. Interestingly, GnRH is reported to regulate Nur77 expression in αT3-1 and LβT2 gonadotrope cell lines through PKA pathway and GnRH receptor promoter via a mechanism involving SF-1 with Nur77 acting as a negative regulator of this response (Sadie, H *et al. Endocrinology*, 2003, 144:1958-71). In a recent study, activation of MAPK pathway involving Raf-1, MEK2 and ERK2 was reported to regulate Nur77 activation resulting in nonapoptotic program cell death (Castro-Obregon, S *et al. J Biol Chem*, 2004). The present inventors have shown that GnRH signaling through MAPK and transcriptional activation of c-fos and c-jun regulate the expression of several specific genes in LSMC and MSMC. This suggests that GnRH-mediated action through

this pathway may regulate nur77 expression thus influencing the outcome of cellular growth arrest and/or apoptosis in leiomyoma.

Recently, a new family of transcriptional co-regulators, the CITED (CBP/p300-interacting transactivator with ED-rich tail) family, was discovered that interact with the first cysteine-histidine-rich region of CBP/p300 (Tien, ES *et al. J Biol Chem*, 2004, 279:24053-63; Kranc, KR *et al. Mol Cell Biol*, 2003, 23:7658-66). The CITED family contains four members and appears to act as key transcriptional modulators in embryogenesis, inflammation, and stress responses (Tien, ES *et al. J Biol Chem*, 2004, 279:24053-63) by affecting the transcriptional activity of many transcription factors ranging from AP2, estrogen receptor, and hypoxia-inducible factor 1 (HIF1) and LIM (Yin, Z *et al. Proc Natl Acad Sci USA*, 2002, 99:10488-10493). The present inventors identified CITED2 among the differentially expressed and regulated genes in leiomyoma, myometrium and their isolated cells, and in response to GnRHa treatment *in vivo* and *in vitro*. Unlike GnRHa therapy which increased CITED2 expression in leiomyoma and myometrium, GnRHa had a biphasic effect on CITED2 expression in MSMC, while inhibiting expression in LSMC. Although *in vitro* culture conditions may directly influence the expression of regulatory molecules that either interact with or regulate CITED2 expression, the exact molecular mechanism resulting in differential expression of CITED2 *in vivo* and *in vitro* by GnRHa requires further investigation. Interestingly, the expression of several growth factors, cytokines and HIF1 are the target of ER, PR regulatory action, and CITED2 acting as a repressor of their expression may serve as an important regulator of processes that regulate inflammatory response, angiogenesis and tissue remodeling in leiomyoma. Additionally, CBP/p300 which serve as promiscuous co-activators for an increasing number of transcription factors resulting in proliferation, differentiation and apoptosis in response to diverse biological factors, including ER- and PR-dependent transcriptional activity, is specifically recruited by Nur77 acting as dimers following PKA activation (Maira, M *et al. Mol and Cell Biol*, 2003, 23;763-776; Kranc, K *et al. Trends Cell Biol*, 1997, 7:230-236; Puri, PL *et al. EMBO J*, 1997, 16:369-383).

In a previous microarray study, it was reported that EGR1, a prototype of a family of zinc-finger transcription factors that includes EGR2, EGR3, EGR4, and NGFI-B (Hjoberg, J *et al. Am J Physiol Lung Cell Mol Physiol*, 2004, 286:L817-825; Thiel, G and Cibelli, G *J Cell Physiol*, 2002, 193:287-92), is differentially expressed in leiomyoma and myometrium (Chegini, N *et al. J Soc Gynecol Investig*, 2003, 10:161-71). Here, the

present inventors provide evidence for the expression of EGR3 and differential regulation in response to GnRHa therapy in leiomyoma and myometrium, as well as in LSMC and MSMC *in vitro*. A recent report demonstrated that EGR1 expression is elevated in leiomyoma compared to corresponding myometrium in women who received GnRHa therapy (Shozu, M *et al. Cancer Research*, 2004, 64:4677-4684) supporting previous microarray data (Chegini, N *et al. J Soc Gynecol Investig*, 2003, 10:161-71). EGRs expression is rapidly and transiently induced by a large number of growth factors, cytokines, polypeptide hormones and injurious stimuli and kinetics of their expression is essentially identical to c-fos proto-oncogene (Hjoberg, J *et al. Am J Physiol Lung Cell Mol Physiol*, 2004, 286:L817-825; Thiel, G and Cibelli, G *J Cell Physiol*, 2002, 193:287-92; Inoue, A *et al. J Mol Endocrinol*, 2004, 32:649-61). In addition, induction of EGR1 occurs primarily at the level of transcription and is mediated, in part, through MAPKs, including ERK, JNK, and p38 pathways (Hjoberg, J *et al. Am J Physiol Lung Cell Mol Physiol*, 2004, 286:L817-825; Thiel, G and Cibelli, G *J Cell Physiol*, 2002, 193:287-92). It has been demonstrated that GnRHa through the activation of MAPK regulates the expression c-fos and c-jun as well as fibronectin, collagen and PAI-1 expression (Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557). In human fibrosarcoma and glioblastoma cells, EGR directly influences the expression of fibronectin, TGF-β1, and PAI-1 and may regulate the expression of PDGF, tissue factor, and membrane type 1 matrix metalloproteinase (Thiel, G and Cibelli, G *J Cell Physiol*, 2002, 193:287-92; Liu, C *et al. J Biol Chem*, 1999, 274:4400-11). Estrogen is also reported to induce EGR3 in various cancer cell lines while is inhibited by progesterone in Schwann cells (Inoue, A *et al. J Mol Endocrinol*, 2004, 32:649-61; Mercier, G *et al. Mol Brain Res*, 2001, 97:137-148). Constitutive transgenic expression of EGR3 has recently been shown to increase thymocytes apoptosis, possibly through potent activation of FasL expression (Xi, H and Kersh, GJ *J Immunol*, 2004, 173:340-8). Given the role of ovarian steroids and a large number of growth factors, cytokines and polypeptide hormones in leiomyoma growth, and suppression by GnRHa, their differential influence on EGR1 and EGR3 expression may represent a mechanism resulting in balance between the rate of cell proliferation and apoptosis as well as tissue turnover, affecting leiomyoma growth and regression.

The present study also provides the first evidence of the expression and regulation of TIEG and TGIF, novel three zinc-finger Kruppel-like transcriptional repressors, and key regulators of TGF-β receptor signaling (Johnsen, SA *et al. Oncogene*, 2002, 21:5783-90;

Cook, T and Urrutia, R *Am J Physiol Gastrointest Liver Physiol*, 2000, 278:G513-21; Ribeiro, A *et al. Hepatology*, 1999, 30:1490-7; Chen, F *et al. Biochem J*, 2003, 371:257-63; Melhuish, TA *et al. J Biol Chem*, 2001, 276:32109-14), by GnRHa in leiomyoma, myometrium, LSMC and MSMC. TIEG regulates TGF-β receptor signaling through a negative feedback mechanism by repressing the inhibitory Smad7 (Johnsen, SA *et al. Oncogene*, 2002, 21:5783-90). In addition, TGIF through direct binding to DNA or interaction with TGF-β-activated Smads represses TGF-β-responsive gene expression (Chen, F *et al. Biochem J*, 2003, 371:257-63; Melhuish, TA *et al. J Biol Chem*, 2001, 276:32109-14). Since GnRHa suppresses TGF-β and TGF-β receptors while enhancing Smad7 expression in leiomyoma and myometrium as well as LSMC and MSMC, differential regulation of TIEG and TGIF may serve as an additional downstream mechanism altering TGF-β autocrine/paracrine actions in leiomyoma. To further understand the regulation of these transcription factors in leiomyoma, the present inventors also provide evidence for their regulation in LSMC and MSMC by TGF-β, further implicating the importance of TGF-β in pathogenesis of leiomyoma (as described in Examples 4-7).

The expression, activation and direct interaction of these and other transcription factors with DNA results in regulation of the expression of various genes whose products influence cell growth, inflammation, angiogenesis, apoptosis and tissue turnover. In previous studies (Chegini, N *et al. J Soc Gynecol Investig*, 2003, 10:161-71; Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557) and the present study, several differentially expressed and regulated genes were identified in leiomyoma, myometrium and LSMC and MSMC whose promoters are the target of these transcription factors. Among these genes are members of cell cycle regulatory proteins that play a central role in leiomyoma growth and regression (Chegini, N "Implication of growth factor and cytokine networks in leiomyomas" In; Cytokines in human reproduction. J. Hill ed. New York, Wiley & Sons Publisher, 2000, 133-162; Maruo, T *et al. Hum Reprod Update*, 2004, 10:207-20; Zhai, YL *et al. Int J Cancer*, 1999, 84:244-50), including p27, p57 and Gas1. The present inventors identified p27, p57 and Gas1 as differentially expressed and regulated in leiomyoma and myometrium as well as LSMC and MSMC and in response to GnRHa treatment. Although p27, p57 and Gas1 function as major regulators of cell cycle progression, several studies have also shown Cip/Kip proteins function as transcriptional cofactors by regulating the activity of NFκ-B, STAT3, Myc, Rb, C/EBP,

CBP/p300, E2F and AP1 (Coqueret, O *Trends Cell Biol*, 2003, 13:65-70). A recent report also suggests that p21, p27 and p57 are involved in regulation of apoptosis (Blagosklonny, MV *Semin Cancer Biol*, 2003, 13:97-105) and their differential regulation in leiomyoma and myometrium is consistent with GnRHa induction of apoptosis related gene in LSMC and MSMC (Chegini, N "Implication of growth factor and cytokine networks in leiomyomas" In; Cytokines in human reproduction. J. Hill ed. New York, Wiley & Sons Publisher, 2000, 133-162; Maruo, T *et al. Hum Reprod Update*, 2004, 10:207-20; Mizutani, T *et al. J Clin Endocrinol Metab*, 1998, 83:1253-1255; Zhai, YL *et al. Int J Cancer*, 1999, 84:244-50). However, the results disclosed herein are the first to document the expression of Gas1 in leiomyoma and myometrium, and regulation in LSMC and MSMC in response to timed-dependent action of GnRHa. GnRHa has been demonstrated to alter cell cycle progression and programmed cell death in several cell types including leiomyoma smooth muscle cells (Chegini, N "Implication of growth factor and cytokine networks in leiomyomas" In; Cytokines in human reproduction. J. Hill ed. New York, Wiley & Sons Publisher, 2000, 133-162; Mizutani, T *et al. J Clin Endocrinol Metab*, 1998, 83:1253-1255; Zhai, YL *et al. Int J Cancer*, 1999, 84:244-50), and these results provide additional support for the involvement of specific cell cycle and apoptotic related genes in leiomyoma growth and regression. How the expression of these genes is regulated and through what mechanism their products influence LSMC and MSMC cell cycle progression and programmed cell death awaits further investigation.

Leiomyoma growth and GnRHa therapy resulting in leiomyoma regression also involves extracellular matrix turnover. In previous studies(Chegini, N *et al. J Soc Gynecol Investig*, 2003, 10:161-71), in the present study, and in recent studies by other groups (Tsibris, JCM *et al. Fertil Steril*, 2002, 78:114-121; Wang, H *et al. Fertil Steril*, 2003, 80:266-76; Weston, G *et al. Mol Hum Reprod*, 2003, 9:541-9; Ahn, WS *et al. Int J Exp Pathol*, 2003, 84:267-79; Quade, BJ *et al. Genes Chromosomes Cancer*, 2004, 40:97-108), several genes in this category were identified displaying differential expression in leiomyoma and myometrium and were targeted by GnRH therapy (Tables 1-4) (Chegini, N "Implication of growth factor and cytokine networks in leiomyomas" In Cytokines in human reproduction. J. Hill ed. New York, Wiley & Sons Publisher, 2000, 133-162; Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557; Dou, Q *et al. Mol Hum Reprod*, 1997, 3:1005-1014; Levens, E *et al. Fertil Steril*, 2004, (In press); Stewart, EA *et al. J Clin Endocrinol Metab*, 1994, 79:900-6). These include the expression of several

collagens, small leucine rich repeat family of proteoglycans, decorin, biglycan, osteomodulin, fibromodulin, versican, and osteoadherin/osteoglycin, fibronectin, desmin and vimentin, several member of proteases such as matrix metalloproteinases (MMPs) and their inhibitors, TIMPs, a disintegrin-like and metalloproteinase proteins (ADAM), *etc.* It has also been reported that GnRHa regulates the expression of fibronectin, collagen type 1, PAI-1, MMPs and TIMPs (Chegini, N "Implication of growth factor and cytokine networks in leiomyomas" In Cytokines in human reproduction. J. Hill ed. New York, Wiley & Sons Publisher, 2000, 133-162; Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557; Dou, Q *et al. Mol Hum Reprod*, 1997, 3:1005-1014), as well as decorin, versican, desmin and vimentin (unpublished data) in leiomyoma and myometrium, involving the activation of MAPK in LSMC and MSMC (Ding, L *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557). Since ECM turnover is a key regulator of the outcome of tissue fibrosis, and many cytokines, chemokines, growth factors and polypeptide hormones through specific intracellular signal transduction and activation of transcription factors influence the expression of ECM and proteases, further investigation is underway to elucidate their regulatory interactions affecting processes that may influence leiomyoma growth and regression.

In summary, in the present study, the inventors provide a comprehensive assessment of the gene expression profile of leiomyoma and matched myometrium during early-mid luteal phase of the menstrual cycle, a period characterized by elevated production of ovarian steroids and maximal leiomyoma growth, compared with tissues obtained from hormonally suppressed patients on GnRHa therapy and in response to the direct action of GnRHa on LSMC and MSMC. The present inventors identified several common and tissue-specific gene clusters in these cohorts suggesting their co-regulation by the same factors and or mechanism(s) in the same cluster. The present inventors validated the expression of several genes whose products are important in signal transduction, transcription, cell cycle regulation, apoptosis and ECM turnover, events critical to development, growth and regression of leiomyoma. Based on these and previous observations, the present inventors propose that the product of these specific genes, by regulating the local inflammatory and apoptotic processes leading to elaboration of profibrotic cytokines production such as TGF-β is central to the establishment and progression of fibrosis in leiomyoma. Provided in Examples 4-7 is further evidence for the role of TGF-β autocrine/paracrine action in this process.

Example 4—Gene Expression Profiles of Leiomyoma and Matched Myometrium Cells In Response to TGF-β1

It has been reported that leiomyoma and myometrium express all the components of the TGF-β system, and it has been shown that TGF-β through Smads and MAPK pathways regulates the expression of a specific number of genes in LSMC and MSMC (Chegini, N. *et al. J Clin Endocrinol Metab*, 1999, 84:4138-43; Chegini, N. *et al. Mol Hum Reprod*, 2002, 8:1071-1078; Chegini, N. *et al. Mol Cell Endocrinol*, 2003, 209:9-16; Xu, J. *et al. J Clin Endocrinol Metab*, 2003, 88:1350-1361; Ding, L. *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557; Tang, X.M. *et al. Mol Hum Reprod*, 1997, 3:233-40). Here, the present inventors performed microarray analysis to further characterize the molecular environment of LSMC and MSMC directed by TGF-β autocrine/paracrine actions. LSMC and MSMC were treated with TGF-β1 (2.5 ng/ml) for 2, 6 and 12 hours, total RNA was isolated and subjected to microarray analysis. Following global normalization and transformation of the gene expression values, supervised learning, discrimination analysis, cross validation and variation filtering, the gene expression values for this study were independently subjected to statistical R programming analysis and ANOVA with false discovery rate selected at $p \leq 0.001$. The analysis identified 310 genes or 2.46% of the genes on the array as differentially expressed and regulated in response to time-dependent action of TGF-β in LSMC and MSMC.

Hierarchical clustering analysis separated these differentially expressed genes into distinctive clusters, with sufficient difference in their patterns allowing each cohort to cluster into their respective subgroup. The differentially expressed and regulated genes were separated into five clusters in response to time-dependent action of TGF-β in LSMC and MSMC, with genes in clusters A and B displaying a late response, genes in cluster D displaying early response, and genes in clusters C and E showing biphasic regulatory behaviors. Further analysis of the variance-normalized mean gene expression values divided the genes into 6 clusters, each displaying a different level of response to time-dependent action of TGF-β, with overlapping behavior between LSMC and MSMC with the exception of genes in clusters E and F.

Comparative analysis between gene expression profiles of LSMC and MSMC in response to TGF-β action, with their corresponding leiomyoma and myometrium (tissues) from the untreated group revealed a substantial variability among their profiles (data not shown). However, gene ontology assessment and division into functional categories indicated that the majority of these genes (60 to 70%) are involved in transcriptional regulation and metabolism, cell cycle regulation, extracellular matrix and adhesion molecules, signal transduction and transcription factors. The time-dependent action of TGF-β on expression the profile of a selective group of these genes in the above clusters representing transcription factors, growth factors, cytokines, signal transduction pathways, ECM/adhesion molecules *etc.* in LSMC and MSMC are presented in Figure 5A-5N.

Example 5—Gene Expression Profiles of LSMC and MSMC In Response to TGF-β Following Pretreatment with TGF-β type II Receptor Antisense

To further evaluate the autocrine/paracrine action of TGF-β in leiomyoma and myometrial microenvironments, LSMC and MSMC were pretreated with TGF-β type II receptor (TGF-β type IIR) antisense oligomers to block/reduce TGF-β receptor signaling. Following pretreatments the cells were treated with or without TGF-β for 2 hours and their total RNA was subjected to microarray analysis. Based on the same data analysis described above with false discovery rate of $p \leq 0.001$, the present inventors identified 54 differentially expressed and regulated genes in response to TGF-β1 (2.5 ng/ml for 2 hours) in LSMC and MSMC pretreated with TGF-β type IIR antisense. Hierarchical cluster analysis distinctively separated these genes into 3 clusters with each cohort separated into their respective subgroups. The genes in clusters A and C displayed different response to TGF-β type IIR antisense treatment, while genes in cluster B showed overlapping behavior in LSMC and MSMC. However, there was an overlapping pattern between the gene expression profiles in TGF-β type IIR sense- and antisense-treated cells that could be due to the inability of antisense treatment to block all the combined action of autocrine/paracrine and exogenously added TGF-β. Interestingly, antisense treatment altered the expression of many genes known to be the target of TGF-β action, including those validated in this study. Gene ontology assessment and division into similar functional categories indicated that the majority of these genes are involved in

transcriptional regulation and metabolism, cell cycle regulation, extracellular matrix and adhesion molecules, and transcription factors.

Example 6—Comparative Analysis of Gene Expression Profiles in Response to TGF-β type II Receptor Antisense and GnRHa Treatments In LSMC and MSMC

Since GnRHa alters the expression of TGF-β and TGF-β receptors expression in leiomyoma and myometrium as well as in LSMC and MSMC, the present inventors compared the gene expression profile of TGF-β type IIR antisense-treated with GnRHa-treated LSMC and MSMC, searching for common genes whose expression are affected by these treatments. Based on the same data analysis described above with false discovery rate selected at $p \leq 0.001$, the present inventors identified 222 genes differentially expressed and regulated in LSMC and MSMC in response to TGF-β type IIR antisense- and GnRHa-treated cells (Tables 7 and 8). Hierarchical clustering analysis separated these genes into 4 clusters displaying different pattern of regulation allowing their separation into respective subgroup. The genes in cluster A, B and D displayed different response to TGF-β type IIR antisense and GnRHa treatments, with genes in cluster C showing overlapping behavior in LSMC and MSMC.

Table 7 is a categorical list of genes identified as differentially expressed in LSMC pretreated with TGF-β type II receptor (TGF-β type IIR) antisense for 24 hours followed by TGF-β treatment for 2 hrs compared to LSMC treated with GnRHa (0.01 μM) for 2, 6, 12 hours. The genes were identified following supervised analysis of their expression values and statistical analysis in R programming and ANOVA with a false-discovery rate of rate of $p \leq 0.001$.

Table 8 is a categorical list of genes identified as differentially expressed in LSMC pretreated with TGF-b type II receptor (TGF-b type IIR) antisense for 24 hrs followed by TGF-b treatment for 2 hrs compared to LSMC treated with GnRHa (0.01 μM) for 2, 6, 12 hours. The genes were identified following supervised analysis of their expression values and statistical analysis in R programming and ANOVA with a false-discovery rate of rate of $p \leq 0.001$

Example 7—Verification of Gene Transcripts in TGF-β-treated LSMC and MSMC

Using Realtime PCR, the present inventors validated the expression of 12 genes in response to time dependent action of TGF-β in LSMC and MSMC (Figures 6A-6R).

They include IL-11, CITED2, Nur77, EGR3, TIEG, TGIF, p27, p57, GAS-1 and GPRK5, whose expression was also validated in leiomyoma and matched myometrium from untreated and GnRHa-treated cohorts as well as LSMC and MSMC treated in vitro with GnRHa. In addition, the present inventors verified the expression of Runx1 and Runx2. As illustrated TGF-β in a time dependent manner differentially regulate the expression of these genes in LSMC and MSMC with a pattern of expression displaying significant overlap between Realtime PCR and microarray analysis (Figures 6A-6R). However, the expression value of GPRK5 and Runx2 genes in microarray analysis of LSMC and MSMC did not meet the standard of analysis and was not included among the list of differentially expressed and regulated genes in response to TGF-β, although Runx2 mRNA is detectable by Realtime PCR (Figures 6A-6R). The results indicated that Runx1 and Runx2 expression not only is the target of TGF-β regulatory action, they are also regulated by GnRHa therapy in leiomyoma and myometrium as well as by GnRHa in LSMC and MSMC *in vitro*, with their time-dependent inhibition in MSMC (Figures 6A-6R).

The present inventors verified the expression of IL-11, TIGF, TIEG, p27 and p57 by Western blotting and their cellular distribution using immunohistochemistry in leiomyoma and myometrium. These findings provide further support for the microarray and Realtime PCR data indicating that the products of these genes are expressed in leiomyoma and myometrium. The present inventors are currently investigating time-dependent and dose-dependent regulation of these genes in response to TGF-β.

By extending previous work on the role of TGF-β in leiomyoma, in this study, the present inventors have provided the first example of gene expression fingerprints of LSMC and MSMC in response to autocrine/paracrine action of TGF-β. The present inventors further characterized the molecular environment of these cells following pretreatment with TGF-β type IIR antisense as a tool to interfere with the autocrine/paracrine action of TGF-β isoforms, and comparatively assessed their expression profiles with GnRHa-treated cells, which also inhibits TGF-β receptor expression in these cells (Dou, Q. *et al. J Clin Endocrinol Metab*, 1996, 81:3222-3230; Chegini, N. *et al. Mol Hum Reprod*, 2002, 8:1071-1078). Since the aim of this study was to capture the early and late autocrine/paracrine action of TGF-β in these cells, the present inventors selected a treatment strategy based on previous observations reflecting TGF-β time-dependent regulation of c-fos, c-jun, fibronectin, collagen type I, and PAI-1 expression (Ding, L. *et*

al. J Clin Endocrinol Metab, 2004, 89:5549-5557). TGF-β regulates the expression of these genes in LSMC and MSMC through TGF-β receptor activation of Smad and MAPK pathways (Schnaper, H.W. *et al. Am J Physiol Renal Physiol*, 2003, 284:F243-252; Xu, J. *et al. J Clin Endocrinol Metab*, 2003, 88:1350-1361; Ding, L. *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557), whose promoters are known to contain TGF-β regulatory elements (Miyazono, K. *et al. Oncogene*, 2004, 23:4232-7; Moustakas, A. *et al. Immunol Lett*, 2002, 82:85-91). This study design is also consistent with other microarry studies profiling gene expression in response to TGF-β action in human dermal fibroblasts, HaCaT kritonocyte cell line and NMuMG, mouse mammary gland epithelial cell line, in which the cells were treated for 1, 2, 6 and 24 hours, displaying a Smad-mediated regulation of selected number of genes (Verrecchia, F. *et al. J Biol Chem*, 2001, 276:17058-17062; Zavadil, J. *et al. Proc Natl Acad Sci USA*, 2001, 98:6686-6691; Xie, L. *et al. Breast Cancer Res*, 2003, 5:R187-R198 25-27).

Cluster and tree-view analysis revealed a considerable similarity in overall gene expression patterns between LSMC and MSMC in response to TGF-β action; however, there was sufficient difference allowing their separation into respective subgroups. The genes in these clusters displayed different regulatory response to TGF-β action in a cell- and time-specific manner, with genes in clusters A and B displaying a late response, genes in cluster D displaying early responsiveness, and clusters C and E showing a biphasic regulatory behavior. These results suggest that the same factors and/or mechanisms co-regulate the expression of these genes in each cluster, possibly due to the presence of common regulatory elements in their promoters. Whether the expression profile of these genes in LSMC and MSMC respond differently to varying concentration of TGF-β, or other TGF-β isoforms is not established. However, the concentration of TGF-β used in this and other studies examining the effect of TGF-βon the expression of other genes (Xu, J. *et al. J Clin Endocrinol Metab*, 2003, 88:1350-1361; Ding, L. *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557; Tang, X.M. *et al. Mol Hum Reprod*, 1997, 3:233-40; Arici, A. and Sozen, I. *Am J Obstet Gynecol*, 2003, 188:76-83; Verrecchia, F. *et al. J Biol Chem*, 2001, 276:17058-17062; Zavadil, J. *et al. Proc Natl Acad Sci USA*, 2001, 98:6686-6691; Xie, L. *et al. Breast Cancer Res*, 2003, 5:R187-R198), is comparable with level of TGF-β produced by these cells, although LSMC produces more TGF-β1 compared to MSMC (Chegini, N. *et al. J Clin Endocrinol Metab*, 1999, 84:4138-43; Chegini, N. *et al. Mol Hum Reprod*, 2002, 8:1071-1078). Moreover, based on the profile of TGF-β isoforms's

expression in leiomyoma, it has previously been proposed that TGF-β1 and TGF-β3 play an more critical role in leiomyoma (Chegini, N. *et al. J Clin Endocrinol Metab*, 1999, 84:4138-43), and *in vitro* studies have indicated a higher growth response to TGF-β1 (personal observations) and TGF-β3 in LSMC compared to MSMC (Lee, B.S. and Nowak, R.A. *J Clin Endocrinol Metab*, 2001, 86:913-920; Arici, A. and Sozen, I. *Fertil Steril*, 2000, 73:1006-1011). However, TGF-β isoforms mediate their actions through TGF-β type IIR, and alterations in the TGF-β receptor system may serve as a more accurate indicator of their overall autocrine/paracrine actions in these and other cell types. It has been shown that leiomyoma over-expresses TGF-β type IIR compared to myometrium (Dou, Q. *et al. J Clin Endocrinol Metab*, 1996, 81:3222-3230; Chegini, N., Luo X, Ding L, Ripley D 2003 The expression of Smads and transforming growth factor beta receptors in leiomyoma and myometrium and the effect of gonadotropin releasing hormone analogue therapy. Mol Cell Endocrinol 209:9-16), and pretreatment of LSMC with TGF-β type IIR antisense oligomers and/or neutralizing antibodies prevented TGF-β receptor-mediated actions (Chegini, N. *et al. Mol Hum Reprod*, 2002, 8:1071-1078; Xu, J. *et al. J Clin Endocrinol Metab*, 2003, 88:1350-1361).

These observations as well as identification of specific genes whose expression exhibited sensitivity to pretreatment with TGF-β type IIR antisense, among them genes containing TGF-β regulatory response elements in their promoters, further support TGF-β receptors mediated signaling in regulating the overall expression of these genes in LSMC and MSMC, and possibly in leiomyoma and myometrium. Lack of response of other TGF-β-targeted genes to TGF-β type IIR antisense pretreatment could be due to inability of antisense to block all the autocrine/paracrine, as well as exogenously added TGF-β. However, the expression of these genes may also be regulated as a consequence of TGF-β receptors overexpression and/or their altered intracellular signaling. Interestingly, activin receptor-like kinases (ALK) ALK1 and ALK5, which serve as TGF-β type I receptors and are activated by TGF-β type II receptors, have been shown to regulate the expression of different genes in endothelial cell in response to TGF-β action (Ota, T. *et al. J Cell Physiol*, 2002, 193:299-318). However, ALK1 functions as a TGF-β type I receptor in endothelial cells, while ALK-5 is expressed in various cells, and through distinct Smad proteins, *i.e.*, Smad1/Smad5 and Smad2/Smad3, respectively, regulate gene expression in response to TGF-β actions (Ota, T. *et al. J Cell Physiol*, 2002, 193:299-318). The present inventors have identified the expression of all the components of the TGF-β receptor system,

including ALK5 and Smad2/3 in leiomyoma and myometrium as well as LSMC and MSMC. However, TGF-β-mediated action through ALK1 could result in the regulation of a different set of genes not involving ALK5. In addition to TGF-β and TGF-β receptors, alteration in Smad expression is also considered to influence the outcome of several disorders targeted by TGF-β including tissue fibrosis (Flanders, K.C. *Int J Exp Pathol*, 2004, 85:47-64).

Gene ontology dividing the differentially expressed and regulated genes into similar functional categories revealed that the majority of the genes targeted in response to TGF-β treatment of LSMC and MSMC are associated with cellular metabolism, cell growth regulation (cell cycle and apoptosis), cell and tissue structure (ECM, adhesion molecules and microfilements), signal transduction and transcription factors. Despite the differences in their profiles, the present inventors found a substantial degree of similarity in functional annotation among the genes identified at tissue (leiomyoma and myometrium) and cellular (LSMC and MSMC) levels in response to TGF-β1. These differences between gene expression profiles of tissues and LSMC/MSMC in response to TGF-β could be due to the contribution of other cell types to the gene pool, and the influence of other autocrine/paracrine regulators on the overall genes expression at the tissue level. Previous studies from this laboratory and others have reported the expression of a few other genes targeted by TGF-β action in LSMC and MSMC. However, to the present inventors' knowledge, this is the first example of a large-scale gene expression profiling of these cells in response to TGF-β. Using quantitative realtime PCR analysis, the presenti inventors validated the expression of several of these genes in response to time-dependent action of TGF-β in LSMC and MSMC, including the expression of 10 genes validated in leiomyoma/myometrium as well as in LSMC/MSMC in response to GnRHa treatment.

The present inventors demonstrated that LSMC express an elevated level of IL-11 compared to MSMC, and its expression is a major target of TGF-β regulatory action. Although the biological significance of IL-11 expression in leiomyoma and myometrial environments, and consequence of its overexpression in leiomyoma await investigation, IL-11, alone, or through interaction with TGF-β, is considered to play a critical role in progression of fibrotic disorders (Leng, S.X. and Elias, J.A. *Int J Biochem Cell Biol*, 1997, 29:1059-1062; Kuhn, C. *et al. Chest*, 2000, 117:260S-262S; Zhu, Z. *et al. Am J Respir Crit Care Med*, 2001, 164:S67-70; Chakir, J. *et al. J Allergy Clin Immunol*, 2003,

111:1293-1298). Other members of the interleukin family, IL-4 and IL-13, and their interactions with TGF-β are also reported to be equally important in this disorder (Wynn, T.A. *Nat Rev Immunol*, 2004, 4:583-594; Wynn, T.A. *Annu Rev Immunol*, 2003, 21:425-456). IL-13 expression has recently been identified in leiomyoma, and it has been discovered that exposure of LSMC to IL-13 upregulates the expression of TGF-β and TGF-β type II receptors in LSMC, suggesting a direct, and/or indirect regulatory function for IL-13 in mediating events leading to progression of tissue fibrosis in leiomyoma (Ding, L., Luo, X. Chegini, N. "The expression of IL-13 and IL-15 in leiomyoma and myometrium and their influence on TGF-b and proteases expression in leiomyoma and myometrial smooth muscle cells and SKLM, leiomyosarcoma cell line" *J Soc Gyncol Invest*, 2004, 00, 00). Other cytokines in this category including IL-4, IL-6, IL-8, IL-15, IL-17, TNF-α and GM-CSF are also expressed in leiomyoma and myometrium (Ding, L., Luo, X. Chegini, N. "The expression of IL-13 and IL-15 in leiomyoma and myometrium and their influence on TGF-b and proteases expression in leiomyoma and myometrial smooth muscle cells and SKLM, leiomyosarcoma cell line" *J Soc Gyncol Invest*, 2004, 00, 00; Chegini, N. "Implication of growth factor and cytokine networks in leiomyomas" In: Cytokines in human reproduction, J Hill ed. Wiley & Sons New York, 2000, 133-162; Chegini, N. *et al. J Soc Gynecol Investig*, 2003, 10:161-71). These cytokines are classified as type1/type2 related subsets and predominance toward type II direction is considered to result in inflammatory/immune responses leading to progression of tissue fibrosis (Zhu, Z. *et al. Am J Respir Crit Care Med*, 2001, 164:S67-70; Chakir, J. *et al. J Allergy Clin Immunol*, 2003, 111:1293-1298; Wynn, T.A. *Nat Rev Immunol*, 2004, 4:583-594; Wynn, T.A. *Annu Rev Immunol*, 2003, 21:425-456; Lee, C.G. *et al. J Exp Med*, 2004, 200:377-389). A recent report has further elaborated the participation of IL-11 and TGF-β, and transcription factor EGR1 in tissue fibrosis, through a mechanism involving regulation of the balance between the rate of cellular apoptosis and inflammatory response (Lee, C.G. *et al. J Exp Med*, 2004, 200:377-389). EGR1 has previously been identified among the differentially expressed genes in leiomyoma and myometrium (Chegini, N. *et al. J Soc Gynecol Investig*, 2003, 10:161-71) and expression of EGR2 and EGR3 in these tissues and regulation of EGR3 in response to TGF-βaction in LSMC and MSMC is demonstrated herein.

Elevated expression and preferential phosphorylation of EGR1 leads to regulation of target genes whose products are involved in apoptosis as well as angiogenesis and cell

survival, including IL-2, TNF-alpha, Flt-1, Fas, Fas ligand, cyclin D1, p15, p21, p53, PDGF-A, angiotensin II-dependent activation of PDGF and TGF-β, VEGF, tissue factor, 5-lipoxygenase, thymidine kinase, superoxide dismutase, intercellular adhesion molecule 1 (ICAM-1), fibronectin, urokinase-type plasminogen activator and matrix metalloproteinase type 1 (Thiel, G. and Cibelli, G. *J Cell Physiol*, 2002, 193:287-292; Khachigian, L.M. *Cell Cycle*, 2004, 3:10-1; Nagamura-Inoue, T. *et al. Int Rev Immunol*, 2001, 20:83-105; Liu, C. *et al. Cancer Gene Ther*, 1998, 5:3-28; Liu, C. *et al. J Biol Chem*, 1999, 274:4400-11; Baoheng, Du. *et al. J Biol Chem*, 2000, 275:39039–39047). The expression of many of these genes has been documented in myometrium and leiomyoma (Blobe, G.C. *et al. N Engl J Med*, 2000, 342:1350-1358), and known to be the target of TGF-βregulatory action. EGR1 also acts as a transcriptional repressor of TGF-β type II receptor through direct interaction with SP1 and Ets-like ERT sites in proximal promoter of the gene (Baoheng, Du. *et al. J Biol Chem*, 2000, 275:39039–39047). Transfection of EGR1 expression vector into a myometrial cell line (KW) expressing low levels of EGR1 is reported to result in a rapid growth inhibition of these cells (Shozu, M. *et al. Cancer Res*, 2004, 64:4677-4684). To the present inventors' knowledge, this is the first report of the regulatory action of TGF-β on EGR3 expression, not only in LSMC and MSMC, but any other cell types. Based on previous and present observations, the present inventors propose that a local inflammatory response mediated through individual and combined actions of TGF-β, IL-13 and IL-11, as well as regulatory function of TGF-β on EGR expression, results on local expression of set of genes whose products promote apoptotic and non-apoptotic cell death, further enhancing an inflammatory reaction that orchestrate various events leading to progression of fibrosis in leiomyoma.

Additional genes identified as differentially expressed and regulated by TGF-β autocrine/paracrine action in LSMC and MSMC in this functional category include TGIF, TIEG, CITED2, Nur77, Runx1 and Runx2. These transcription factors possess key regulatory functions in the expression of a wide range of genes in response to various stimuli specifically TGF-β. The expression of TGIF, TIEG, CITED2 and Nur77 is highly regulated in LSMC and MSMC, and with the exception of CITED2, TGF-β transiently increased their expression in a time-dependent manner. TGIF is a transcriptional co-repressor that directly associates with Smads and inhibits Smad-mediated transcriptional activation by competing with p300 for Smad association (Chen, F. *et al. Biochem J*, 2003, 371:257-263; Wotton, D. *et al. Cell Growth Differ*, 2001, 12:457-63). CITED2, induced

by multiple cytokines, growth factors and hypoxia, also interacts with p300 and function as a coactivator for transcription factor AP-2 (Tien, E.S. *et al. J Biol Chem*, 2004, 279:24053-63). CITED2-mediated action is reported to result in down-regulation of MMP-1 and MMP-13 through interactions with CBP/p300 and other transcription factors such as c-fos, Ets-1, NFκB, and Smads that control MMPs promoter activities (Yokota, H. *et al. J Biol Chem*, 2003, 278:47275-47280; Shi, Y. and Massague, J. *Cell*, 2003, 113:685-700). TGF-β targets the expression of these transcription factors and MMPs in many cell types, including LSMC and MSMC (Ding, L. *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557; Shi, Y. and Massague, J. *Cell*, 2003, 113:685-700; Ma, C. and Chegini, N. *Mol Hum Reprod*, 1999, 5:950-954), thus their differential regulation and interactions with CITED2 and TGIF may serve in regulating the outcome of TGF-β autocrine/paracrine actions in leiomyoma involving cell growth, inflammation, apoptosis and tissue turnover. Unlike TGIF, TIEG is rapidly induced by TGF-β and enhances TGF-β actions through Smad2/3 activation (Johnsen, S.A. *et al. Oncogene*, 2002, 21:5783-90; Cook, T. and Urrutia, R. *Am J Physiol Gastrointest Liver Physiol*, 2000, 278:G513-521; Ribeiro, A. *et al. Hepatology*, 1999, 30:1490-1497). However, TIEG has no effect on gene transcription in the absence of Smad4, or due to overexpression of Smad7, although it is capable of increasing Smad2/3 activity in the absence of Smad7 (Shi, Y. and Massague, J. *Cell*, 2003, 113:685-700; Johnsen, S.A. *et al. Oncogene*, 2002, 21:5783-90). It was shown that TGF-β induced a rapid, but transient expression of TIEG in LSMC and MSMC, and the expression of Smad2/3, Smad4 and Smad7 and their differential regulation by TGF-β has been demonstrated in these cells (Xu, J. *et al. J Clin Endocrinol Metab*, 2003, 88:1350-1361; Ding, L. *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557). Based on these observations, the present inventors further propose that TGF-β through a mechanism involving TGIF, TIEG and Smads self regulates its own autocrine/paracrine action in leiomyoma/ myometrium. Estrogen has also been shown to increase TIEG expression in breast tumor cell (Johnsen, S.A. *et al. Oncogene*, 2002, 21:5783-90; Sorbello, V. *et al. Int J Biol Markers*, 2003, 18:123-9). Since estrogen, a major growth-promoting factor for leiomyoma, induces TGF-β expression in LSMC and MSMC (Chegini, N. *et al. J Clin Endocrinol Metab*, 1999, 84:4138-43; Chegini, N. *et al. Mol Hum Reprod*, 2002, 8:1071-1078), E2-induced TGF-β or estrogen directly may regulate TIEG expression in leiomyoma. TIEG is also reported to trigger apoptotic cell programs by a mechanism involving the formation of reactive oxygen species (Ribeiro,

A. *et al. Hepatology*, 1999, 30:1490-1497), often created as a result of local inflammatory response. Whether TGF-β-induced TIEG through the above mechanism results in apoptotic response in leiomyoma is not known; however, formation of reactive oxygen species may enhance local inflammatory response serving as an additional mediator of tissue fibrosis in leiomyoma.

With respect to Nur77, it regulates the expression of a group of genes whose products are involved in cell cycle regulation, differentiation, apoptosis, and malignant transformation (Rajpal, A. *et al. EMBO J*, 2003, 22:6526-36; Castro-Obregon, S. *et al. J Biol Chem*, 2004, 279:17543-17553). Evidence has been provided that Nur77 is the target of regulatory action of TGF-βin LSMC and MSMC, with pattern of expression resembling that observed in leiomyoma and myometrium, respectively (Chegini, N. *et al. J Soc Gynecol Investig*, 2003, 10:161-71). Although the nature and functional significance of Nur77 expression in leiomyoma, and regulation by TGF-β is unknown, malignant transformation in leiomyoma is rare, suggesting Nur77 may function as regulator of cell cycle in leiomyoma and myometrium. In addition to Nur77, the present inventors discovered that the expression of various genes functionally associated with cell cycle regulation and apoptosis are influenced by TGF-β autocrine/paracrine action, and balance of their expression may become a critical factor in leiomyoma growth and regression. Additional transcription factors whose expression was the target of TGF-β action in LSMC and MSMC are Runx1 and Runx2. This family of transcriptional factors consisting of Runx1 to Runx3, are integral components of signaling cascades mediated by TGF-β and bone morphogenetic proteins regulating various biological processes, including cell growth and differentiation, hematopoiesis and angiogenesis (Miyazono, K. *et al. Oncogene*, 2004, 23:4232-7; Shi, Y. and Massague, J. *Cell*, 2003, 113:685-700; Levanon, D. and Groner, Y. *Oncogene*, 2004, 23:4211-4219; McCarthy, T.L. *et al. J Biol Chem*, 2003, 278:43121-43119; Ito, Y. and Miyazono, K. *Curr Opin Genet Dev*, 2003, 13:43-47). The present inventors provided the first evidence for regulatory action of GnRHa therapy and GnRHa direct action on Runx1 and Runx2 expression in leiomyoma, myometrium as well as LSMC and MSMC, with GnRHa significantly inhibiting their expression, specifically in MSMC. Although Runx2 is expressed at low levels in leiomyoma and myometrium, Runx1 and Runx2 expression in LSMC and MSMC displayed a rapid response to TGF-β action *in vitro*, with Runx1 showing a significantly higher response. TGF-β activation of Smad and MAPK cascades regulates the expression

of Runx2; however, interaction with Smad3 causes repression of Runx2 and downstream transcription activation of specific genes (Miyazono, K. *et al. Oncogene*, 2004, 23:4232-7; Shi, Y. and Massague, J. *Cell*, 2003, 113:685-700; Ito, Y. and Miyazono, K. *Curr Opin Genet Dev*, 2003, 13:43-47). It has recently been reported that TGF-β and GnRH activate the MAPK pathway (Ding, L. *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557), and GnRHa alter TGF-β-activated Smad in LSMC and MSMC (Xu, J. *et al. J Clin Endocrinol Metab*, 2003, 88:1350-1361), signaling cascade that may regulate Runx1 and Runx2 expression in these cells. Differential regulation of Runx1 and Runx2 by TGF-β and GnRHa imply their potential biological implication, specifically in regulating TGF-β action in leiomyoma microenvironment. This is particularly interesting since estrogen is also reported to enhance Runx2 activity, through a mechanism involving TGF-β type I receptor gene promoter, which contains several Runx binding sequences (McCarthy, T.L. *et al. J Biol Chem*, 2003, 278:43121-43119). Together, the identification of these and several other key transcription factors in LSMC and LSMC, and their regulation by TGF-β serving as integral components of inflammatory, cell cycle and apoptotic processes, further support the present inventors' hypothesis that a regulatory balance between these events is a key factor in progression of fibrosis mediated by TGF-β in leiomyoma.

Such balance between cell proliferation and apoptosis is critical to tissue homeostasis and central to leiomyoma growth and regression. Since both positive and negative signals determine the outcome of these events, the present inventors searched and identified several genes in this category in previous studies and in the current study as differentially expressed and regulated in leiomyoma and myometrium, as well as in LSMC and MSMC in response to TGF-β. The primary focus here was placed on p27Kip1, p57Kip2 and Gas1 expression, because of their regulation by GnRHa. It was found that TGF-β suppressed the expression of these genes in LSMC, and in a biphasic fashion accompanied by suppression of GAS1 expression in MSMC. TGF-β is known to regulate the expression of several cell cycle regulatory proteins including p27, which bind cyclin-dependent kinase (CDK), and by inhibiting catalytic activity of CDK-cyclin complex, regulate cell cycle progression and apoptosis (Reed, S.I. *Nat Rev Mol Cell Biol*, 2003, 4:855-64). However, TGF-β regulation of p57 expression is limited (Miyazono, K. *et al. Oncogene*, 2004, 23:4232-7; Moustakas, A. *et al. Immunol Lett*, 2002, 82:85-91; Kim, S.J. and Letterio, J. *Leukemia*, 2003, 17:1731-7) and available data suggests that TGF-β enhances p57 degradation through ubiquitin-proteasome pathway and Smad-

mediated signaling (Nishimori, S. *et al. J Biol Chem*, 2001, 276:10700-10705). TGF-β-induced p57 degradation, CDK2 activation and cell proliferation is blocked by proteasome inhibitors and/or by overexpression of Smad7 (Nishimori, S. *et al. J Biol Chem*, 2001, 276:10700-10705; Yokoo, T. *et al. J Biol Chem*, 2003, 278:52919-52923; Brown, K.A. *et al. Breast Cancer Res*, 2004, 6:R130-R139; Kawaguchi, K. *et al. Hum Pathol.*, 2004, 2004;35:61-8). TGF-β-induced cell growth is also influenced by c-myc and the expression and activities of G1, G2, CDK and cyclins, and their inhibitors p15INκ4b and p21 (Miyazono, K. *et al. Oncogene*, 2004, 23:4232-7; Moustakas, A. *et al. Immunol Lett*, 2002, 82:85-91; Shi, Y. and Massague, J. *Cell*, 2003, 113:685-700), and they were identified among differentially expressed and regulated genes in LSMC and MSMC by TGF-β (Chegini, N. *et al. J Soc Gynecol Investig*, 2003, 10:161-71). With respect to Gas1, to the present inventors' knowledge, this observation is the first to demonstrate Gas1 expression in human uterine tissue and its regulation by TGF-β. GAS1 acts as a negative regulator of the cell cycle and has been positively correlated with the inhibition of endothelial cell apoptosis and the integrity of resting endothelium (Spagnuolo, R. *et al. Blood*, 2004, 103:3005-12). Similar to p15, p21 and p27, myc suppresses the expression of GAS1 by limiting myc-max heterodimers binding to their promoters, (Gartel, A.L. and Shchors, K. *Exp Cell Res*, 2003, 283:17-21; Lee, T.C. *et al. Proc Natl Acad Sci USA*, 1997, 94:12886-91). GAS1 is also reported to suppress growth and tumorigenicity of human tumor cells, and overexpression of MDM2, or p53 mutation inhibits Gas1-mediated action (Evdokiou, A. and Cowled, P.A. *Exp Cell Res*, 1998, 240:359-67). The present inventors have identified max and MDM2 expression in LSMC and MSMC and their regulation by TGF-β, suggesting their potential interactions in leiomyoma cellular environment. It was previously reported that TGF-β isoforms stimulate DNA synthesis, but not cell division in LSMC and MSMC, suggesting that p27, p57 and Gas1, as well as the products of other cell cycle regulators, may influence the effect of TGF-β action on leiomyoma cell growth late in the S to M phases of the cell cycle transition. Collectively, the identification of several genes in this category, whose products regulate cell cycle progression as target of TGF-β autocrine/paracrine action in LSMC and MSMC, further indicate the importance of TGF-β in regulating the balance between cell proliferation, cell cycle arrest and apoptosis whose outcome directs leiomyoma growth and/or regression.

Expression and activation of various components of signal transduction pathways are essential for mediating the cellular actions of growth factors, cytokines, chemokines, polypeptide hormones, and adhesion molecules. The present inventors identified several genes functionally belonging to this category as differentially expressed and regulated in LSMC and MSMC in response to TGF-β action, among them are member of family of Ras/Rho, Smads and MAPK, guanine nucleotide binding protein alpha, GTP-binding protein overexpressed in skeletal muscle, PTK2 protein tyrosine kinase 2, S100 calcium-binding protein A5, adenylate cyclase 9, CDC-like kinase 2, Cdc42 effector protein 4, retinoic acid induced 3, receptor tyrosine kinase-like orphan receptor 1, LIM protein and LIM domin kinase 2, phosphodiesterase 4D (cAMP-specific), protein phosphatase alpha, serine/threonine kinase 17a (apoptosis-inducing), focal adhesion kinase 2, STATs, *etc.* Although, Smad and MAPK pathways are known to be recruited and activated by TGF-β receptors, including in LSMC and MSMC, the components of other pathways are not the target of TGF-β. However, many growth factors, cytokines, chemokines, polypeptide hormones and adhesion molecules, expressed by LSMC and MSMC, either alone or through crosstalk with TGF-β receptor signaling may activate various components of the other pathways (Blobe, G.C. *et al. N Engl J Med*, 2000, 342:1350-1358; Chegini, N. "Implication of growth factor and cytokine networks in leiomyomas" In: Cytokines in human reproduction, J Hill ed. Wiley & Sons New York, 2000, 133-162; Chegini, N. *et al. J Soc Gynecol Investig*, 2003, 10:161-71), although only the expression and activation of a few of these molecules has been demonstrated in leiomyoma and myometrium, and in LSMC and MSMC. Since GPRK5 expression was detected in leiomyoma and myometrium and was the target of GnRHa action in LSMC and MSMC, the present inventors further investigated and found GPRK5 expression is regulated by TGF-β. The biological implication of GPRK5 and regulation by TGF-β in LSMC and MSMC is unclear; however, GPKs serve as negative regulators of GPCR mediated biological responses through the generation of second messengers, such as cAMP and calcium/calmodulin, and down-regulation of their activity (desensitization) (Luo, J. and Benovic, J.L. *J Biol Chem*, 2003, 278:50908-14; Miyagawa, Y. *et al. Biochem Biophys Res Commun*, 2003, 300:669-73; Cornelius, K. *et al. J. Biol. Chem*, 2001, 276:1911-1915). Activation of calcium/calmodulin is reported to alter Smad function, with inhibition of calmodulin resulting in an increase in activin-dependent induction of target genes, whereas its overexpression decreased activin- and TGF-βaction (Miyazono, K. *et*

al. Oncogene, 2004, 23:4232-7; Moustakas, A. *et al. Immunol Lett*, 2002, 82:85-91; Shi, Y. and Massague, J. *Cell*, 2003, 113:685-700). The result suggests that GPRK may act as downstream regulator of TGF-β receptor singling possibly through modulation of PKC, MAPK and/or calmodulin and hence influencing TGF-β autocrine/paracrine action in leiomyoma.

Tissue remodeling is also a critical step in progression of fibrotic disorders and modulation of ECM, adhesion molecules and protease expression, and phenotypic changes toward a myofibroblastic phenotype are essential components of this process (Blobe, G.C. *et al. N Engl J Med*, 2000, 342:1350-1358; Gabbiani, G. *J Pathol*, 2003, 200:500-3; Phan, S.H. *Chest*, 2002, 122:286S-289S; Shephard, P. *et al. Thromb Haemost*, 2004, 92:262-74; Gauldie, J. *et al. Curr Top Pathol*, 1999, 93:35-45). In this study and the previous study, the presenti inventors identified the expression of several genes in this category in leiomyoma and myometrium, as well as LSMC and MSMC including fibronectin, collagens, decorin, versican, desmin, vimentin, fibromodulin, several member of intergrin family, desmoplakin, extracellular matrix protein 1, enhancer of filamentation 1, porin, SPARC-like 1, syndecan 4, endothelial cell-specific molecule 1, as well as MMPs, TIMPs and ADAMs (Chegini, N. *et al. J Soc Gynecol Investig*, 2003, 10:161-71). The expression of fibronectin, vimentin, collagen type 1, fibromodulin, MMP1, MMP2 and MMP9, TIMPs in leiomyoma and myometrium has been demonstrated and showed that TGF-β, through the activation of MAPK, regulates the expression of some of these genes (Ding, L. *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557; Yokota, H. *et al. J Biol Chem*, 2003, 278:47275-47280; Dou, Q. *et al. Mol Hum Reprod*, 1997, 3:1005-14). Of particular interest are the elevated expression of decorin, vimentin and fibromodulin in leiomyoma since they are considered to regulate the outcome of tissue fibrosis and their ability to bind TGF-β, thus controlling TGF-β autocrine/paracrine action (Blobe, G.C. *et al. N Engl J Med*, 2000, 342:1350-1358; Chegini, N. "Implication of growth factor and cytokine networks in leiomyomas" In: Cytokines in human reproduction, J Hill ed. Wiley & Sons New York, 2000, 133-162; Levens E, Luo X, Ding L, Williams RS, Chegini N "Differential Expression of fibromodulin and Abl-interactor 2 in leiomyoma and myometrium and regulation by gonadotropin releasing hormone analogue (GnRHa) therapy" *Fertil Steril*, 2004, (In press); Chakravarti, S. *Glycoconj J*, 2002, 19:287-93). Since leiomyoma is believed to derive from transformation of myometrial connective tissue fibroblast and/or smooth muscle cells, the expression of vimentin in

leiomyoma/LSMC imply that these cells have adopted a myofibroblastic characteristic. While granulation tissue myofibroblasts are derived from local fibroblasts, other cell types including smooth muscle cells have the potential to acquire a myofibroblastic phenotype (Lee, C.G. *et al. J Exp Med*, 2004, 200:377-389; Gabbiani, G. *J Pathol*, 2003, 200:500-3; Phan, S.H. *Chest*, 2002, 122:286S-289S; Shephard, P. *et al. Thromb Haemost*, 2004, 92:262-74). These cells express various cytokines including GM-CSF, IL-11 and TGF-β of which GM-CSF is considered to participate in fibroblasts transformation into myofibroblasts and enhancing their TGF-β expression (Gabbiani, G. *J Pathol*, 2003, 200:500-3; Phan, S.H. *Chest*, 2002, 122:286S-289S; Shephard, P. *et al. Thromb Haemost*, 2004, 92:262-74). It has been shown that GM-CSF is a key regulator of TGF-β in LSMC, and their interaction and as well as the involvement of other cytokines such as IL-11 and IL-13 regulate various events leading to leiomyoma formation and progression of fibrosis (Ding, L. *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557; Ding L, Luo X Chegini N "The expression of IL-13 and IL-15 in leiomyoma and myometrium and their influence on TGF-b and proteases expression in leiomyoma and myometrial smooth muscle cells and SKLM, leiomyosarcoma cell line" *J Soc Gyncol Invest*, 2004, 00, 00). IL-11 either alone or through the induction of TGF-β is reported to alter myofibroblasts ECM turnover resulting in the progression of tissue fibrosis (Lee, C.G. *et al. J Exp Med*, 2004, 200:377-389; Bamba, S. *et al. Am J Physiol Gastrointest Liver Physiol*, 2003, 285:G529-38). Despite the importance of tissue turnover in the pathophysiology of leiomyoma, little data are currently available of the extent of ECM expression and the difference that may exist compared to myometrium, that contribute to the fibrotic characteristic of leiomyoma.

In conclusion, as a continuation of work with TGF-β, the present inventors have provided the first large-scale example of gene expression profile of LSMC and MSMC identifying specific cluster of genes whose expression is targeted by autocrine/paracrine action of TGF-β. The present inventors validated the expression of a selective number of these genes functionally recognized to regulate inflammatory response, angiogenesis, cell cycle, apoptotic and non-apoptotic cell death, and ECM matrix turnover, events that are central to leiomyoma pathobiology. Based on the present work and previous work with TGF-β, the present inventors propose that the individual and combined action of TGF-β with other profibrotic cytokines such as IL-11, orchestrate local inflammatory responses mediated through and influenced by the expression of genes whose products regulate cell

cycle progression, angiogenesis, apoptosis and tissue turnover, providing an environment leading to the progression of fibrosis.

Example 8—Differential Expression of Fibromodulin and Abl-interactor 2 in Leiomyoma and Myometrium and Regulation by Gonadotropin Releasing Hormone Analogue (GnRHa) Therapy

To validate the expression of fibromodulin and Abl-interactor 2 (Abi-2) that were identified as being differentially expressed in leiomyomata and myometrium and were regulated by GnRHa therapy. Fibromodulin is considered to have an anti-fibrotic role in wound repair and may be a biologically relevant modulator of TGF-beta activity during scar formation. Abl-interactor 2 encodes a non-receptor tyrosine kinase, c-Abl, that has been implicated in a variety of cellular processes including cell growth, reorganization of cytoskeleton, cell death and stress responses. Accordingly, a prospective study determining the tissue gene expression profile of myometrium and elimyoma using Real-time polymerase chain reaction (PCR) was carried out. Portions of leiomyoma and matched unaffected myometrium were collected from premenopausal women (N=27) who were scheduled to undergo hysterectomy for indications related to symptomatic leiomyoma. Seven of the patients received GnRHa therapy for three months prior to surgery. The untreated patients did not receive any medications (including hormonal therapy) during the 3 months prior to surgery.

Based on endometrial histology and the patient's last menstrual period, the tissue samples were identified as being from the proliferative (N=8) or the secretory (N=12) phase of the menstrual cycle. Total RNA was isolated and subjected to Real-time PCR. The results were analyzed using unpaired Student-test and Tuckey test (ANOVA) with a probability level of $P<0.05$ considered significant. These results for the first time document expression of fibromodulin and Abi-2 in leiomyoma and myometrium and provide evidence that the expression of these genes is influenced by ovarian steroids and possibly by a direct action of GnRHa on myometrial and leiomyoma cells.

Materials and Methods

The following materials and methods describe those utilized in Examples 9-13. All the materials for Realtime PCR, immunoblotting and immunohistochemistry were purchased from APPLIED BIOSYSTEM (Foster City, CA), BIORAD (Hercules, CA),

and VECTOR Laboratories (Burlingame, CA), respectively. Leuprolide acetate (GnRHa) was purchased from SIGMA Chemical (St Louis, MO), human recombinant TGF-β1, polyclonal antibody to CCN4 (WISP-1) were purchased from R&D System (Minneapolis, MN). Polyclonal antibodies to CTGF (CCN2), NOV (CCN-3), fubulin-1C and SA100A4 were purchased from SANTA CRUZ Biotechnology (Santa Cruz, CA). U0126, MEK1/2 synthetic inhibitor was purchased from CALBIOCHEM (San Diago, CA).

Portions of leiomyoma and matched myometrium were collected from premenopausal women (N=27) who were scheduled to undergo hysterectomy for symptomatic uterine leiomyomas at the University of Florida affiliated Shands Hospital. Of these patients seven received GnRHa therapy for a period of three months prior to surgery. The untreated patients did not receive any medications during the 3 months prior to surgery and, based on endometrial histology and patient last menstrual cycle, they were from proliferative (N=8) and secretory (N=12) phases of the menstrual cycle. To maintain a standard, leiomyomas used in this study were 2 to 3 cm in diameter. Prior approval was obtained from the University of Florida Institutional Review Board for the experimental protocol of this study.

Isolation and Culture of Leiomyoma and Myometrial Smooth Muscle Cells. Leiomyoma and myometrial smooth muscle cells (LSMC and MSMC) were isolated and cultured as previously described (Chegini, N. *et al. Mol Hum Reprod*, 2002, 8:1071-1078). Prior to use in these experiments, the primary cell cultures were characterized using immunofluroscence microscopy and antibodies to □smooth muscle actin, desmin and vimentin (Chegini, N. *et al. Mol Hum Reprod*, 2002, 8:1071-1078). LSMC and MSMC were cultured in 6-well plates at an approximate density of 10^6 cells/well in DMEM-supplemented media containing 10% FBS. After reaching visual confluence, the cells were washed in serum-free media and incubated for 24 hrs under serum-free, phenol red-free condition (Chegini, N. *et al. Mol Hum Reprod*, 2002, 8:1071-1078; Ding, L. *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557). These cells were used for the following experiments.

The Expression of CCNs, Fibulin-1C and S100A4 and Regulation by TGF-beta and GnRHa. To determine whether TGF-beta and GnRHa influence the expression of CCNs, fibulin-1C and S100A4, LSMC and MSMC cultured as above were treated with TGF-β1 (2.5 ng/ml) or GnRHa (0.1μM) for 2, 6 and 12 hrs (Ding, L. *et al. J Clin*

Endocrinol Metab, 2004, 89:5549-5557). Since TGF-beta and GnRHa action in LSMC and MSMC is mediated in part through activation of MAPK pathway (Ding, L. *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557), the present inventors further determined whether inhibition of MAPK activation alters TGF-beta and GnRHa effects on CCNs, fibulin-1C and S100A4 expression. LSMC and MSMC were cultured as above and following pretreatment with U0126 (20 μg/ml), a synthetic inhibitor of ERK1/2, for 2 hrs (Ding, L. *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557), the cells were treated with TGF-beta1 (2.5 ng/ml) or GnRHa (0.1μM) for 2hrs.

Activation of Smad also serves a major signaling pathway for TGF-β mediated action in LSMC and MSMC (Shi, Y. and Massague, J. *Cell*, 2003, 113:685-700; Xu, J. *et al. J Clin Endocrinol Metab*, 2003, 88:1350-1361). To determine whether TGF-β action in regulating the expression of CCNs, fibulin-1C and S100A4 is mediated through Smad pathway, LSMC and MSMC were cultured as above and transfected with Smad3 SiRNA designed using Dharmacon Inc (Lafayette, CO) tool with the target sequence of 5'-UCCGCAUGAGCUUCGUCAAAdTdT-3' as previously described (Kim, B.C. *et al. J Biol Chem.*, 2004, 279:28458-28465). LSMC and MSMC at 80% confluence were transfected with SiRNA using transfectamine 2000 reagent according to the manufacturer's instructions (Inveritogen, Carlsbad, CA), with 200 pmol of SiRNA and 10 μl of transfection reagent for 48 hrs. The cells were then treated with TGF-β1 (2.5 ng/ml) for 2 hrs. Untreated or cells treated with scrambled Smad3 SiRNA were used a negative control. Total RNA was isolated from the treated and untreated controls cells and subjected to Realtime PCR.

Realtime PCR. Total RNA was isolated using Trizol Reagent (invitrogen) and the level of TGF-β1, TGF-β3, CCNs, fibulin-1C and S100A4 mRNA expression was determined by Realtime PCR as previously described using Taqman and ABI-Prism 7700 (Applied Biosystems) and Sequence Detection System 1.91 software (Ding, L. *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557). Results were analyzed using comparative method following normalization of expression values to the 18S rRNA expression as previously described (Ding, L. *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557).

Western Blot Analysis and Immunohistochemical Localization. For Western blotting, total protein was isolated from small portions of GnRHa-treated and untreated leiomyoma and myometrium as previously described (Xu, J. *et al. J Clin Endocrinol*

Metab, 2003, 88:1350-1361; Ding, L. *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557). The homogenates' protein contents were determined, and an equal amount was subjected to SDS-PAGE and transferred to polyvinyldiene difluoride membrane. The blots were incubated with anti-CCN2, CCN3, CCN4, fibulin-1C, and S100A4 antibodies for 1 hr at room temperature. The membranes were exposed to corresponding HRP-conjugated IgG and immunostained proteins were visualized using enhanced chemiluminesence reagents (AMERSHAM-PHARMACIA Biotech, Piscataway, NJ) as previously described (Xu, J. *et al. J Clin Endocrinol Metab*, 2003, 88:1350-1361; Ding, L. *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557).

For immunohistochemical localization, tissue sections were prepared from formalin-fixed and paraffin-embedded leiomyoma and myometrium and subjected to standard processing. The sections were then immunostained using antibodies to CCN2, CCN3, CCN4, fibulin-1C, and S100A4 at 5μg of IgG/ml for 2-3 hrs at room temperature. Following further processing including incubation with biotinylated secondary antibodies and avidin-conjugated HRP (ABC ELITE kit, VECTOR Laboratories, Burlingame, CA), the chromogenic reaction was detected with 3,3'-diaminobenzidine tetrahydrochloride solution. Omission of primary antibodies, or incubation of tissue sections with non-immune mouse-rabbit and -goat IgGs instead of primary antibodies at the same concentration during immunostaining served as controls (Xu, J. *et al. J Clin Endocrinol Metab*, 2003, 88:1350-1361).

All the experiments were performed at least three times in duplicate using independent cell cultures. Where appropriate the results are expressed as mean ± SEM and statistically analyzed using unpaired Student t-test and ANOVA. A probability level of $P<0.05$ was considered significant.

Example 9—Expression of CCNs, Fibulin-1C and S100A4 in Leiomyoma and Myometrium and the effect of GnRHa Therapy

Using Realtime PCR the present inventors validated the expression of CCN2 (CTGF), CCN3 (NOV), CCN4 (WISP-1), fibulin-1C and S100A4 mRNA in leiomyoma and myometrium, demonstrating a significantly lower expression of CCN2, CCN3 and S100A4, with higher expression of fibulin 1C in leiomyoma as compared to myometrium (Figures 8A-8E; $p<0.05$). The level of CCN4 mRNA displayed a trend toward lower

expression as compared to myometrium, but these levels did not reach statistical significance. GnRHa therapy resulted in significant reduction in CCN3, CCN4, and S100A4 expression in myometrium. Conversely, GnRHa therapy did significantly affect the expression of the above genes in leiomyoma with the exception of CCN2 ($p<0.05$; Figures 8A-8E).

As illustrated in Figure 9, leiomyoma and matched myometrium from proliferative and secretory phase of the menstrual cycle express variable levels of CCN2, CCN3, CCN4 and fibulin-1C proteins; however, quantitating their levels was not attempted in this study. The SA100A4 antibody was not useful for Western analysis and several attempts failed to detect any immunoreactive proteins in either tissue or cell extracts. Immunohistochemically, CCN2, CCN3, CCN4, fibulin-1C and S100A4 were localized in leiomyoma and myometrial smooth muscle cells, connective tissue fibroblasts and vasculature (Figures 10A-10L). The present inventors observed mostly cytoplasmic localization with a considerable heterogeneity in immunostaining intensity among various cell types. Incubation with normal rabbit (Figure 10K) or goat (Figure 10L) sera resulted in a considerable reduction in immunostaining intensity associated with these cells.

Example 10—Correlation of CCNs with TGF-β Expression

The present inventors have previously reported that leiomyoma and LSMC express elevated levels of TGF-β isoforms (TGF-β1, β2 and β3) as compared to myometrium and MSMC (Chegini, N. *et al. J Clin Endocrinol Metab*, 1999, 84:4138-4143; Chegini, N. *et al. Mol Hum Reprod*, 2002, 8:1071-1078; Chegini, N. *et al. Mol Cell Endocrinol*, 2003, 209:9-16; Xu, J. *et al. J Clin Endocrinol Metab*, 2003, 88:1350-1361; Ding, L. *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557; Tang, X.M. *et al. Mol Hum Reprod*, 1997, 3:233-240; Arici, A. and Sozen, I. *Am J Obstet Gynecol*, 2003, 188:76-83; Lee, B.S. and Nowak, R.A. *J Clin Endocrinol Metab*, 2001, 86:913-920; Arici, A. and Sozen, I. *Fertil Steril*, 2000, 73:1006-1011). Here, the present inventors confirmed these results showing that leiomyoma expressed a higher level of TGF-β1 compared to TGF-β3, with elevated levels as compared to myometrium ($p<0.05$; Figures 11A and 11B). In addition, leiomyoma express significantly higher levels of total and active TGF-β1 as compared to myometrium ($p<0.05$, Figures 11A and 11B). Since TGF-β action on tissue

fibrosis is considered to be indirect and mediated through the induction of CCN2, the present inventors compared the expression of CCN2 with that of TGF-β1 and TGF-β3 in leiomyoma and myometrium. As shown in Figures 11A-11B and 8A-8E, not only the expression CCN2, but also the expression of CCN3 and CCN4 were inversely correlating with the expression of TGF-β1 and TGF-β3 in leiomyoma and myometrium.

Example 11—The Expression of CCNs, Fibulin1C and S100A4 in LSMC and MSMC and regulation by TGF-β

To evaluate whether TGF-β regulates the expression of CCN2 in leiomyoma and myometrium, the present inventors isolated LSMC and MSMC from these tissues and showed that these cells express CCNs, fibulin1-C and S100A4 and regulated by TGF-β1 (Figures 12A-12E). As shown in Figures 12A-12E, TGF-β in a cell- and time-dependent manner significantly increased the expression of CCN2 by 10 to 25 fold, and CCN4 by two fold, while inhibiting the expression of CCN3 ($P<0.05$). However, TGF-β1 had a limited effect on the expression of fibulin-1C and S100A4, moderately inhibiting their expression in LSMC and MSMC, while increasing fibulin-1C expression in MSMC ($p<0.05$; Figures 12A-12E).

Example 12—The effect of GnRHa on the Expression of CCNs, Fibulin1C and S100A4 in LSMC and MSMC

Conventional and microarray studies, including the inventors' own, have identified the expression profile of several genes targeted by GnRHa in leiomyoma and myometrial smooth muscle cells (Luo, X. *et al. Endocrinology*, 2005, 146:1074-1095; Luo, X. *et al. Endocrinology*, 2005, 146:1096-1118). The present inventors further assessed the direct action of GnRHa on CCNs, fibulin-1C and S100A4 expression following treatment of serum-starved LSMC and MSMC with GnRHa. As illustrated in Figures 13A-13E, GnRHa (0.1 μM) treatment for 2, 6 and 12 hrs in a time- and cell-dependent manner inhibited the expression of CCN2, CCN3, CCN4, fibluin-1C and S100A4 in LSMC and MSMC, with an increased expression of S100A4 in LSMC after 2 and 6 hrs of treatment as compared to MSMC ($p<0.05$).

Example 13—Inhibition of MAPK and Smad3 pathways on TGF-β and GnRHa-mediated Action

TGF-β and GnRH recruit and activate Smad and MAPK signaling pathways, respectively targeting the expression of many genes including fibronectin, collagen, MMPs, TIMPs, plasminogen activator inhibitor (PAI-1), c-fos and c-jun in LSMC and MSMC (Xu, J. *et al. J Clin Endocrinol Metab*, 2003, 88:1350-1361; Ding, L. *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557; Arici, A. and Sozen, I. *Fertil Steril*, 2000, 73:1006-1011; Dou, Q. *et al. Mol Hum Reprod*, 1997, 3:1005-1014; Ma, C. and Chegini, N. *Mol Hum Reprod*, 1999, 5:950-954; Luo, X. *et al. Endocrinology*, 2005, 146:1074-1095; Luo, X. *et al. Endocrinology*, 2005, 146:1096-1118). To determine whether TGF-β and GnRHa regulate the expression of CCNs, fibulin-1C and S100A4 in LSMC and MSMC through these pathways, the cells were pretreated with MEK1/2 inhibitor (U0126). As shown in Figures 14A-14E pretreatment with U0126 altered the basal expression of CCN2, CCN3, CCN4, fibulin-1C and S100A4 in LSMC and MSMC, with a limited effect on TGF-β-mediated action on CCN2, but inhibited CCN3 expression in MSMC, and CCN4, fibulin-1C and S100A4 expression in both LSMC and MSMC ($p<0.05$). Pretreatment with U0126 also altered GnRHa-mediated action on CCN2, CCN3, CCN4, fibulin-1C and S100A4 expression in LSMC and MSMC in cell specific manner (Figures 14A-14E).

Transfection of LSMC and MSMC with Smad3 SiRNA, but not scrambled SiRNA significantly reduced the expression of Smad3 mRNA in LSMC and MSMC. Transfection with Smad3 SiRNA had a limited effect on the expression of CCN2, CCN4, fibulin-1C or S100A4 expression, although it increased the expression of CCN3 in both MSMC and LSMC (Figures 15A-15E). Treatment of Smad3 SiRNA-transfected cells with TGF-β1 for 2 hrs resulted in a significant enhancement of TGF-β1-mediated action on CCNs, fibulin-1C and S100A4 in both LSMC and MSMC (Figures 15A-15E).

In the present study, the present inventors demonstrated that leiomyoma and myometrium expresses several components of CCN family, as well as fibulin-1C and S100A4. The present inventors showed that leiomyoma expresses significantly lower levels of CCN2, CCN3 and S100A4, while expressing more fibulin-1C as compared to myometrium, with several cell types including LSMC and MSMC as their major source of local expression. The present inventors also provided the first evidence that GnRHa

therapy alters the expression of CCN2 without affecting CCN3, CCN4 or fibulin-1C expression. The present inventors extended these observations and further demonstrated the expression of these genes in LSMC and MSMC and their regulation by TGF-β1 and GnRH through Smad and MAPK signaling pathway, respectively.

With respect to leiomyoma and myometrial expression of CCNs, fibulin-1C and S100A4 a limited correlation between levels of their expression and the phases of the menstrual cycle was found. Other studies have also reported a lack of menstrual cycle-dependent and lower expression of CCN1 (Cyr61), CCN2 and CCN5 in leiomyoma as compared to myometrium, except with higher expression of CCN5 in tissues from proliferative phase of the menstrual cycle and lowest expression detected during menstrual period (Sampath, D. *et al. J Clin Endocrinol Metab*, 2001, 86:1707-1715; Weston, G. *et al. Mol Hum Reprod*, 2003, 9:541-549; Mason, H.R. *et al. Mol Hum Reprod*, 2004, 10:181-187). Estrogen has been reported to regulate the expression of CCN5 in rat uterus (Mason, H.R. *et al. Mol Hum Reprod*, 2004, 10:181-187) and in human breast cancer cell lines (Sampath, D. *et al. Endocrine*, 2002, 18:147-159), as well as the expression of CCN1 in myometrial, but not in leiomyoma's explant cultures, whereas progesterone receptor agonist, R5020, alone or in combination with E2 had no effect (Sampath, D. *et al. J Clin Endocrinol Metab*, 2001, 86:1707-1715; Sampath, D. *et al. Endocrine*, 2002, 18:147-159; Sampath, D. *et al. Endocrinology*, 2001, 142:2540-2548). Considering that leiomyoma overexpresses estrogen and progesterone receptors as compared to myometrium, the expression profile of CCNs in these tissues suggests either a lack of, or an equal regulatory function for ovarian steroids. Since GnRHa therapy creates a hypoestrogenic condition, alteration in the expression of these genes in GnRHa-treated group imply a regulatory function for ovarian steroids. However, GnRHa therapy only affected the expression of CCN2, suggesting factors other than ovarian steroids may influence the expression of other members of CCN family in leiomyoma and myometrium. In this context, bFGF has been shown to increase the expression of CCN1 in myometrial, but not leiomyoma explants (Sampath, D. *et al. J Clin Endocrinol Metab*, 2001, 86:1707-1715). Unlike bFGF action on CCN1 expression, the present inventors found that TGF-β1 is equally effective in regulating the expression of CCN2, CCN3 and CCN4 in LSMC and MSMC, by increasing the expression of CCN2 and CCN4, while inhibiting CCN3.

TGF-β is a key profibrotic cytokine whose action on tissue fibrosis is considered to be indirect and mediated through the induction of CCN2 (Schnaper, H.W. *et al. Am J Physiol Renal Physiol*, 2003, 284:F243-F252; Ihn, H. *Curr Opin Rheumatol*, 2002, 14:681-685; Leask, A. and Abraham, D.J. *Biochem Cell Biol*, 2003, 81:355-363). Leiomyomas have several characteristic features typical of fibrotic disorder, including overexpression of TGF-β, TGF-β receptors and Smads as compared to normal myometrium (Dou, Q. *et al. J Clin Endocrinol Metab*, 1996, 81:3222-3230; Chegini, N. *et al. J Clin Endocrinol Metab*, 1999, 84:4138-4143; Chegini, N. *et al. Mol Hum Reprod*, 2002, 8:1071-1078; Chegini, N. *et al. Mol Cell Endocrinol*, 2003, 209:9-16; Xu, J. *et al. J Clin Endocrinol Metab*, 2003, 88:1350-1361; Ding, L. *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557; Tang, X.M. *et al. Mol Hum Reprod*, 1997, 3:233-240; Arici, A. and Sozen, I. *Am J Obstet Gynecol*, 2003, 188:76-83; Lee, B.S. and Nowak, R.A. *J Clin Endocrinol Metab*, 2001, 86:913-920; Arici, A. and Sozen, I. *Fertil Steril*, 2000, 73:1006-1011). Based on their expression profiles the present inventors have previously proposed that TGF-β1 and TGF-β3 play a more critical role in leiomyoma as compared to TGF-β2 (Dou, Q. *et al. J Clin Endocrinol Metab*, 1996, 81:3222-3230; Chegini, N. *et al. J Clin Endocrinol Metab*, 1999, 84:4138-4143; Chegini, N. *et al. Mol Hum Reprod*, 2002, 8:1071-1078; Chegini, N. *et al. Mol Cell Endocrinol*, 2003, 209:9-16; Xu, J. *et al. J Clin Endocrinol Metab*, 2003, 88:1350-1361; Ding, L. *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557). The present inventors provided further evidence in support of the inventors' previous observations and showed that leiomyoma express significantly higher levels of TGF-β1 and TGF-β3 as compared to matched myometrium, and with significantly higher TGF-β1 expression compared to TGF-β3. However, the expression profile of TGF-β1 and TGF-β3 in leiomyoma was inversely correlated, not only with CCN2 (CTGF), but also with CCN3 and CCN4 expression. Since most evidence supporting the involvement of CCN2 as a downstream signal in mediating TGF-β-induced tissue fibrosis comes from in vitro studies (Ihn, H. *Curr Opin Rheumatol*, 2002, 14:681-685; Leask, A. and Abraham, D.J. *Biochem Cell Biol*, 2003, 81:355-363), the present inventors isolated LSMC and MSMC from these tissues and showed, as expected, that TGF-β1 significantly increased the expression of CCN2 in these cells. The present inventors also found that TGF-β1 positively regulates the expression of CCN4, while suppressing CCN3 expression in these cells. To the present inventors' knowledge, this is

the first study to demonstrate a differential regulatory function for TGF-β1 on CCN2, CCN3 and CCN4 expression in LSMC and MSMC, although TGF-β is known for regulating the expression of CCN2 in several cell types, with a few documented examples of regulation of CCN3 (Schnaper, H.W. *et al. Am J Physiol Renal Physiol*, 2003, 284:F243-F252; Ihn, H. *Curr Opin Rheumatol*, 2002, 14:681-685; Leask, A. and Abraham, D.J. *Biochem Cell Biol*, 2003, 81:355-363; Perbal, B. *Lancet*, 2004, 363:62-64; Brigstock, D.R. *J Endocrinol*, 2003, 178:169-175; Perbal, B. *Mol Pathol*, 2001, 54:57-79). To the present inventors' knowledge, this study is also the first to provide evidence for divergence between the expression of TGF-β isoforms and CCNs expression and regulation at tissue and cellular levels originating from these tissues. In hypertrophic scars gene expression profiling also indicated a lower expression of CCN2 accompanied by elevated expression of TGF-β1 as compared to normal skin (Tsou, R. *et al. J Burn Care Rehabil*, 2000, 21:541-550). The results of these studies indicate that a direct correlation between TGF-β and CCN2 expression may not serve as a common feature of all fibrotic disorders as previously proposed (Ihn, H. *Curr Opin Rheumatol*, 2002, 14:681-685; Leask, A. and Abraham, D.J. *Biochem Cell Biol*, 2003, 81:355-363).

TGF-β regulates its own expression in LSMC and MSMC and acting through downstream signaling from Smad and MAPK pathways regulates the expression of many other genes in different functional categories including cell cycle, transcription factors, cell and tissue structure, signal transduction and apoptosis (Dou, Q. *et al. J Clin Endocrinol Metab*, 1996, 81:3222-3230; Chegini, N. *et al. J Clin Endocrinol Metab*, 1999, 84:4138-4143; Xu, J. *et al. J Clin Endocrinol Metab*, 2003, 88:1350-1361; Ding, L. *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557; Arici, A. and Sozen, I. *Fertil Steril*, 2000, 73:1006-1011; Dou, Q. *et al. Mol Hum Reprod*, 1997, 3:1005-1014; Ma, C. and Chegini, N. *Mol Hum Reprod*, 1999, 5:950-954; Luo, X. *et al. Endocrinology*, 2005, 146:1074-1095; Luo, X. *et al. Endocrinology*, 2005, 146:1096-1118). Here, the present inventors demonstrated that pretreatment of LSMC and MSMC with U0126, a synthetic inhibitor of MEK1/2 inhibits the basal expression of CCNs expression and reverses TGF-β1 action. However, treatment of Smad3 SiRNA-transfected LSMC and MSMC with TGF-β1 resulted in a significant increase in CCNs expression. Although the results provide further evidence that components of both MAPK and Smad pathways are involved in mediating TGF-β action on the expression of CCNs (Ihn, H. *Curr Opin*

Rheumatol, 2002, 14:681-685; Leask, A. and Abraham, D.J. *Biochem Cell Biol*, 2003, 81:355-363; Perbal, B. *Lancet*, 2004, 363:62-64; Brigstock, D.R. *J Endocrinol*, 2003, 178:169-175; Perbal, B. *Mol Pathol*, 2001, 54:57-79), including in LSMC and MSMC, a sharp increase in the expression of these genes in Smad3 SiRNA-transfected cells following TGF-β treatment was unexpected. The present inventors propose that crosstalk with components of other signaling pathways activated by TGF-β receptors may have opposing effect on TGF-β-induced CCNs, fibulin-1C and S100A4 expression in LSMC and MSMC. A recent study has reported that inhibition of ERK and c-jun NH(2)-terminal kinase (JNK), but not of p38 MAPK and PI3K, blocked TGF-β1-induced CCN2 expression and Smad2/3 phosphorylation in airway smooth muscle cells (Xie, S. *et al. Am J Physiol Lung Cell Mol Physiol*, 2005, 288:L68-L76). However, the inhibitory action of TGF-β on CCN4 expression in NCI H295R, adrenocortical cell line has been reported to be mediated through c-Jun in a Smad-independent manner (Lafont, J. *et al. J Biol Chem*, 2002, 277:41220-41229). The present inventors have recently reported that TGF-β through MEK1/2 regulates the expression of c-Jun in LSMC and MSMC (Ding, L. *et al. J Clin Endocrinol Metab*, 2004, 89:5549-5557), further supporting the involvement of multiple signaling pathways in TGF-β regulation of CCNs expression in LSMC and MSMC. Further consideration for TGF-β enhancement of CCNs expression in Smad3 SiRNA-transfected LSMC and MSMC may relate to elevated expression of Smad3 in leiomyoma (Xu, J. *et al. J Clin Endocrinol Metab*, 2003, 88:1350-1361), which similar to the expression of TGF-β1/β3, it is inversely correlate with CCNs expression. Such a condition may explain why the inhibition of Smad3 expression resulted in an increase in CCNs expression in LSMC and MSMC. Interestingly, plasminogen activator inhibitor (PAI-1) mRNA expression, a well known gene targeted by TGF-β was significantly inhibited following treatment of Smad3 SiRNA-transfected LSMC and MSMC with TGF-β (unpublished observation). In addition to TGF-β, other cytokines such as IL-4 and IL-13 that are expressed in leiomyoma (Ding, L. *et al. J Soc Gyncol Invest*, 2004, 11:319A) also reported to attenuate TGF-β1-induced CCN2 expression by inhibiting TGF-β-stimulated ERK1/2 and Smad2/3 activation, while TNF-α and IL-1β reduced TGF-β-induced CCN2 without affecting TGF-β-induced Smad2/3 (Xie, S. *et al. Am J Physiol Lung Cell Mol Physiol*, 2005, 288:L68-L76). A functional Smad binding site and TGF-β responsive enhancer (TGFβRE) in CCN2 promoter has been found to be

necessary for basal promoter activity in normal fibroblasts, whereas Smad element is not required for high CCN2 promoter activity in scleroderma fibroblasts (Leask, A. and Abraham, D.J. *Biochem Cell Biol*, 2003, 81:355-363).

These results with Smad3 SiRNA transfected LSMC and MSMC contrast with reports indicating the involvement of Smad pathway activation in TGF-β-induced CCN2 expression in other cell types (Ihn, H. *Curr Opin Rheumatol*, 2002, 14:681-685; Leask, A. and Abraham, D.J. *Biochem Cell Biol*, 2003, 81:355-363; Perbal, B. *Lancet*, 2004, 363:62-64; Brigstock, D.R. *J Endocrinol*, 2003, 178:169-175; Perbal, B. *Mol Pathol*, 2001, 54:57-79; Chen, Y. *et al. Kidney International*, 2002, 62:1149-1159). Although transfection with Smad3 SiRNA resulted in a significant inhibition of Smad3 mRNA expression in LSMC and MSMC, Smad3 inhibition coincided with significant increase, not only in CCN2 expression, but also CCN3, CCN4, fibulin-1C and S100A4 expression following TGF-β treatment. The mechanism underlying TGF-β induction of these genes is not clear from this study; however, TGF-β-induced CCN2 expression in dermal fibroblasts has been reported to involve a functional Smad binding site in the CTGF promoter since deletion or mutation at this site abolished the ability of TGF-β to induce CTGF promoter activity (Leask, A. and Abraham, D.J. *Biochem Cell Biol*, 2003, 81:355-363; Chen, Y. *et al. Kidney International*, 2002, 62:1149-1159; Holmes, A. *et al. J Biol Chem*, 2001, 276:10594-10601). Mutation of Smad element also reduced constitutive CTGF promoter activity, suggesting that the promoter is necessary for both basal and TGF-β-induced CTGF transcription (Leask, A. and Abraham, D.J. *Biochem Cell Biol*, 2003, 81:355-363; Chen, Y. *et al. Kidney International*, 2002, 62:1149-1159). However, in normal and scleroderma dermal fibroblasts mutation of Smad element is reported to affect TGF-β-induced, but not basal CTGF promoter activity (Chen, Y. *et al. Kidney International*, 2002, 62:1149-1159; Holmes, A. *et al. J Biol Chem*, 2001, 276:10594-10601). Smads alone is considered not activate transcription rather acting through recruitment of transcription factors to the promoter of their target genes and synergistic interactions with other signaling cascades they activate gene expression. Among the signaling pathway that interacts with Smads is MAPK (Shi, Y. and Massague, J. *Cell*, 2003, 113:685-700). The present inventors found that MEK1/2 inhibitor, U0126, in a cell specific manner reduced basal and TGF-β-induced CCN4, fibluin-1C and S100A4, but not TGF-β-induced CCN2 expression in LSMC and MSMC. Previous reports in other

cells types indicated that preincubation with U0126, as well as tyrosine kinase, serine/threonine and protein kinase C inhibitors reduced the basal and TGF-β-induced CTGF promoter activity (Leask, A. and Abraham, D.J. *Biochem Cell Biol*, 2003, 81:355-363; Chen, Y. *et al. Kidney International*, 2002, 62:1149-1159; Holmes, A. *et al. J Biol Chem*, 2001, 276:10594-10601). Interestingly, MEK1 inhibitor (PD98059) did not affect TGF-β-induced CTGF, suggesting that the TGF-β induction of CTGF in mesangial cells requires MEK2, but not MEK1 (Chen, Y. *et al. Kidney International*, 2002, 62:1149-1159).

The present inventors also identified the expression of fibulin-1C and S100A4 in leiomyoma and myometrium, and in LSMC and MSMC and found that GnRHa therapy at tissue level and in vitro in a time- and cell-dependent manner altered their expression in LSMC and MSMC. TGF-β1 had a limited effect on the expression of fibulin-1C and S100A4 in these cells; it inhibited fibulin-1C and S100A4 in LSMC, while increasing fibulin-1C expression in MSMC. To the present inventors' knowledge, this is the first study to provide evidence for the expression of fibulin-1C and S100A4 at tissue level and their regulation in cell derived from these tissues in vitro. While this study was completed, a report showed that leiomyoma and myometrium expresses several members of S100 family including S100A4 using standard RT-PCR, and further demonstrated that S100A11 act as a suppressor of LSMC proliferation (Kanamori, T. *et al. Mol Hum Reprod*, 2004, 10:735-742). Although the biological significance of S100A4 in leiomyoma and myometrium is not clear from the present inventors' study, S100A4 expression has been associated with elevated levels of wild-type p53, and their physical interactions stimulate cells entry into the S phase of the cell cycle (Kanamori, T. *et al. Mol Hum Reprod*, 2004, 10:735-742; Grigorian, M. *et al. J Biol Chem*, 2001, 276:22699-22708). Furthermore, transfection of S100A4-negative cells with S100A4 constructs resulted in clonal death that was prevented by co-transfection with the anti-apoptotic gene bcl-2, which control calcium entry in different subcellular compartments (Chen, H. *et al. Biochem Biophys Res Commun*, 2001, 286:1212-1217; Brooke, J.S. *et al. BMC Cell Biol*, 2002, 3:2). Similar to CCN3 pro-angiogenic activities (Perbal, B. *Lancet*, 2004, 363:62-64; Brigstock, D.R. *J Endocrinol*, 2003, 178:169-175; Perbal, B. *Mol Pathol*, 2001, 54:57-79), S100A4 also promotes angiogenesis by acting directly as an angiogenic factor (Barraclough, R. *Biochim Biophys Acta*, 1998, 1448:190-199; Chen, H. *et al. Biochem*

Biophys Res Commun, 2001, 286:1212-1217). Thus, the inhibitory action of GnRHa on CCN3 and S100A4 expression in leiomyoma may represent a mechanism by which GnRHa therapy regresses leiomyoma growth.

The interaction between fibulin-1C and CCN3 has been considered as an important step in CCN signaling involving ECM, cytoskeleton proteins and calcium (Perbal, B. *et al. Proc Natl Acad Sci USA*, 1999, 96:869-874; Argraves, W.S. *et al. EMBO Rep*, 2003, 4:1127-1131; Timpl, R. *et al. Nat Rev Mol Cell Biol*, 2003, 4:479-489; Tran, H. *et al. J Biol Chem*, 1995, 270:19458-19464). Similar to CCN3, fibulin-1C also contains a calcium-binding type II EGF-like domain enabling fibulin-1C to interact with extracellular domain of heparin-binding EGF (HB-EGF) (Perbal, B. *et al. Proc Natl Acad Sci USA*, 1999, 96:869-874; Argraves, W.S. *et al. EMBO Rep*, 2003, 4:1127-1131; Timpl, R. *et al. Nat Rev Mol Cell Biol*, 2003, 4:479-489; Tran, H. *et al. J Biol Chem*, 1995, 270:19458-19464; Tran, H. *et al. J Biol Chem*, 1997, 272:22600-22606). This EGF-like domain is also present in fibronectin and their interaction is considered to result in modification of calcium levels in surrounding cellular environment (Chegini, N. "Implication of growth factor and cytokine networks in leiomyomas" In: Cytokines in human reproduction, J Hill ed. Wiley & Sons, New York, pp. 133-162, 2000). Yeast two-hybrid screens have indicated that latent TGF-β binding protein (LTBP-3) also interacts with proHB-EGF through the EGF-like domains, and interaction among HB-EGF, LTBP-3 and fibulin-1C to serve as a novel function for HB-EGF action between cell and ECM (Grigorian, M. *et al. J Biol Chem*, 2001, 276:22699-22708). Since EGF, HB-EGF, TGF-BP and their receptors as well as fibronectin are expressed in leiomyoma and myometrium (Sherbet, G.V. and Lakshmi, M.S. *Anticancer Res*, 1998, 18:2415-2421), it is likely that their interactions may also influence communication between cellular and ECM compartment in leiomyoma. CCN3 has also been reported to interact with Notch1, a member of a family of highly conserved transmembrane receptors, involved in differentiation, proliferation and apoptosis, fundamental biological processes during embryonic development (Perbal, B. *Lancet*, 2004, 363:62-64; Brigstock, D.R. *J Endocrinol*, 2003, 178:169-175; Perbal, B. *Mol Pathol*, 2001, 54:57-79; Lin, C.G. *et al. J Biol Chem*, 2003, 278:24200-24208; Yu, C. *et al. J Pathol*, 2003, 201:609-615; Sakamoto, K. *et al. J Biol Chem*, 2002, 277:29399-29405; Soon, L.L. *et al. J Biol Chem*, 2003, 278:11465-11470; Margalit, O. *et al. Br J Cancer*, 2003, 89:314-319; Xie, D. *et al.*

Cancer Res, 2001, 61:8917-8923; Saxena, N. *et al. Mol Cell Biochem*, 2001, 228:99-104). CCN3 is expressed in many different types of tumors and shows positive or negative effects on tumorigenesis and metastasis, however S100A4 is not tumorigenic rather it is elevated during metastasis suggesting a role in tumor progression (Brooke, J.S. *et al. BMC Cell Biol*, 2002, 3:2; Davies, M. *et al. DNA Cell Biol*, 1995, 14:825-832). Immunohistochemically, CCN2, CCN3 and CCN4 as well as fibulin 1C and S100A4 were detected in association with ECM and cytoplasmic compartments of various cell types in leiomyoma and myometrium with significant overlap in their distribution. CCN3 is detected in ECM, culture conditioned media, cytoplasm and nucleus, while S100A4 is essentially a cytoplasmic protein, although it is also secreted (Perbal, B. *Lancet*, 2004, 363:62-64; Brigstock, D.R. *J Endocrinol*, 2003, 178:169-175; Perbal, B. *Mol Pathol*, 2001, 54:57-79; Duarte, W.R. *et al. Biochem Biophys Res Commun*, 1999, 255:416-420). The results suggest that CCNs, fibulin-1C and S100A4 could interact intra- and extra-cellularly, influencing various cellar events during physiological and pathological conditions. For instance CCN3 through interaction with S100A4 might alter cytoskeletal organization, facilitate cell motility and cell proliferation, since CCN3 decreases adhesive capacity while increasing motility of Ewing's transfected cells (Margalit, O. *et al. Br J Cancer*, 2003, 89:314-319), and S100A4 affecting cytoskeleton assembly (Heizmann, C.W. and Cox, J.A. *Biometals*, 1998, 11:383-397; Barraclough, R. *Biochim Biophys Acta*, 1998, 1448:190-199). Inhibition of S100A4 has also been reported to decrease matrix metalloproteinases expression a mechanism that may account for S100A4 reduction in cellular migration (Merzak, A. *et al. Neuropathol Appl Neurobiol*, 1994, 20:614-619; Bjornland, K. *et al. Cancer Res*, 1999, 59:4702-4708).

In conclusion, the present inventors have provided further evidence that leiomyoma expresses elevated levels ofTGF-β1 and TGF-β3 compared to myometrium whose expression inversely correlates with CCN2 as well as CCN3 and CCN4 expression in leiomyoma. The expression of CCNs as well as fibulin-1C and S100A4 is targeted by GnRHa therapy, and under in vitro condition TGF-β acting through MAPK/ERK and Smad pathways differential regulates their expression in LSMC and MSMC. Taken together, to the present inventors' knowledge, this study is the first to provide evidence for divergence of TGF-β and CCNs expression and regulation at cell and tissue levels

from the same origin implying that CCN2 may not represent a common feature of fibrotic disorder associated with TGF-β overexpression.

Materials and Methods

The following materials and methods describe those utilized in Examples 14-16. The materials for Realtime PCR, Western blotting and immunohistochemistry were purchased from Applied Biosystem (Foster City, CA), BioRad (Hercules, CA), and Vector Laboratories (Burlingame, CA), respectively as previously described (Ding, L. *et al. J Clin Endocrinol Metab.*, 2004, 89:5549-5557; Xu, J. *et al. J Clin Endocrinol Metab.*, 2003, 88:1350-1361). Polyclonal antibody generated in goat against recombinant FMOD was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Portions of leiomyoma and matched myometrium were collected from premenopausal women (N=27) who were scheduled to undergo hysterectomy for symptomatic uterine leiomyomas at the University of Florida affiliated Shands Hospital. Of these patients, seven received GnRHa therapy for a period of three months prior to surgery. The untreated patients did not receive any medications during the previous 3 months prior to surgery and based on endometrial histology and the patient's last menstrual period they were identified as being from proliferative (N=8) or secretory (N=12) phases of the menstrual cycle. To maintain a standard, leiomyomas used in this study were 2 to 3 cm in diameter. Prior approval was obtained from the University of Florida Institutional Review Board for the experimental protocol of this study. Following collection, total RNA and protein was isolated from these tissues and subjected to Realtime PCR, Western blotting or processed for immunohisochemistry and cell culturing as previously described (Ding, L. *et al. J Clin Endocrinol Metab.*, 2004, 89:5549-5557; Xu, J. *et al. J Clin Endocrinol Metab.*, 2003, 88:1350-1361).

Realtime PCR. Briefly, total RNA was isolated from leiomyoma and matched myometrium using Trizol Reagent (INVITROGEN, Carlsbad, CA) and complimentary DNA was generated from 2 μg of total RNA using Taqman reverse transcription reagent. The newly synthesized cDNA was used for PCR performed in 96-well optical reaction plates with cDNA equivalent to 100ng RNA in a volume of 50μl reaction containing 1x Taqman Universal Master Mix, optimized concentrations of FAM-labeled probe and specific forward and reverse primer for FMOD selected from Assay on Demand (APPLIED BIOSYSTEMS). Controls included RNA subjected to RT-PCR without

reverse transcriptase and PCR with water replacing cDNA. The results were analyzed using a comparative method and the values were normalized to the 18S rRNA expression and converted into fold change based on a doubling of PCR product in each PCR cycle, according to the manufacturer's guidelines as previously described (Ding, L. *et al. J Clin Endocrinol Metab.*, 2004, 89:5549-5557; Luo, X. *et al. Endocrinology*, 2005, 146:1074-1096).

Western Blot Analysis and Immunohistochemistry. For Western blotting small pieces of tissues were lysed in a lysis buffer, centrifuged and the supernatants were collected and their total protein content was determined using a conventional method (Pierce, Rockford, IL) as previously described (Xu, J. *et al. J Clin Endocrinol Metab.*, 2003, 88:1350-1361; Ding, L. *et al. J Clin Endocrinol Metab.*, 2004, 89:5549-5557). Equal amounts of sample proteins were subjected to PAGE, transferred to polyvinyldiene difluoride (PVDF) membranes, and following further processing, the blots were incubated with FMOD antibody for 1 hr at room temperature. The blots were washed with washing buffer and exposed to corresponding HRP-conjugated IgG, and immunostained proteins were visualized using enhanced chemiluminescence reagents (Amersham-Pharmacia Biotech, Piscataway, NJ).

For immunohistochemistry, tissue sections were prepared from formalin-fixed and paraffin embedded leiomyoma and myometrium and following standard processing immunostained using antibodies to FMOD at 5μg of IgG/ml for 2-3 hrs at room temperature. Following further standard processing, chromogenic reaction was detected with 3,3'-diaminobenzidine tetrahydrochloride solution (Xu, J. *et al. J Clin Endocrinol Metab.*, 2003, 88:1350-1361). Omission of primary antibody, or incubation of tissue sections with non-immune goat IgG instead of primary antibody at the same concentration served as controls.

The Expression and Regulation of Fibromodulin in LSMC and MSMC by TGF-beta and GnRHa. Leiomyoma and myometrial smooth muscle cells (LSMC and MSMC) were isolated, characterized and cultured as previously described (Chegini, N. et al. Mol Hum Reprod., 2002, 8:1071-1078). LSMC and MSMC were cultured in 6-well plates at an approximate density of 10^6 cells/well in DMEM-supplemented media containing 10% FBS. After reaching visual confluence, the cells were washed in serum-free media and

incubated for 24 hrs under serum-free, phenol red-free conditions (Chegini, N. et al. Mol Hum Reprod., 2002, 8:1071-1078).

To determine whether TGF-β and GnRHa influence the expression of FMOD, LSMC and MSMC cultured as above were treated with TGF-β1 (2.5 ng/ml) or GnRHa (0.1μM) for 2, 6 and 12 hrs (Xu, J. *et al. J Clin Endocrinol Metab.*, 2003, 88:1350-1361; Ding, L. *et al. J Clin Endocrinol Metab.*, 2004, 89:5549-5557). Since TGF-β mediates its action in part through activation of the MAPK pathway (Ding, L. *et al. J Clin Endocrinol Metab.*, 2004, 89:5549-5557), the present inventors determined whether inhibition of the MAPK pathway alter TGF-β mediated action in regulating the expression of FMOD. LSMC and MSMC were cultured as above and following pretreatment with U0126 (20 μM), a synthetic inhibitor of ERK1/2, for 2 hrs, the cells were treated with TGF-β1 or GnRHa for 2hrs (Ding, L. *et al. J Clin Endocrinol Metab.*, 2004, 89:5549-5557). Activation of Smad also serves as a major signaling pathway for TGF-β mediated action including in LSMC and MSMC (Xu, J. *et al. J Clin Endocrinol Metab.*, 2003, 88:1350-1361). To determine whether TGF-β mediated action through the Smad pathway regulates the expression of FMOD, LSMC and MSMC were cultured as above and transfected with Smad3 SiRNA as previously described (Luo, X. *et al. Endocrinology*, 2005, 146:1097-1118). LSMC and MSMC at 80% confluence were transfected with 200 pmol of SiRNA using transfectamine 2000 reagent (10 μl) according to the manufacturer's instructions (INVITROGEN, Carlsbad, CA) for 48 hrs. The cells were then treated with TGF-β1 (2.5 ng/ml) for 2 hrs. Untreated or cells treated with scrambled Smad3 SiRNA were used as a negative control. Total RNA was isolated from the treated and untreated controls cells and subjected to Realtime PCR.

Where appropriate, the results are expressed as mean ± SEM and statistically analyzed using unpaired Student t-test and variance (ANOVA) using Tukey test. A probability level of $P<0.05$ was considered significant.

Example 14—Expression of FMOD in Leiomyoma and Myometrium

Using Realtime PCR, the present inventors demonstrated that leiomyoma and matched myometrium used for microarray analysis express FMOD mRNA with a considerable overlap between microarray analysis and Realtime PCR data. The present inventors evaluated the relative expression of FMOD and the influence of the menstrual

cycle using total RNA isolated from leiomyoma and matched myometrium from proliferative (N=8) and secretory (N=12) phases of the menstrual cycle with Realtime PCR. The results indicated that FMOD is expressed at a significantly higher level in leiomyoma as compared to matched myometrium from the proliferative phase of the menstrual cycle ($p<0.05$; Figure 16). There was a trend toward a lower expression of FMOD in leiomyoma compared to myometrium from the secretory phase, however these values did not reach statistical significance (Figure 16). The relative level of FMOD expression was significantly elevated in myometrium from the secretory phase compared to proliferative phase ($p<0.05$) with a trend toward lower expression in leiomyoma (Figure 16). The expression of FMOD was significantly reduced in both leiomyoma and myometrium in women who received GnRHa therapy (N=7), reaching the levels observed in myometrium from the proliferative phase (P=0.05; Figure 16).

To further assess the expression of FMOD, total protein was isolated from these tissues and subjected to Western blot analysis. As shown in Figure 17, leiomyoma (L) and matched myometrium (M) from proliferative and secretory phases of the menstrual cycle contain immunoreactive FMOD and with higher intensity in L compared with M in tissue from the proliferative phase, with an increase in intensity in tissues from the secretory phase. There was a reduction in FMOD immunoreactive intensity in L and M from the GnRHa treated group compared to tissues from the secretory phase (Figure 17). Immunoreactive FMOD was also localized in leiomyoma and myometrial tissue sections with staining associated with myometrial and leiomyoma smooth muscle cells, as well as connective tissue fibroblasts and vasculature (Figures 18A-18D). Incubation of tissue sections with non-immune goat IgGs instead of primary antibody at the same concentration served as control and showed a substantial reduction in staining intensity associated with these cells.

Example 15—Expression of FMOD in LSMC and MSMC and Regulation by TGF-β

The present inventors have recently characterized the expression profile of LSMC and MSMC in response to TGF-β and GnRHa using gene microarray which indicated that the expression of several components of ECM including FMOD are the target of their regulatory action (Luo, X. *et al. Endocrinology*, 2005, 146:1097-1118). To further evaluate the influence of TGF-β on FMOD expression in leiomyoma and myometrium,

the present inventors isolated LSMC and MSMC and following treatment with TGF-β1 (2.5ng/ml) determined the expression of FMOD in these cells. As shown in Figures 19A-19D, treatment with TGF-β1 in a cell- and time-dependent manner significantly increased the expression of FMOD in MSMC with a gradual reduction in expression reaching control levels after 12 hrs ($P<0.05$). TGF-β had either no effect, or inhibited FMOD expression in LSMC after 12 hrs of treatment (Figures 19A-19D; $P<0.05$). Treatment of LSMC and MSMC with GnRHa (0.1 μM) for 2 and 6 hrs had no significant effect on FMOD expression; however, it inhibited FMOD after 12 hrs of treatment (Figures 19A-19D; $P<0.05$).

Example 16—Inhibition of MAPK and Smad3 Pathways on TGF-β- and GnRHa-Mediated Actions

TGF-β recruits and activates several intracellular signaling pathways, specifically Smad and MAPK pathways. TGF-β through the activation of these pathways regulates the expression of many genes including fibronectin and collagen in LSMC and MSMC (Xu, J. *et al. J Clin Endocrinol Metab.*, 2003, 88:1350-1361; Ding, L. *et al. J Clin Endocrinol Metab.*, 2004, 89:5549-5557; Luo, X. *et al. Endocrinology*, 2005, 146:1097-1118). To determine whether TGF-β regulates the expression of FMOD through these pathways, LSMC and MSMC were pretreated with U0126 followed by treatment with TGF-β1 (2.5 ng/ml) for 2 hrs. As shown in Figures 19A-19D, pretreatment with U0126 increased the basal expression of FMOD in LSMC and MSMC and TGF-β-mediated action in LSMC, while inhibiting TGF-β-mediated action in MSMC ($p<0.05$). Pretreatment with U0126 also increased the expression of FMOD in MSMC and LSMC treated with GnRHa as compared to untreated control and U0126-treated cells, respectively (Figures 19A-19D; $P<0.05$).

Transfection of LSMC and MSMC with Smad3 SiRNA, but not scrambled SiRNA significantly inhibited the expression of Smad3 in both cell types, and resulted in a trend toward increased basal expression of FMOD in MSMC and LSMC (Figures 19A-19D). However Smad3 SiRNA transfection significantly reduced TGF-β-induced FMOD in MSMC reaching control levels, without affecting LSMC (Figures 19A-19D; $P<0.05$).

Using microarray gene expression profiling, the present inventors have identified fibromodulin (FMOD) among the differentially expressed genes in leiomyoma and

myometrium and in LSMC and MSMC treated with TGF-β1 (Luo, X. *et al. Endocrinology*, 2005, 146:1074-1096; Luo, X. *et al. Endocrinology*, 2005, 146:1097-1118). In the present study, the present inventors validated the expression of FMOD using Realtime PCR showing a considerable overlap with microarray observations. The present inventors extended this work and demonstrated the menstrual cycle-dependent expression of FMOD in leiomyoma and myometrium. These results indicated that the expression of FMOD is significantly higher in leiomyoma compared to myometrium from the proliferative, but not the secretory phase of the menstrual cycle, suggesting a regulatory function for ovarian steroids on FMOD expression. The influence of the menstrual cycle on the expression of FMOD appears to be tissue specific, because of an increase in myometrial expression of FMOD from the secretory phase compared to the proliferative phase, with lower levels in leiomyoma. Since GnRHa therapy creates a hypoestrogenic condition, these results, as well as a significant reduction in the expression of FMOD in both leiomyoma and myometrium in women who received GnRHa therapy, further support the involvement of ovarian steroids in regulating FMOD expression in these tissues. The present inventors also demonstrated the expression of FMOD in LSMC and MSMC, and showed differential regulation by TGF-β1 and GnRHa through Smad and MAPK signaling pathways, respectively.

The biological significance of FMOD expression in leiomyoma and myometrium await detailed investigation, however, FMOD was found in association with several cell types in leiomyoma and myometrium and was differentially regulated by TGF-β in MSMC and to a certain extend in LSMC. Fibromodulin is a collagen-binding protein widely expressed in many connective tissues and appears to play an important role in ECM remodeling, specifically in tissues that undergo extensive tissue turnover such as cervix during ripening, fetal wound healing, atherosclerosis and bleomycin-induced lung fibrosis (Westergren-Thorsson, G. *et al. Biochim Biophys Acta.*, 1998, 1406:203-213; Strom, A. *et al. Histol Histopathol.*, 2004, 19:337-347; Soo, C. *et al. Am J Pathol.*, 2000, 157:423-433; Venkatesan, N. *et al. Am J Respir Crit Care Med*, 2000, 161:2066-2073). Fibromodulin is a member of the proteoglycan family including biglycan, decorin, lumican and chondroadherin small molecules with important roles in binding to other matrix molecules either to aid fibrillogenesis or act as bridging molecules between various tissue elements (Blochberger, T.C. *et al. J Biol Chem*, 1992, 267: 347-352;

Noonan, D.M. and Hassell, J.R. *Kidney Int*, 1993, 43:53-60; Yanagishita, M. *Acta Pathol Jpn*, 1993, 43:283-293). It has been reported that for each collagen molecule there is at least one FMOD binding site, however these sites are limited in number and are highly specific (Hedbom, E. and Heinegård, D. *J Biol Chem*, 1993, 268:27307-27312). Evidence suggests that FMOD regulates the formation of the collagen fibrils network through its interaction with collagen types I, II and XII (Font, B. *et al. Matrix Biol*, 1996, 15:341-348), whose expressions have been documented in leiomyoma and myometrium (Stewart, E.A. *et al. J Clin Endocrinol Metab.*, 1994, 79:900-906; Stewart, E.A. *et al. J Soc Gynecol Investig.*, 1998, 5:44-47; Ding, L. *et al. J Clin Endocrinol Metab.*, 2004, 89:5549-5557; Leppert, P.C. *et al. Fertil Steril.*, 2004, 82(Suppl 3):1182-1187). Fibromodulin, like decorin, binds to type I and type II collagens and through interaction with TGF-β regulates the local biological activity and retention of TGF-β within the ECM (Fukushima, D. *et al. J Biol Chem*, 1993, 268:22710-22715; Hildebrand, A. *et al. Biochem J*, 1994, 302:527-534). Since leiomyoma and myometrium express biglycan and decorin (Luo, X. *et al. Endocrinology*, 2005, 146:1074-1096; Luo, X. *et al. Endocrinology*, 2005, 146:1097-1118; personal observations), alteration in the expression of FMOD could influence the organization of collagen and local availability of TGF-β, thus influencing the outcome of fibrosis in leiomyoma.

Leiomyomas have several characteristic features typical of fibrotic disorders, including overexpression of TGF-β, TGF-β receptors and Smads as compared to normal myometrium (Dou, Q. *et al. Mol Hum Reprod.*, 1997, 3:1005-1014; Chegini, N. *et al. Mol Hum Reprod.*, 2002, 8:1071-1078; Chegini, N. *et al. J Soc Gynecol Investig.*, 2003, 10:161-171; Chegini, N. *et al. Mol Cell Endocrinol.*, 2003, 209:9-16; Chegini, N. and Kornberg, L. *J Soc Gynecol Investig.*, 2003, 10:21-26; Xu, J. *et al. J Clin Endocrinol Metab.*, 2003, 88:1350-1361). Since leiomyoma also express a higher level of FMOD compared to myometrium the present inventors expected a positive regulatory function for TGF-β on the expression of FMOD in LSMC as compared to MSMC. However, under culture conditions of the present inventors' study, TGF-β resulted in a significant increase (5-10 fold) in FMOD expression in MSMC which declined to control levels, compared to a slight reduction in the expression in LSMC in a time-dependent manner. How TGF-β causes differential regulation of FMOD expression in MSMC and LSMC is unclear from this study and requires detailed investigation; however, it is clear that TGF-

β mediated signaling though MAPK/ERK and Smad in MSMC are involved in differential regulation of TGF-β action in these cells. In other tissues such as the cervix during ripening, the expression of collagen type I and III, versican, biglycan, decorin and FMOD as well as TGF-β1 are reported to induce no significant change in small proteoglycans expression despite an almost 50% decrease in their concentration (Westergren-Thorsson, G. *et al. Biochim Biophys Acta.*, 1998, 1406:203-213). However, in a rat model that transits from scarless fetal-type repair to adult-type repair, the expression of FMOD is reported to decrease as compared to TGF-β and TGF-β receptors, and when compared to adult wound healing (Soo, C. *et al. Am J Pathol.*, 2000, 157:423-433). These results in a rat model of wound healing and scar tissue formation is comparable to the present inventors' observations in leiomyoma, suggesting that FMOD may act as a biologically relevant modulator of TGF-β activity during tissue fibrosis. TGF-β1 is reported to modulate the synthesis and accumulation of decorin, biglycan, and FMOD in cartilage explants cultured under conditions in which aggrecan synthesis remains relatively constant, with FMOD content most rapidly augmented in response to TGF-β1 (Burton-Wurster, N. *et al. Osteoarthritis Cartilage*, 2003, 11:167-176). In addition to TGF-β regulation of FMOD in dermal skin fibroblasts, CTGF has also been reported to increase the expression of FMOD, as well as the expression of type I and III collagens and basic fibroblast growth factor, without influencing the expression of HSP47, decorin, biglycan, and versican (Wang, J.F. *et al. Wound Repair Regen.*, 2003, 11:220-229). In the gene expression profiling studies described herein, the present inventors found a significantly lower expression of CTGF in leiomyoma as compared to matched myometrium, however it was increased in TGF-β-treated LSMC and MSMC (Luo, X. *et al. Endocrinology*, 2005, 146:1097-1118). These results suggest that these cytokines could influence FMOD expression at the tissue level differently when compared to their action in vitro. Furthermore, the present inventors have reported that TGF-β self-regulates its own expression and the expression of CTGF and TGF-β through the activation of MAPK pathway regulates the expression of type I collagen and fibronectin in LSMC and MSMC (Ding *et. al.*, 2004). In mouse uterus, analysis of decorin, biglycan, lumican and FMOD expression from day I to day 7 of pregnancy indicated that decorin was present together with lesser amounts of lumican in the stroma before the onset of decidualization, whereas biglycan and FMOD were almost absent

(San Martin, S. *et al. Reproduction*, 2003, 125:585-595). Fibromodulin was weakly expressed in the non-decidualized stroma, but only after implantation (San Martin, S. *et al. Reproduction*, 2003, 125:585-595).

Fibromodulin expression has been found only in mitotic, but not in mitomycin C-induced postmitotic skin fibroblasts, or in endothelial cells and keratinocytes, and is considered to serve as a specific marker for mitotic activity which could indicate cell ageing (Petri, J.B. *et al. Mol Cell Biol Res Commun.*, 1999, 1:59-65). Interestingly, matrix metallporteinases (MMPs) such as MMP-2, -8 and -9, and specifically MMP-13 are reported to effectively cleave FMOD in fresh articular cartilage, and the cleaved product was found to be identical to that observed in cleaved FMOD from cartilage explant cultures treated with IL-1 (Heathfield, T.F. *et al. J Biol Chem.*, 2004, 279:6286-6295). Since leiomyoma and myometrium express several MMPs including 2, 8, 9 and 13, and proinflammatory cytokines such as IL-1, they may target FMOD degradation in a manner similar to that demonstrated in other tissues (Dou, Q. *et al. Mol Hum Reprod.*, 1997, 3:1005-1014, Lee, B.S. *et al. J Clin Endocrinol Metab.*, 1998, 83:219-223, Tang, X.M. *et al. Mol Hum Reprod.*, 1997, 3:233-240; Palmer, S.S. *et al. J Soc Gynecol Investig.*, 1998, 5:203-209). Fibromodulin deficiency is reported to lead to a significant reduction in tendon stiffness in FMOD (-/-) mice, with irregular collagen fibrils and increased frequency of small diameter fibrils, suggesting that FMOD is required early in collagen fibrillogenesis (Chakravarti, S. *Glycoconj J.*, 2002, 19:287-293). Thus, altered expression of FMOD would be expected to impact the organization of collagen in various fibrotic disorders such as leiomyoma.

In summary, these results document the first example of expression of FMOD in leiomyoma and myometrium and provide evidence for direct regulatory action of GnRHa and TGF-β on its expression in LSMC and MSMC. Since FMOD acts as key regulator of connective tissue remodeling its differential expression in leiomyoma and myometrium may influence leiomyoma fibrotic characteristics.

All patents, patent applications, provisional applications, and publications referred to or cited herein, whether supra or infra, are incorporated by reference in their entirety, including all figures, tables, and sequences, to the extent they are not inconsistent with the explicit teachings of this specification.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application.

Table 1

Gene Accession #	Gene Symbol	Change in Expression LYM vs MYM (P≤0.02)	Gene Accession #	Gene Symbol	Change in Expression LYM vs MYM (P≤0.02)
Transcription factors			***Intracellular***		
AB020634	NFAT5	+	***transducers/modulators***		
M97388	DR1	+	AB007881	SMG1	+
U26914	RREB1	+	AB004904	SOCS3	+
AF040253	SUPT5H	-	D89094	PDE5A	+
AB002386	EZH1	-	Z50053	GUCY1A2	+
L38933	HUMGT198A	-	X95632	ABI2	+
AB022785	ASH2L	-	Y13493	DYRK2	+
AB014558	CRY2	-	D88532	PIK3R3	+
Cell cycle regulators			Y18206	PPP1R3D	+
X60188	MAPK3	-	M96995	GRB2	+
U66469	CGRRF1	-	AF015254	AURKB	+
Cell adhesion receptors/proteins			U02680	PTK9	+
AF106861	ATRN	+	AF052135	STAMBP	+
Z29083	TPBG	+	U46461	DVL1	+
AB002382	CTNND1	-	AB003698	CDC7	+
Extracellular transport/carrier			AI961669	ARFGEF2	+
proteins			X70218	PPP4C	+
U09210	SLC18A3	+	X99325	STK25	+
Oncogenes and tumor suppressors			L36151	PIK4CA	-
X57110	CBL	+	AL049970	PRKRIR	-
M16038	LYN	+	AI671547	RAB9A	-
X60287	MAX	+	AF103905	RAPGEF3	-
U96078	HYAL1	-	X95735	ZYX	-
Stress response proteins			M33552	LSP1	-
W28616	HSPCB	+	X62048	WEE1	-
X83573	ARSE	-	S76965	PKIA	-
D87953	NDRG1	-	U25771	ARF4L	-
Membrane channels and			AF035299	DOK1	+
transporters			***Protein turnover***		
AF027153	SLC5A3	+	X87212	CTSC	+
M55531	SLC2A5	+	AL080090	ANAPC10	+
X57303	SLC7A1	+	AJ132583	NPEPPS	-
X91906	CLCN5	-	AF099149	ARIH2	-
Extracellular matrix proteins			***Cell receptors (by activities)***		
U05291	FMOD	-	AF084645	NR1I2	+
AB011792	ECM2	-	AB020639	ESRRG	+
Trafficking/targeting proteins			***Cytoskeleton/motility***		
D89618	KPNA3	+	***proteins***		
AC004472	VCP	+	AB008515	NOL7	+
AA890010	SEC22L1	+	AI056696	CETN3	-
L43964	PSEN2	+	***Functionally unclassified***		
X97074	AP2S1	+	AF035444	PHLDA2	+

Gene Accession #	Gene Symbol	Change in Expression LYM vs MYM (P≤0.02)	Gene Accession #	Gene Symbol	Change in Expression LYM vs MYM (P≤0.02)
AA192359	TNPO3	+	U79299	OLFM1	+
U32315	STX3A	-	U22963	MR1	+
Metabolism			U15552	HSU15552	+
D50840	UGCG	+	AB015332	AKAP8L	+
M21186	CYBA	+	AF068195	UBADC1	+
AC005329	NDUFS7	+	AB011542	EGFL5	+
U44111	HNMT	+	Z78368	C1orf8	-
M84443	GALK2	+	AF053356	LRCH4	-
X14608	PCCA	+	AF009426	C18orf1	-
AF014402	PPAP2A	+	***not classified***		
AF035555	HADH2	+	AB011096	SARM1	+
U84371	AK2	+	AJ236885	ZNF148	+
AA526497	UQCRH	+	N42007	NUP50	+
AI557064	NDUFV2	+	Z48570	DDX24	+
D55654	MDH1	+	M19650	CNP	+
AL049954	AHCYL1	-	AB002348	KIAA0350	+
AA420624	MAOA	-	AB014564	KIAA0664	-
M93107	BDH	-	M29551	PPP3CB	-
Post-translational modification			AB020699	KIAA0892	-
U84404	UBE3A	-	AB002370	KIAA0372	-
Translation			AB023181	DLGAP4	-
L36055	EIF4EBP1	+	AB011106	ATRNL1	-
Apoptosis associated proteins			D88152	SLC33A1	-
Z70519	TNFRSF6	+	AF082657	ERAL1	+
AJ006288	BCL10	+	AB023163	HIP14	-
U04806	FLT3LG	-	AF040964	C4orf15	+
RNA processing, turnover, and transport			U33838	RELA	+
			M22919	MYL6	-
U40763	PPIG	+	U93869	POLR3F	+
AB007510	PRPF8	-	X59417	PSMA6	+
X85237	SF3A1	-	AJ224326	RPE	+
U76421	ADARB1	-	U60644	PLD3	+
Cell receptors (by ligand)			AB018257	ZNF294	-
J03171	IFNAR1	+			
M33210	NDRG1	-			
AJ225028	GABBR1	-			
D15050	TCF8	-			
AF030339	PLXNC1	-			

Table 2

Gene Accesssion#	Gene Symbol	Change in Expression LYM vs MYM (P≤0.02)	Gene Accesssion#	Gene Symbol	Change in Expression LYM vs MYM (P≤0.02)
Cell surface/Matrix Protein			***Growth Factor/Cyt/Chemo/Polypept-Horm***		
D26579	ADAM8	+	U79716	RELN	+
Transcription Factor			M63582	TRH	+
U15655	ERF	+	M13982	IL4	+
L39059	TAF1C	+	X52599	NGFB	+
M96577	E2F1	+	***Intracellular iransducers/modulators***		
AF025654	RNGTT	-	U39064	MAPKK6	+
U15642	E2F5	-	X82260	RANGAP1	+
AB015132	KLF7	-	Z15108	PRKCZ	+
U63810	CIAO1	-	R54564	MINK	+
U52960	SURB7	-	U09284	LIMS1	+
U65093	CITED2	-	U12779	MAPKAPK2	+
AJ001183	SOX10	-	U18420	RAB5C	+
Cell cycle			AL050268	RAB1A	-
U03106	CDK1A	+	AB005047	SH3BP5	-
L23959	TFDP1	-	X52213	LTK	-
M80629	CDC2L5	-		GNEF1	-
X77794	CCNG1	-	D85758	ERH	-
Cell adhesion receptors/proteins			AF014398	IMPA2	-
			AJ011736	GRAP2	-
AF007194	Mucin 3	+	U59913	SMAD5	-
X15606	ICAM2	-	X17576	NCK1	-
D14705	CTNNA1	-	U48730	STAT5B	-
S66213	ITGA6	-	U17743	MAP2K4	-
Oncogenes and tumor suppressors			U43885	GAB1	-
			Protein turnover		
U96078	HYAL1	-	D49742	HABP2	+
Stress response proteins			U80034	MIPEP	-
AI972631	ARS2	-	***Cytoskeleton/motility proteins***		
Membrane channels and transporters			W27148	MAP1B	-
			DNA synthesis, recombination, repair		
X89066	TRPC1	-	X91992	ALKBH	-
AB021981	SLC35A3	-	Y15572	RAD51L3	-
D50312	KCNJ8	-	AF007871	DYT1	-
Extracellular matrix proteins			AF058696	NBS1	-
			Functionally unclassified		
U37283	MFAP5	-	AI924594	TSPAN-2	-
Trafficking/targeting proteins			Z68747	mitochondrial ribosomal protein S31	-
AF002163	AP3D1	+			
X96783	SYT5	-	AB018285	zinc finger protein	-
Metabolism			***Not classified***		
AJ004832	NTE	+	D42085	NUP93	-
AF062529	NUDT3	+	D87437	C1orf16	+

D38537	PPOX	+	X77548	NCOA4	-
AI345944	NDUFB1	-	D79990	RASSF2	-
AI766078	COQ7	-	U05861	AKR1C1	-
D14710	ATP5A1	-	L49054	MLF1	-
Post-translational modification			AB007884	ARHGEF9	-
			AF044896	C1orf38	-
U31525	GYG	-	AJ223352	HIST1H2BK	-
Apoptosis associated proteins			AA043348	HSPA4	-
			Z85986	C6orf69	-
Y09392	TNFRSF25	+	W26677	FLJ35827	+
AF015451	CFLAR	-	AB011133	MAST3	+
M16441	LTA	-	AB018274	LARP	+
RNA processing, turnover, and transport			U92896	EFNA2	+
			AF064801	RNF139	+
L35013	SF3B4	+	U47924	GRCA	-
AJ007509	HNRPUL1	+	AB007896	KIAA0436	-
AF016369	PRPF4	-	AJ002428	VDAC1	-
M96954	TIA1	-			
Chromatin proteins					
AF045184	SKIIP	-			
Cell Surface receptors					
X06614	RARA	+			
AF109134	OGFR	+			
D16827	SSTR5	-			
X61615	LIFR	-			
M64347	FGFR3	-			
M15169	ADRB2	-			
U23850	ITPR1	-			

Table 3

Gene Accesssion #	Gene Symbol	Change in Expression (p≤0.02)	Gene Accesssion #	Gene Symbol	Change in Expression (p≤0.02)
Cell surface antigens			***GTP/GDP/G-protein/GTPase***		
X84746	ABO	+	***modulators***		
AF004876	YIF1	+	D13988	GDI2	-
Transcription/activators/repressors			U18420	RAB5C	+
X98253	ZNF183	-	U34806	GPR15	+
D38251	POLR2E	-	U18550	GPR3	-
U22431	HIF1A	-	***Amino- and***		
AB002332	CLOCK	+	***carboxypeptidases***		
U33838	RELA	-	L13977	PRCP	-
U15306	NFX1	-	***Metalloproteinases***		
AF040253	SUPT5H	+	U80034	MIPEP	-
L19067	RELA	+	***Proteosomal proteins***		
M74099	CUTL1	+	D26600	PSMB4	-
U48436	FMR2	+	AB009398	PSMD13	-
AA478904	KLF7	+	X59417	PSMA6	-
M69043	NFKBIA	-	D26598	PSMB3	-
Cell cycle-regulating kinases			D38048	PSMB7	-
U17743	MAP2K4	-	***Cytoskeleton/motility proteins***		
D88357	CDC2	-	AB007862	PCNT2	+
L04658	CDK5	-	U48734	ACTN4	+
X66357	CDK3	+	U01828	MAP2	+
M74091	CCNC	-	U39226	MYO7A	+
L23959	TFDP1	-	AI540958	DNCL1	+
Cell adhesion receptors/proteins			AF020267	MYO9B	+
X69819	ICAM3	-	U43959	ADD2	+
Z29083	TPBG	-	AL096717	EML2	+
AF007194	Mucin 3, Intestinal	+	AI961040	TUBGCP2	+
Oncogenes and tumor suppressors			***Extracellular matrix and***		
J03069	MYCL2	+	***carrier proteins***		
X72631	NR1D1	+	M12625	LCAT	+
U09577	HYAL2	-	AF093118	FBLN5	+
AI743606	RAB8A	-	M20776	COL6A1	-
U04313	SERPINB5	+	U80034	MIPEP	-
AF013168	TSC1	+	AB006190	AQP7	+
Trafficking/targeting proteins			AB021981	SLC35A3	-
X99459	AP3S2	-	U90313	GSTO1	-
AW044624	RER1	-	X67301	IGHM	-
U60644	PLD3	-	M92303	CACNB1	+
AA890010	SEC22L1	-	X91906	CLCN5	+
AC004472	VCP	-	AB023173	ATP11B	+
AF034546	SNX3	-	M20471	CLTA	-
Z12830	SSR1	-	U27467	BCL2A1	+
AF044671	GABARAP	-	U30872	CENPF	-
Metabolism			AI857458	UCN	-
AC005329	NDUFS7	-	D87432	SLC7A6	+
M22976	CYB5	+	N80906	CST6	+
AF047181	NDUFB5	-	D38535	ITIH4	+

Gene Accesssion #	Gene Symbol	Change in Expression (p≤0.02)	Gene Accesssion #	Gene Symbol	Change in Expression (p≤0.02)
D16294	ACAA2	-	M31767	MGMT	+
AI345944	NDUFB1	-	AB007884	ARHGEF9	-
D14710	ATP5A1	-	AC004472	KIAA1539	-
X06994	CYC1	-	***Functionally unclassified***		
AI540957	QP-C	-	W28869	TEGT	-
AI557064	NDUFV2	-	Z68747	MRPS31	-
U19822	ACACA	+	L07758	PWP1	-
AF047469	ASNA1	-	AJ007014	NCBP2	-
Protein modification enzymes			U72508	B7	-
D29643	DDOST	-	AA524058	C6orf74	-
AD000092	CALR	-	D86062	C21orf33	-
AF035280	EIF2B2	-	D87343	DSCR3	+
L36055	EIF4EBP1	-	AF042384	BC-2	-
L34600	MTIF2	-	AF068195	UBADC1	-
D28483	RBMS2	-	AL021937	RFPL3S	+
RNA processing/ turnover/ transport			U80744	TNRC5	-
			AF035444	PHLDA2	-
U51334	TAF15	+	***not classified***		
D59253	NP25	-	AL031177	APG4A	+
Z48501	PABPC1	-	AB007884	ARHGEF9	-
L36529	THOC1	+	AC004472	KIAA1539	+
AF083190	DNAJC8	+	AF040964	C4orf15	-
D28423	SFRS3	-	D87742	FLJ39207	+
Growth factors/cytokines/ chemokines			AB006628	FCHO1	+
			AB014592	KIAA0692	+
J00219	IFNG	+	AB023214	ZBTB1	+
U32324	IL11RA	+	AB028964	FOXJ3	+
Z70519	TNFRSF6	-	U54999	GPSM2	+
X04571	EGF	+	L49054	MLF1	-
X72308	CCL7	+	AA926959	CKS1B	+
X78686	CXCL5	+	NM_00635	Ras-Like	
J04513	FGF2	-		Protein Tc4	-
S74221	IK	-	AB002292	ARHGEF10	+
U43368	VEGFB	+	M24899	THRA	+
AL021155	NPPA	+	U92896	EFNA2	+
Intracellular transducers/modulators			AJ222967	CTNS	+
			AL031983	OR2H3	+
X75958	NTRK2	+	U05681	BCL3	+
S76475	NTRK3	+	AF014398	IMPA2	-
U43885	GAB1	-	X67325	IFI27	-
X84709	FADD	-	U90907	PIK3R3	-
M96995	GRB2	-	AF030107	RGS13	+
U46461	DVL1	-	AL049634	PTPNS1L2	+
AF051323	SCAP2	-	AF091071	RER1	+
X66363	PCTK1	-	AC005525	IGSF4C	+
AB018330	CAMKK2	+	U49278	UBE2V1	-
L13616	PTK2	-	U39318	UBE2D3	+
U02680	PTK9	-	AF075599	UBE2M	-

Gene Accesssion #	Gene Symbol	Change in Expression ($p \leq 0.02$)	Gene Accesssion #	Gene Symbol	Change in Expression ($p \leq 0.02$)
X72964	CETN2	-	AJ002428	VDAC1	-
Y17711	CBARA1	-	U84388	CRADD	-
U51004	HINT1	-	X63657	FVT1	+
U94747	HAN11	-			
U78733	SMAD2	-			

Table 4

Gene Accesssion #	Gene Symbol	Change in Expression (p≤0.02)	Gene Accesssion #	Gene Symbol	Change in Expression (p≤0.02)
Cell surface/Matrix protein			***Trafficking/targeting proteins***		
AF106861	ATRN	-	AF002163	AP3D1	-
AJ001683	KLRC4	-	D63476	ARHGEF7	-
D26579	ADAM8	-	U00957	AKAP10	+
M33308	VCL	+	X07315	NUTF2	+
U12255	FCGRT	+	***DNA replications***		
Transcription Factors			J05249	RPA2	+
AJ001183	SOX10	-	L20046	ERCC5	+
AB004066	BHLHB2	-	L26336	HSPA2	+
AF012108	NCOA3	-	L26339	RCD-8	+
AF025654	RNGTT	-	L78833	VAT1	+
AF035262	SMARCE1	-	M62302	GDF1	+
D42123	CRIP2	-	M84820	RXRB	+
D80003	NCOA6	-	***Other functional protein***		
L19067	RELA	+	M20681	SLC2A3	-
L19871	ATF3	+	AA631972	NK4	-
L38933	HUMGT198A	+	AB026891	SLC7A11	-
L39059	TAF1C	+	AF047472	BUB3	-
L49380	SF1	+	AI972631	ARS2	-
M81601	TCEA1	+	AL008726		-
U37251	ZNF177	+	AL050254	FBXO7	-
U63810	CIAO1	+	D44466	PSMD1	-
U68727	PKNOX1	+	D87953	NDRG1	-
X99720	PRCC	+	L43964	PSEN2	+
Metabolism			M76558	CACNA1D	+
AF104421	UROD	-	M83664	HLA-DPB1	+
AL049954	AHCYL1	-	M95178	ACTN1	+
D16294	ACAA2	-	U40705	TERF1	+
D16481	HADHB	-	U59913	SMAD5	+
D28137	BST2	-	U72263	EXT2	+
D38537	PPOX	-	X01703	TUBA3	+
D55639	KYNU	-	X14487	KRT10	+
U25849	ACP1	+	X51602	FLT1	+
U91316	BACH	+	X58199	ADD2	+
X58965	NME2	+	X76538	MPV17	+
X76228	ATP6V1E1	+	X78338	ABCC1	+
RNA processing transport			Z24727	TPM1	+
AA205857	SNRPD3	-	***Functionally unclassified***		
AB007510	PRPF8	-	AA923149	WSB2	-
AB017019	HNRPDL	-	AB002322	SRRM2	-
AL008726	ZSWIM3	-	AB007879	CP110	-
U40763	PPIG	+	AB007890	LKAP	-
Growth factor/chemokine and receptors			AB007915	KIAA0446	-
			AB007931	RBAF600	-
X78686	CXCL5	-	AB011133	MAST3	-
X81882	CUL5	-	AB011151	BDG29	-
D13168	EDNRB	-	AB014515	N4BP1	-

Gene Accesssion #	Gene Symbol	Change in Expression ($p \leq 0.02$)	Gene Accesssion #	Gene Symbol	Change in Expression ($p \leq 0.02$)
D14582	EPIM	-	AB014564	KIAA0664	-
D26070	ITPR1	-	AB014599	BICD2	-
J03278	PDGFRB	-	AB018344	DDX46	-
J03634	INHBA	-	AB023186	PEPP3	-
M91211	AGER	+	AB028995	PPM1E	-
S67368	GABRB2	+	AB028998	TENC1	-
U23850	ITPR1	+	AB029012	EST1B	-
U78110	NRTN	+	AF051941	NME6	-
X06614	RARA	+	AF058696	NBS1	
X60592	TNFRSF5	+	AL031228	VPS52	-
X64116	PVR	+	AL031282	FLJ13052	-
Non-receptor protein kinases			AL046940	FLJ46603	-
AI341656	LIM	-	D29677	HELZ	-
L13738	ACK1	+	D50645	SDF2	-
L27071	TXK	+	D50920	THRAP4	-
X54637	TYK2	+	D79990	RASSF2	-
Non-receptor phosphatases			D87119	TRIB2	-
AI739548		-	***Not classified***		
J03805	PPP2CB	-	S59184	RYK	+
L36151	PIK4CA	+	U01062	ITPR3	+
M29893	RALA	+	U12597	TRAF2	+
M64929	PPP2R2A	+	U41737		+
X68277	DUSP1	+	U85611	CIB1	+
Nuclear receptors			U89358	L3MBTL	+
AB020639	ESRRG	-	U93869	POLR3F	+
AF084645	NR1I2	-	W25974	MTX1	+
AF109134	OGFR	-	W27949	HEBP2	+
X75918	NR4A2	+	X16281	ZNF44	+
Translation/post-trans modification			X52851	PPIA	+
			X65784	SPG7	+
D84273	NARS	-	X92814	HRASLS3	+
M34539	FKBP1A	+	XM29054		+
Death receptor proteins/adaptors			Y09305	DYRK4	+
				GEF	+
AF006041	DAXX	-	NM_003242	Protein Kinase	
U04806	FLT3LG	+		Pitslre, Alpha,	
U50062	RIPK1	+		Proto-Oncogene	
X98176	CASP8	+		N-Cym,	
Chaperones/ heat shock proteins				Single-Stranded DNA-Binding	
W28616	HSPCB	-		Protein Mssp-	+
L26336	HSPA2	+			
X04106	CAPNS1	+			
Cell signaling/EC communication					
AI658639	ENSA	-			
L19605	ANXA11	+			
M32886	SRI	+			

Gene Accession #	Gene Symbol	Change in Expression ($p \leq 0.02$)	Gene Accession #	Gene Symbol	Change in Expression ($p \leq 0.02$)
U37283	MFAP5	+			
U79716	RELN	+			
Adaptor/receptor-associated proteins					
AF015767	BRE	-			
U09284	LIMS1	+			
GTP/GDP and G-protein GTPase activity modulators					
AB002349	RALGPS1	-			
AI961929	ARHGAP1	-			
M85169	PSCD1	+			
U57629	RPGR	+			

Table 5

Gene Symbol	Gene Name	Ref#9	Ref#11	Ref#12	Ref#14
BCL10	B-cell CLL/lymphoma 10	-	+	-	-
CDH2	Cadherin 2A	+	-	-	-
F13A1	Coagulation factor XIII	-	-	+	-
CRH	Corticotropin Releasing Hormone	-	+	-	-
ECM2	Extracellular Matrix Protein 2	+	-	-	-
HOXD4	Homeo box D4	-	-	-	+
ENO1	c-myc binding protein	-	-	-	+
PIPPIN	Ortholog of rat Pippin	-	-	-	+
PPIB	Peptidylprolyl isomerase B	-	-	-	+
RY1	Putative ucleic acid binding protein	-	-	-	+
TYMS	Thymidylate synthetase	+	+	-	+

Ref#9: Tsibris, J. *et al. Fertil Steril*, 2002, 78:114-121

Ref#11: Wang, H. *et al. Fertil Steril*, 2003, 80:266-276

Ref#12: Weston, G. *et al. Mol Hum Reprod*, 2003, 9:541-549

Ref#14: Quade, B.J. *et al. Cancer*, 2004, 40:97-108

Table 6

Gene Accesssion #	Gene Symbol	Gene Accesssion #	Gene Symbol
	Transcription activators/repressors		***Intracellular kinases (non-receptor)***
AJ000041	HOXC11	AF-068864	PAK3
NM_001130	AES	L13616	PTK2
NM_006164	NFE2L2	NM_003177	SYK
	Cell cycle-regulating kinases	NM_002822	PTK9
M84489	MAPK1	NM_012290	TLK1
	Oncogene/tumor suppressors		***GPs/GTPase activity modulators***
NM_002315	LMO1		
M24898	NR1D1	M28212	RAB6A
NM_002350	LYN	AF030107	RGS13
	Membrane channels and transporters		***Kinase activators/inhibitors***
		X82240	TCL1A
NM_006358	SLC25A17	NM_003629	PIK3R3
	Trafficking		***Cytoskeleton/motility proteins***
NM_005829	AP3S2		
	Metabolism	X58199	ADD2
NM_001355	DDT		***Functionally unclassified***
NM_000819	GART	NM_004487	GOLGB1
NM_004317	ASNA1	NM_004337	C8orf1
	Translation/post-translational	NM_006992	B7
NM_006156	NEDD8		***Not classified***
NM_003758	EIF3S1	NM_021964	ZNF148
	Death receptor-associated proteins	NM_021999	ITM2B
		NM_014629	ARHGEF10
AF015956	DAXX	NM_030913	SEMA6C
	RNA processing/turnovert	NM_012263	TTLL1
NM_002568	PABPC1	NM_020150	SARA1
	Neuropeptides/growth factors		PPIA
NM_003353	UCN		RPE
NM_002006	FGF2		MAFK
	Extracellular communication		LRIG2
NM_001405	EFNA2		DKFZP586F242
NM_004279	EEEF1E1		KIAA0290
	Intracellular transducers/effectors		Homeotic Protein Hox5.4
NM_005079	TPD52		
NM_006012	CLPP		

Table 7

Gene Accession#	Gene Symbol	GnRHa 2h vs TGF-βRII antisense p≤0.001	Gene Accession#	Gene Symbol	GnRHa 6h vs TGF-βRII antisense p≤0.001
BC003576	ACTN1	-	AK023082	GORASP2	+
Adenylyl Cyclase-AP2		+	AF077204	GTPBP1	+
M12271	ADH1A	+	BC035837	HAS1	+
AB014605	AIP1	+	AK097824	HSPA2	+
BC000171	AMD1	-	BC009696	IFITM2	+
AK092006	ANXA2	+	AC005369	IK	+
BC001429	ANXA5	-	L25851	ITGAE	+
AK098588	APEX1	+	AF003521	JAG2	-
AF038954	ATP6V1G1	-	BC002646	JUN	-
AB020680	BAG5	-	AF081484	K-ALPHA-1	-
AF019413	BF	+	AF056022	KATNA1	+
AB004066	BHLHB2	-	AK025504	KIAA0251	-
BC009050	BTG1	+	AB002301	KIAA0303	-
AB030905	CBX3	+	AB014528	KIAA0628	+
BC008816	CCBP2	+	AB014548	KIAA0648	+
BC032518	CCNG2	+	AB040969	KIAA1536	-
AU130185	CDH6	+	AB040972	KIAA1539	-
AJ011497	CLDN7	-	AF061809	KRT16	+
AJ006267	CLPX	+	BC009971	KRTHA3B	+
BC005159	COL6A1	-	AB014581	L3MBTL	+
AK098615	CRY1	-	AF000177	LSM1	+
AL833597	CSF2RA	-	AB025186	MAPRE3	-
AF013611	CTSW	-	AB018266	MATR3	-
AK025446	DKFZP564M182	-	AC005943	MBD3	-
AJ005821	DMXL1	-	AY032603	MCM3	-
AF088046	DNAJA2	-	AF508978	MTA1	-
BC039596	DNM2	-	AK130664	MTHFD2	-
AF139463	EGR2	-	AB023192	NISCH	+
N66802	EGR3	-	AC004663	NOTCH3	-
AF001434	EHD1	-	AB005060	NRG2	+
AF208852	EIF4A2	+	AK025458	NUCB1	-
BC000738	EMD	-		NCOR 2	-
AF103905	EPAC	+	AF109134	OGFR	+
AF052181	EPIM	+	AJ238420	PDGFA	-
BC003384	FKBP2	+	AB005754	POLS	-
AF085357	FLOT1	+	AB051763	POR	-
AY358917	FSTL3	-	AA846273	PRCC	+
L13698	GAS1	+	AF044206	PTGS2	-
AF169253	GATA2	-	AY449732	PTHR1	+
AF144713	GDI2	+	BC002438	RAB4A	+
AC000051	GGT1	+	AF080561	RBM14	-
NM_000855	GUCY1A2	+	BC003608	RBPMS	-
X83412	HAB1	+	AL031228	RING1	+
AF103884	HB-1	+	AB078417	RIS1	+

Gene Accession#	Gene Symbol	GnRHa 2h vs TGF-βRII antisense p≤0.001	Gene Accession#	Gene Symbol	GnRHa 6h vs TGF-βRII antisense p≤0.001
AF264785	HES1	-	AK096243	RPN2	+
BC022283	HFL3	+	D10570	RUNX1	-
	IGF I	+	BC002829	S100A2	+
D86989	IGL2	+	AB011096	SARM1	+
AF038953	ITM2A	-	BC020740	SGCD	-
NM_005354	JUND	-	AC004000	SLC25A5	-
AB014765	JWA	+	AY142112	SLC4A3	+
AB002308	KIAA0310	-	AF053134	SNCB	+
AB014548	KIAA0648	+	AB061546	SRP14	+
AK129875	LAPTM4A	+	AK125542	SRPX	+
AB017498	LRP5	+	AB015718	STK10	+
AF027964	MADH2	+	BC012085	STK38	+
AK026690	MADH3	+	AF064804	SUPT3H	+
AB025247	MAFF	-	BC000125	TGFB1	-
AB025186	MAPRE3	-	AI290070	THBS1	+
AB017335	MAZ	-	AY117678	TPT1	+
AF061261	MBNL2	+	AF062174	TRIAD3	-
BC012396	MGC40157	+	BC014243	TYK2	-
AF125532	MKNK2	+	AB003730	UBC	+
BC001122	MSH2	+	AB014610	USP52	+
AF508978	MTA1	-	BC030810	ZNF230	-
AF057354	MTMR1	+	AJ245587	ZNF248	+
NM_005593	MYF5	-	BI547129	ZW10	-
AB011179	NCDN	-	AC006020	AASS	+
AF047181	NDUFB5	+	AF245699	AGTR1	+
AB014887	ORM1	+	AC002366	AMELX	+
BC009610	PC4	+	D12775	AMPD3	+
AK023529	PCBP2	-	AB084454	ANGPT1	+
AB029821	PEMT	-	AF019225	APOL1	+
AF254253	PHKG1	+	BC014450	B7	+
AF220656	PHLDA1	-	AB004066	BHLHB2	-
AF025439	PKM2	-	AB062484	CALD1	+
A18757	PLAUR	-	AB023172	CARD8	+
AB006746	PLSCR1	-	BC002609	CBX1	-
A24059	PNLIP	+	AF213700	CDKN1B	+
AB005754	POLS	-	AF018081	COL18A1	+
AF042385	PPIE	+	BC000326	COPB2	+
BC047502	PPP1R3D	+	AF062536	CUL1	-
AK091875	PPP2CB	-	NM_005491	CXorf6	-
AI800682	PTPN21	-	AC004634	DTR	-
BC028038	PTPRD	+	AA053720	EDIL3	+
BC001390	QP-C	+	AF174496	EEF1A1	+
BC003608	RBPMS	-	AF139463	EGR2	-
AF019413	RDBP	+	N66802	EGR3	-
AF086557	RPL10A	+	AF000670	ELF4	-

Gene Accession#	Gene Symbol	GnRHa 2h vs TGF-βRII antisense p≤0.001	Gene Accession#	Gene Symbol	GnRHa 6h vs TGF-βRII antisense p≤0.001
AB007147	RPS2	+	AF083633	EXTL1	-
BC011645	RRAD	-	BC001786	FKBP4	-
D10570	RUNX1	-	AY358917	FSTL3	-
AB028976	SAMD4	-	AB014560	G3BP2	-
AF070614	SCHIP1	-	AK022142	GAB1	+
BC005927	SERPINE1	-	AF169253	GATA2	-
AJ000051	SF1	-	AL031659	GHRH	+
AK097315	SF3B4	-	BC026329	GJA1	+
BC004534	SFPQ	-	AF052693	GJB5	+
AL110214	SFRS6	-	AF493902	GNA13	+
AB020410	SHH	+	K03460	H2-ALPHA	-
AB001328	SLC15A1	+	AF264785	HES1	-
AF519179	SMOX	-	AB017018	HNRPDL	+
AK096917	SREBF2	-	AF056979	IFNGR1	-
AF261072	TCBAP0758	+	AC005369	IK	+
BC003151	TCFL1	+	AJ271736	IL9R	+
BC000125	TGFB1	-	AF007140	ILF3	+
AF050110	TIEG	-	AY351902	IQGAP2	+
AF087143	TOP2B	+	AB007893	KIAA0433	+
AC002481	TUSC4	+	AB014528	KIAA0628	+
AC002400	UBPH	+	AB028956	KIAA1033	-
AF060538	VAMP1	+	AB014581	L3MBTL	+
AF134726	VARS2	-	BC016618	LCP2	+
BC000165	VDAC2	-	AF211969	LENG4	-
AF007132	ABHD5	-	AF004230	LILRB1	+
AL831821	ACADSB	+	BC017263	LMAN2	+
AJ306929	AFURS1	-	AF055581	LNK	-
AB031083	AKR1C1	+	AK095843	LOC169834	+
AC002366	AMELX	+	AB025247	MAFF	-
AB084454	ANGPT1	+	AC005943	MBD3	-
AF168956	APLP2	-	BC012396	MGC40157	+
AF047432	ARF6	+	AF508978	MTA1	-
AK000379	ASNS	-	AK130664	MTHFD2	-
AF022224	BAG1	+	NM_005593	MYF5	-
BC019307	BCL2L1	+	AB020673	MYH11	+
AC006378	BET1	+	BC005318	MYL1	+
AB004066	BHLHB2	-	AB014887	ORM1	+
AF002697	BNIP3	-	AK125499	P5	+
AL021917	BTN3A3	+	AJ238420	PDGFA	-
AB059429	BUCS1	+	AK055119	PDK2	-
AJ420534	C6orf145	-	AB051763	POR	-
AF111344	CASP10	+	AF042385	PPIE	+
AK022697	CBARA1	-	AF345987	PRKCG	+
BC009356	CDC42EP1	-	M95929	PRRX1	-
AF002713	CENPB	-	AF119836	RAB6A	+

Gene Accession#	Gene Symbol	GnRHa 2h vs TGF-βRII antisense p≤0.001	Gene Accession#	Gene Symbol	GnRHa 6h vs TGF-βRII antisense p≤0.001
AK128741	CHD4	+	AF019413	RDBP	+
AF136185	COL17A1	+	AF055026	RPIP8	+
AB014764	COPS7A	-	BC020740	SGCD	+
AF452623	CRELD1	-	AF519179	SMOX	-
AK098615	CRY1	-	AF391283	SSA1	-
AL833597	CSF2RA	-	BC012088	TAF10	-
AB014595	CUL4B	+	BC000125	TGFB1	-
AB015051	DAXX	-	AF050110	TIEG	-
AJ313463	DF	+	AY065346	TNFAIP1	-
BC015800	DXYS155E	+	AF019413	TNXB	-
BC014410	EFEMP1	-	AK025459	TRA1	+
AF139463	EGR2	-	AJ440721	TXNDC5	+
BC028412	ELL2	+	AB062290	TYMS	+
AK092872	ERCC2	+	BC000379	UBB	+
AK000818	FLJ20811	+	AB003730	UBC	+
AK074486	FLJ90005	-	AF002224,	UBE3A	-
AK130009	FRZB	+	AF001787	UCP3	+
AJ251501	GAD2	+	AF135372	VAMP2	-
AC004976	GARS	-	AB029013	WHSC1	-
AK094782	GLUD1	-	AB023214	ZBTB1	-
AF070597	GNB1	-	AF060865	ZNF205	+
			AF055077	ZNF42	+

Table 8

Gene Accesion#	Gene Symbol	GnRHa 2h vs TGF-βRII antisense p≤0.001	Gene Accesion#	Gene Symbol	GnRHa 6h vs TGF-βRII antisense p≤0.001
AK000002	ABCC10	+	AK074531	PRR3	-
AF129756	AIF1	+	AF332577	PSMA6	+
AA114994	ARGBP2	+	AK023775	PTPRF	-
BC014450	B7	-	AF263016	PTPRR	-
AB005298	BAI2	-	BC001390	QP-C	+
AF090947	BBS4	+	BC015460	QPCT	+
AB038670	BDNF	+	AF000231	RAB11A	-
AC006378	BET1	+	AK055170	RAE1	+
AB018271	BPAG1	+	AF127761	RBM8A	+
AC000391	BRD3	+	AF155595	RCOR	-
AF016270	BRD8	+	BX537448	SEC14L1	-
AJ420534	C6orf145	-	AF153609	SGK	-
AB029331	C6orf18	+	AF078544	SLC25A14	+
AF072164	C9orf33	+	BC009409	TACSTD2	+
AC002543	CAPZA2	-	AF142482	TEAD3	-
BC015799	CASP7	+	BC000866	TIMP1	+
BC036787	CTF1	-	AF017146	TOP3B	+
AF280107	CYP3A5	+	BC016804	TRAM2	-
BC000485	DDC	-	BC014243	TYK2	-
AB018284	EIF5B	+	AB028980	USP24	+
AF253417	EPHX1	-	AB017103	YWHAE	-
AI879202	ETHE1	-	BC000292	ACTG1	+
BC001325	FUBP3	-	AF023476	ADAM12	+
AB058690	GPS2	+	AF001042	ADARB1	-
AY136740	GPSM2	+	AB018327	ADNP	+
NM_000855	GUCY1A2	+	AF245699	AGTR1	+
X83412	HAB1	+	AF129756	AIF1	+
	HERV-K(HML6)	-	D45915	ALK	+
AF299094	HSF1	-	AK057883	AP2M1	+
AY136751	HTR2B	+	AK023088	ARL6IP	-
BC015335	ICT1	+	AF001307	ARNT	+
AF011889	IDS	+	AB018271	BPAG1	+
BC002793	IFNAR2	-	AK096489	BZW1	+
AF117108	IMP-3	+	AB029331	C6orf18	+
AF003837	JAG1	+	AF037335	CA12	+
AF072467	JRK	+	AF070589	CACNA1C	-
AF361886	KEAP1	-	BC005334	CETN2	+
AB014564	KIAA0664	-	AY497547	CMKLR1	+
BC034041	LMO2	+	NM_001886	CRYBA4	+
AK074703	LOC89944	+	AF361370	DIA1	+
AF000177	LSM1	+	AF498961	DRD1	+
AK025599	MAN1A1	+	AK057845	EFNA1	+
AK124738	MAP4K5	+	AI879202	ETHE1	-
AK025602	MGC2747	+	AC002389	GAPDS	+

Gene Accesion#	Gene Symbol	GnRHa 2h vs TGF-βRII antisense p≤0.001	Gene Accesion#	Gene Symbol	GnRHa 6h vs TGF-βRII antisense p≤0.001
AB037859	MKL1	-	AF015257	GPR30	+
AF102544	MOCS3	-	AF103803	H41	-
BC006491	MPZ	+	X83412	HAB1	+
AB037663	MYLK	+	BC005240	HAX1	+
AF113003	NCOR2	-	AK058013	HPGD	+
AF044958	NDUFB8	+	BC000290	IGHMBP2	+
BC002421	NEF3	+	BC015752	IRF4	+
AB010710	OLR1	-	AK074047	ITGAX	+
AY189737	OVGP1	+	AF135158	JIK	+
AB014608	PARC	+	AF233882	JUP	-
AL133335	PFDN4	+	AB020638	KIAA0831	+
AJ419231	PHC2	-	AF115510	LRRFIP1	-
AF006501	POLR2F	+	AF010193	MADH7	-
AK095191	POU6F1	-	AL137667	MAPK8	+
AF045569	PRKCH	+	AK025602	MGC2747	+
NM_006256	PRKCL2	+	AF125532	MKNK2	+
AF007157	PRNPIP	-	BC006491	MPZ	+
	N-Cym	+	AB051340	MRPL23	+
AK074531	PRR3	-	AF113003	NCOR2	-
AF332577	PSMA6	+	AF013160	NDUFS2	+
AF000231	RAB11A	-		E6-Ap,	-
AF125393	RAB27A	+	Papillomavirus		+
BC002585	RAB7L1	+	BC011539	ORC1L	+
D38076	RANBP1	-	BC000398	PAFAH1B2	+
AB112074	RBBP6	+	AL117618	PDHB	+
BC007102	RQCD1	-	AB002107	PER1	-
AF072825	RREB1	+	BC062602	PNN	-
AC004381	SAH	+	AK095191	POU6F1	-
AF015224	SCGB2A2	+	BC013154	PPP2R5E	-
AF029081	SFN	+	AK055139	PTK2	-
AK127319	SLC16A3	-	AF218026	PTOV1	-
BC041164	SMPD1	-	AF008591	RAC3	-
AB046845	SMURF1	-	AL701206	RARG	+
AB030036	ST14	+	AF127761	RBM8A	-
AF070532	SUPT6H	-	AF155595	RCOR	+
AJ549245	TAF1	+	AB007148	RPS3A	-
BC029891	TFEC	+	BC007102	RQCD1	-
BC000866	TIMP1	+	BC005927	SERPINE1	+
AF139460	ZNF288	+	AB007897	SETBP1	+
BC015961	ADM	+	BC009362	SETDB1	+
AF129756	AIF1	+	AF029081	SFN	+
AY341427	AP2B1	+	AF368279	SGTA	-
BC004537	ATP6V0C	-	AK000416	SLC16A5	+
BC008861	ATP6V0D1	-	AF078544	SLC25A14	+
AB009598	B3GAT3	+	AK127096	SLC30A3	+

Gene Accesion#	Gene Symbol	GnRHa 2h vs TGF-βRII antisense p≤0.001	Gene Accesion#	Gene Symbol	GnRHa 6h vs TGF-βRII antisense p≤0.001
AB029331	C6orf18	+	AY142112	SLC4A3	+
AF078803	CAMK2B	+	BC009409	TACSTD2	+
BC015799	CASP7	+	AB006630	TCF20	-
AB025105	CDH1	+	AF142482	TEAD3	+
AB001090	CDH13	+	BC000866	TIMP1	+
AB037187	CHST7	+	BC029516	TNP1	+
AK122769	CKMT2	+	AF038009	TPST1	-
AB032372	CKTSF1B1	+	AY245544	TRB2	+
AF000959	CLDN5	+	AF104927	TTLL1	+
AF053318	CNOT8	+	BX537824	TXNIP	+
BC022069	CRABP1	+	AB002155	UPK1B	+
BC003015	DGCR14	+	AF122922	WIF1	
BC038231	DUSP8	+			
BC020746	DXS1283E	+			
J03066	EN2	+			
BC002706	ERBB3	-			
BC002706	ERBB3	-			
AI879202	ETHE1	-			
AF241235	FXYD2	+			
AF124491	GIT2	+			
	Glial Growth Factor 2	+			
AL133324	GSS	+			
AB032481	HOXD13	+			
AF299094	HSF1	-			
AF441399	HSGP25L2G	+			
AF275719	HSPCB	+			
AB030304	HUMGT198A	+			
BC014972	IL2RG	+			
AB012853	ING1L	+			
AF361886	KEAP1	-			
BC005407	KIAA0169	+			
BC014932	KIAA0280	-			
AB007887	KIAA0427	-			
AB028953	KIAA1030	+			
BC014781	LCAT	+			
AB016485	LDB1	-			
AF072814	M96	+			
AF010193	MADH7	-			
AL137667	MAPK8	+			
AY032603	MCM3	-			
AL137295	MLLT10	+			
AB051340	MRPL23	+			
AB046613	MYL6	+			
NM_004998	MYO1E	+			
AF113003	NCOR2	-			

Gene Accesion#	Gene Symbol	GnRHa 2h vs TGF-βRII antisense p≤0.001	Gene Accesion#	Gene Symbol	GnRHa 6h vs TGF-βRII antisense p≤0.001
AF013160	NDUFS2	+			
AF020351	NDUFS4	+			
BC013789	NHLH1	+			
	Nuclear Factor 1A	+			
BC011539	ORC1L	+			
AB014887	ORM1	+			
BC006268	PEX7	+			
AK093558	PFDN1	+			
AL133335	PFDN4	+			
BC009899	PIK3R4	+			
BC037246	PNMT	+			
AF055028	POLR2B	+			
BC031043	PRH1	+			
AB026491	PRKCABP	+			

Table 9

Category	Group	Gene Symbol	Gene Name
	All Group	FBLN5	fibulin 5
	All Group	ECM2	extracellular matrix protein 2, female organ and adipocyte specific
Cell adhesion molecule	Other cell adhesion molecule	SDC4	syndecan 4
Cell adhesion molecule	Kinase modulator	ICAM2	intercellular adhesion molecule 2
Extracellular matrix	Extracellular matrix glycoprotein	THBS1	thrombospondin 1
Extracellular matrix	Extracellular matrix structural protein	COL7A1	collagen, type VII, alpha 1
Extracellular matrix	Other extracellular matrix	FMOD	fibromodulin
Extracellular matrix	Extracellular matrix structural protein	COL18A1	collagen, type XVIII, alpha 1
Kinase	Protein kinase	WEE1	WEE1 homolog (S. pombe)
Molecular function unclassified	Miscellaneous function	TNFRSF5	tumor necrosis factor receptor superfamily 5
Transcription factor	Miscellaneous function	NCOA6	nuclear receptor coactivator 6
Molecular function unclassified	Miscellaneous function	GAS1	growth arrest-specific 1
Molecular function unknown	Molecular function unknown	ESM1	endothelial cell-specific molecule 1
Oxidoreductase	Oxygenase	HMOX1	heme oxygenase (decycling) 1
Protease	Cysteine-type protease	CASP8	caspase 8, apoptosis-related cysteine protease
Protease	Cam family adhesion molecule	ADAM17	a disintegrin and metalloproteinase domain 17 (tumor necrosis factor, alpha, converting enzyme)
Receptor	G-protein coupled receptor	GPR30	G protein-coupled receptor 30
Receptor	Cytokine receptor	TNFRSF6	tumor necrosis factor receptor superfamily 6
Select regulatory molecule	Kinase modulator	CCND2	cyclin D2
Select regulatory molecule	Protease inhibitor	CST7	cystatin F (leukocystatin)
Select regulatory molecule	Protease inhibitor	CST6	cystatin E/M
Select regulatory molecule	Kinase modulator	CCNE1	cyclin E1

Signaling molecule	Protein/peptide hormone	EDN1	endothelin 1
Signaling molecule	Protein/peptide hormone	STC2	stanniocalcin 2
Signaling molecule	Cytokine	IL11	interleukin 11
Signaling molecule	Chemokine	CCL3	chemokine (C-C motif) ligand 3
Signaling molecule	Cytokine	IL15	interleukin 15
Signaling molecule	Other signaling molecule	CTNNB1	catenin (cadherin-associated protein), b1
Signaling molecule	Other signaling molecule	HUMGT198A	GT198, complete ORF
Signaling molecule	Cytokine	CXCL10	chemokine (C-X-C motif) ligand 10
Signaling molecule	Growth factor	CXCL12	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)
Signaling molecule	Cytokine	IL17	interleukin 17 (cytotoxic T-lymphocyte-associated serine esterase 8)
Signaling molecule	Chemokine	CXCL5	chemokine (C-X-C motif) ligand 5
Signaling molecule	Cytokine	IL13	interleukin 13
Synthase and synthetase	Synthase	TYMS	thymidylate synthetase
Transcription factor	Zinc finger transcription factor	TIEG	TGFB inducible early growth response
Transcription factor	Homeobox transcription factor	TGIF	TGFB-induced factor (TALE family homeobox)
Transcription factor	Other transcription factor	RUNX3	runt-related transcription factor 3
Transcription factor	Zinc finger transcription factor	LHX1	LIM homeobox 1
Transcription factor	Other transcription factor	E2F1	E2F transcription factor 1
Transcription factor	Zinc finger transcription factor	EGR3	early growth response 3
Transcription factor	Transcription cofactor	CITED2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2
Transcription factor	Transcription cofactor	EP300	E1A binding protein p300
Transcription factor	Nuclear hormone receptor	NR4A1	nuclear receptor subfamily 4, group A, member 1
Transcription factor	Other transcription factor	RUNX1	runt-related transcription factor 1 (acute myeloid

			leukemia 1; aml1 oncogene)
Transferase	Methyltransferase	MGMT	O-6-methylguanine-DNA methyltransferase

Claims

We claim:

1. A method for identifying a modulator of at least one gene that is differentially-expressed in fibrotic tissue or during fibrogenesis, or a polypeptide encoded by the differentially-expressed gene, in a cell population, comprising: (a) contacting the cell population with a test agent under conditions effective for the test agent to modulate a differentially-expressed gene, to modulate the biological activity of the polypeptide encoded by the differentially-expressed gene; and (b) determining whether the test agent modulates the expression of the gene or biological activity of the polypeptide encoded by the gene.

2. The method of claim 1, wherein said determining step comprises detecting mRNA or the polypeptide encoded by the differentially-expressed gene.

3. The method of claim 1, wherein the cell population comprises cells of the female reproductive tract.

4. The method of claim 1, wherein the cell population comprises endometrial cells of the female reproductive tract.

5. The method of claim 1, wherein the cell population comprises human cells.

6. The method of claim 1, wherein the at least one differentially expressed gene includes at least one gene selected from the group consisting of docking protein 1, 62 kD (downstream of tyrosine kinase 1); centromere protein A (17 kD); catenin (cadherin-associated protein), beta 1 (88 kD); nuclear receptor subfamily 1, group I, member 2; v-rel avian reticuloendotheliosis viral oncogene homolog A; LGN Protein; CDC28 protein kinase 1; hypothetical protein; solute carrier family 17 (sodium phosphate), member 1; FOS-like antigen-1; nuclear matrix protein p84; LERK-6 (EPLG6); visinin-like 1; phosphodiesterase 10A; KH-type splicing regulatory protein (FUSE binding protein 2); Polyposis locus (DP1 gene) mRNA; microtubule-associated protein 2; CDC5 (cell division cycle 5, S pombe, homolog)-like; Centromere autoantigen C (CENPC) mRNA; RNA guanylyltransferase and 5'-phosphatase; Nijmegen breakage syndrome 1 (nibrin); ribonuclease, RNase A family, 4; keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris); basic helix-loop-helix domain containing, class B, 2; dual specificity phosphatase 1; annexin A11; putative receptor protein; Human endogenous retrovirus HERV-K(HML6); mitogen-activated protein kinase kinase kinase 12; TXK tyrosine kinase; kynureninase (L-kynurenine hydrolase); ubiquitin specific protease 4 (proto-oncogene); peroxisome biogenesis factor 13; olfactory

receptor, family 2, subfamily F, member 1; membrane protein, palmitoylated 3 (MAGUK p55 subfamily member 3); origin recognition complex, subunit 1 (yeast homolog)-like; dTDP-D-glucose 4,6-dehydratase; cytochrome c oxidase subunit VIa polypeptide 2; gamma-tubulin complex protein 2; Monocyte chemotactic protein-3; myelin transcription factor 1; inhibitor of growth family, member 1-like; thyroid hormone receptor, alpha myosin-binding protein C, slow-type; fragile X mental retardation 2; sonic hedgehog (Drosophila) homolog; 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2; SFRS protein kinase 2; excision repair cross-complementing rodent repair deficiency; cyclin-dependent kinase 5, regulatory subunit 1 (p35); poly(A)-specific ribonuclease (deadenylation nuclease); solute carrier family 12 (potassium/chloride transporters), member 4; Pseudogene for metallothionein; natriuretic peptide precursor A; intercellular adhesion molecule 2; apoptosis antagonizing transcription factor; similar to rat HREV107; major histocompatibility complex, class II, DP beta 1; MpV17 transgene, murine homolog, glomerulosclerosis; uroporphyrinogen decarboxylase; proteasome (prosome, macropain) 26S subunit, ATPase, 1; fms-related tyrosine kinase 3 ligand; actin, gamma 1; Protein Kinase Pitslre, Alpha, Alt. Splice 1-Feb; nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; pyruvate kinase, muscle; telomeric repeat binding factor 2; cell division cycle 2, G1 to S and G2 to M; ADP-ribosylation factor 3; NRF1 Protein; H factor (complement)-like 3; serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 6; mRNA of muscle specific gene M9; solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3; ribosomal protein L36a; suppressor of Ty (S. cerevisiae) 4 homolog 1; amino-terminal enhancer of split; ubiquitin A-52 residue ribosomal protein fusion product 1; hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase; chaperonin containing TCP1, subunit 2 (beta); tyrosine kinase with immunoglobulin and epidermal growth factor homology; domains; Fc fragment of IgG, receptor, transporter, alpha; NRD1 convertase; ADP-ribosylation factor 5; transcription elongation factor A (SII), 1; like mouse brain protein E46; titin; fibromodulin; and Abi-interactor 2 (Abi-2).

7. The method of claim 1, wherein the at least one differentially expressed gene includes at least one gene selected from the group consisting of CDKN1B, CDKN1C, CTGF, fibromodulin, and Abi-2.

8. The method of claim 1, wherein the at least one differentially expressed gene includes at least one of IL-11, IL-13, EGR1, EGR2, EGR3, CITED2, P300, E2F1, E2F2,

E2F3, E2F4, E2F5, MCP3, CXCL5, CCL7, SMAD3, TYMS, GT198, SMAD7, NCOR2, TIMP-1, and ADAM17.

9. The method of claim 1, wherein the at least one differentially-expressed gene includes at least one of those genes listed in Table 9.

10. The method of claim 1, wherein the at least one differentially-expressed gene includes at least one gene selected from the group consisting of stanniocalcin 2, interleukin 11, disintegrin and metalloproteinase domain 17, early growth response 3, fibromodulin, collagen type XVIII alpha 1, and interleukin 13.

11. The method of claim 1, wherein the at least one differentially expressed gene includes a plurality of genes comprising stanniocalcin 2, interleukin 11, disintegrin and metalloproteinase domain 17, early growth response 3, fibromodulin, collagen type XVIII alpha 1, and interleukin 13.

12. A method for detecting a fibrotic disorder in a subject by: (a) providing a biological sample obtained from the subject; (b) analyzing the expression of at least one gene that is differentially expressed in the fibrotic disorder of interest as compared to normal tissue; and (c) correlating the expression of the at least one differentially expressed gene with the presence or absence of the fibrotic disorder in the subject.

13. The method of claim 12, wherein the fibrotic disorder is a fibrotic disorder of the female reproductive tract.

14. The method of claim 12, wherein the fibrotic disorder is a uterine fibrosis.

15. The method of claim 12, wherein the fibrotic disorder is a fibrotic disorder of the female reproductive tract selected from the group consisting of leiomyoma, endometriosis, ovarian hyperstimulation syndrome, adhesion, and endometrial cancer.

16. The method of claim 12, wherein the sample comprises smooth muscle cells.

17. The method of claim 12, wherein the sample comprises endometrium or peritoneal fluid.

18. The method of claim 12, wherein the normal tissue comprises myometrium.

19. The method of claim 12, wherein the at least one differentially expressed gene includes at least one gene selected from the group consisting of docking protein 1, 62 kD (downstream of tyrosine kinase 1); centromere protein A (17 kD); catenin (cadherin-associated protein), beta 1 (88 kD); nuclear receptor subfamily 1, group I, member 2; v-rel avian reticuloendotheliosis viral oncogene homolog A; LGN Protein; CDC28 protein kinase 1; hypothetical protein; solute carrier family 17 (sodium phosphate), member 1; FOS-like

antigen-1; nuclear matrix protein p84; LERK-6 (EPLG6); visinin-like 1; phosphodiesterase 10A; KH-type splicing regulatory protein (FUSE binding protein 2); Polyposis locus (DP1 gene) mRNA; microtubule-associated protein 2; CDC5 (cell division cycle 5, S pombe, homolog)-like; Centromere autoantigen C (CENPC) mRNA; RNA guanylyltransferase and 5'-phosphatase; Nijmegen breakage syndrome 1 (nibrin); ribonuclease, RNase A family, 4; keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris); basic helix-loop-helix domain containing, class B, 2; dual specificity phosphatase 1; annexin A11; putative receptor protein; Human endogenous retrovirus HERV-K(HML6); mitogen-activated protein kinase kinase kinase 12; TXK tyrosine kinase; kynureninase (L-kynurenine hydrolase); ubiquitin specific protease 4 (proto-oncogene); peroxisome biogenesis factor 13; olfactory receptor, family 2, subfamily F, member 1; membrane protein, palmitoylated 3 (MAGUK p55 subfamily member 3); origin recognition complex, subunit 1 (yeast homolog)-like; dTDP-D-glucose 4,6-dehydratase; cytochrome c oxidase subunit VIa polypeptide 2; gamma-tubulin complex protein 2; Monocyte chemotactic protein-3; myelin transcription factor 1; inhibitor of growth family, member 1-like; thyroid hormone receptor, alpha myosin-binding protein C, slow-type; fragile X mental retardation 2; sonic hedgehog (Drosophila) homolog; 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2; SFRS protein kinase 2; excision repair cross-complementing rodent repair deficiency; cyclin-dependent kinase 5, regulatory subunit 1 (p35); poly(A)-specific ribonuclease (deadenylation nuclease); solute carrier family 12 (potassium/chloride transporters), member 4; Pseudogene for metallothionein; natriuretic peptide precursor A; intercellular adhesion molecule 2; apoptosis antagonizing transcription factor; similar to rat HREV107; major histocompatibility complex, class II, DP beta 1; MpV17 transgene, murine homolog, glomerulosclerosis; uroporphyrinogen decarboxylase; proteasome (prosome, macropain) 26S subunit, ATPase, 1; fms-related tyrosine kinase 3 ligand; actin, gamma 1; Protein Kinase Pitslre, Alpha, Alt. Splice 1-Feb; nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; pyruvate kinase, muscle; telomeric repeat binding factor 2; cell division cycle 2, G1 to S and G2 to M; ADP-ribosylation factor 3; NRF1 Protein; H factor (complement)-like 3; serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 6; mRNA of muscle specific gene M9; solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3; ribosomal protein L36a; suppressor of Ty (S. cerevisiae) 4 homolog 1; amino-terminal enhancer of split; ubiquitin A-52 residue ribosomal protein fusion product 1; hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase; chaperonin containing TCP1, subunit 2

(beta); tyrosine kinase with immunoglobulin and epidermal growth factor homology; domains; Fc fragment of IgG, receptor, transporter, alpha; NRD1 convertase; ADP-ribosylation factor 5; transcription elongation factor A (SII), 1; like mouse brain protein E46; titin; fibromodulin; and Abi-interactor 2 (Abi-2).

20. The method of claim 12, wherein the at least one differentially expressed gene includes at least one gene selected from the group consisting of CDKN1B, CDKN1C, CTGF, fibromodulin, and Abi-2.

21. The method of claim 12, wherein the at least one differentially expressed gene includes at least one of IL-11, IL-13, EGR1, EGR2, EGR3, CITED2, P300, E2F1, E2F2, E2F3, E2F4, E2F5, MCP3, CXCL5, CCL7, SMAD3, TYMS, GT198, SMAD7, NCOR2, TIMP-1, and ADAM17, wherein elevated expression of IL-11, IL-13, EGR1, EGR2, EGR3, CITED2, P300, E2F1, E2F2, E2F3, E2F4, E2F5, MCP3, CXCL5, CCL7, SMAD3, TYMS, and/or GT198 is indicative of a fibrotic disorder; and wherein reduced expression of SMAD7, NCOR2, TIMP-1, and/or ADAM17 is indicative of a fibrotic disorder.

22. The method of claim 12, wherein the at least one differentially expressed gene includes at least one of those genes listed in Table 9.

23. The method of claim 12, wherein the at least one differentially expressed gene includes at least one gene selected from the group consisting of stanniocalcin 2, interleukin 11, disintegrin and metalloproteinase domain 17, early growth response 3, fibromodulin, collagen type XVIII alpha 1, and interleukin 13.

24. The method of claim 12, wherein the at least one differentially expressed gene includes a plurality of genes comprising stanniocalcin 2, interleukin 11, disintegrin and metalloproteinase domain 17, early growth response 3, fibromodulin, collagen type XVIII alpha 1, and interleukin 13.

25. The method of claim 12, wherein the subject is human.

26. The method of claim 12, further comprises diagnosing the subject based on said correlating.

27. A method for modulating gene expression in fibrotic tissue, comprising contacting the fibrotic tissue *in vitro* or *in vivo* with an agent that modulates expression of at least one differentially expressed gene in the tissue.

28. The method of claim 27, wherein the agent is a TGF-beta signaling inhibitor.

29. The method of claim 27, wherein the agent is a TGF-beta II receptor inhibitor.

30. The method of claim 27, wherein the agent is a TGF-beta signaling inhibitor, and wherein the agent comprises interfering RNA.

31. The method of claim 27, wherein the at least one differentially expressed gene includes at least one gene selected from the group consisting of docking protein 1, 62 kD (downstream of tyrosine kinase 1); centromere protein A (17 kD); catenin (cadherin-associated protein), beta 1 (88 kD); nuclear receptor subfamily 1, group I, member 2; v-rel avian reticuloendotheliosis viral oncogene homolog A; LGN Protein; CDC28 protein kinase 1; hypothetical protein; solute carrier family 17 (sodium phosphate), member 1; FOS-like antigen-1; nuclear matrix protein p84; LERK-6 (EPLG6); visinin-like 1; phosphodiesterase 10A; KH-type splicing regulatory protein (FUSE binding protein 2); Polyposis locus (DP1 gene) mRNA; microtubule-associated protein 2; CDC5 (cell division cycle 5, S pombe, homolog)-like; Centromere autoantigen C (CENPC) mRNA; RNA guanylyltransferase and 5'-phosphatase; Nijmegen breakage syndrome 1 (nibrin); ribonuclease, RNase A family, 4; keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris); basic helix-loop-helix domain containing, class B, 2; dual specificity phosphatase 1; annexin A11; putative receptor protein; Human endogenous retrovirus HERV-K(HML6); mitogen-activated protein kinase kinase kinase 12; TXK tyrosine kinase; kynureninase (L-kynurenine hydrolase); ubiquitin specific protease 4 (proto-oncogene); peroxisome biogenesis factor 13; olfactory receptor, family 2, subfamily F, member 1; membrane protein, palmitoylated 3 (MAGUK p55 subfamily member 3); origin recognition complex, subunit 1 (yeast homolog)-like; dTDP-D-glucose 4,6-dehydratase; cytochrome c oxidase subunit VIa polypeptide 2; gamma-tubulin complex protein 2; Monocyte chemotactic protein-3; myelin transcription factor 1; inhibitor of growth family, member 1-like; thyroid hormone receptor, alpha myosin-binding protein C, slow-type; fragile X mental retardation 2; sonic hedgehog (Drosophila) homolog; 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2; SFRS protein kinase 2; excision repair cross-complementing rodent repair deficiency; cyclin-dependent kinase 5, regulatory subunit 1 (p35); poly(A)-specific ribonuclease (deadenylation nuclease); solute carrier family 12 (potassium/chloride transporters), member 4; Pseudogene for metallothionein; natriuretic peptide precursor A; intercellular adhesion molecule 2; apoptosis antagonizing transcription factor; similar to rat HREV107; major histocompatibility complex, class II, DP beta 1; MpV17 transgene, murine homolog, glomerulosclerosis; uroporphyrinogen decarboxylase; proteasome (prosome, macropain) 26S subunit, ATPase, 1; fms-related tyrosine kinase 3 ligand; actin, gamma 1; Protein Kinase Pitslre, Alpha, Alt. Splice 1-Feb; nuclear factor of

kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; pyruvate kinase, muscle; telomeric repeat binding factor 2; cell division cycle 2, G1 to S and G2 to M; ADP-ribosylation factor 3; NRF1 Protein; H factor (complement)-like 3; serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 6; mRNA of muscle specific gene M9; solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3; ribosomal protein L36a; suppressor of Ty (S. cerevisiae) 4 homolog 1; amino-terminal enhancer of split; ubiquitin A-52 residue ribosomal protein fusion product 1; hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase; chaperonin containing TCP1, subunit 2 (beta); tyrosine kinase with immunoglobulin and epidermal growth factor homology; domains; Fc fragment of IgG, receptor, transporter, alpha; NRD1 convertase; ADP-ribosylation factor 5; transcription elongation factor A (SII), 1; like mouse brain protein E46; titin; fibromodulin; and Abi-interactor 2 (Abi-2).

32. The method of claim 27, wherein the at least one differentially expressed gene includes at least one gene selected from the group consisting of CDKN1B, CDKN1C, CTGF, fibromodulin, and Abi-2.

33. The method of claim 27, wherein the at least one differentially expressed gene includes at least one of IL-11, IL-13, EGR1, EGR2, EGR3, CITED2, P300, E2F1, E2F2, E2F3, E2F4, E2F5, MCP3, CXCL5, CCL7, SMAD3, TYMS, GT198, SMAD7, NCOR2, TIMP-1, and ADAM17.

34. The method of claim 27, wherein the at least one differentially expressed gene includes at least one of those genes listed in Table 9.

35. The method of claim 27, wherein the at least one differentially expressed gene includes at least one gene selected from the group consisting of stanniocalcin 2, interleukin 11, disintegrin and metalloproteinase domain 17, early growth response 3, fibromodulin, collagen type XVIII alpha 1, and interleukin 13.

36. The method of claim 27, wherein the at least one differentially expressed gene includes a plurality of genes comprising stanniocalcin 2, interleukin 11, disintegrin and metalloproteinase domain 17, early growth response 3, fibromodulin, collagen type XVIII alpha 1, and interleukin 13.

37. The method of claim 27, wherein the agent is a selective estrogen receptor modulator (SERM).

38. The method of claim 27, wherein the agent is a selective progesterone receptor modulator (SPRM).

39. The method of claim 27, wherein the agent is a mast cell inhibitor.

40. The method of claim 27, wherein the agent has a pyrazolopyridine scaffold, a pyrazole scaffold, an imadazpyridine scaffold, a triazole scaffold, a pyridopyrimidine scaffold, or an isothiazole scaffold.

41. The method of claim 27, wherein the agent is a GnRh agonist or antagonist.

42. The method of claim 27, wherein the agent is at least one selected from the group consisting of roloxifene; asoprisnil (J867); RU486; SB-505124; SB-431542; tranlist; cystatin C (CystC); SD-208; LY550410; LY580276; pitavastatin; 1,-5 naphthyridine amiothiazole derivative; 1,5 naphthyridine pyrazole derivative; and ursolic acid.

43. An array comprising a substrate having a plurality of addresses, wherein each address disposed thereon has a capture probe that can specifically bind at least one polynucleotide that is differentially expressed in fibrotic disorders, or a complement thereof.

44. The array of claim 43, wherein the at least one polynucleotide is selected from the group consisting of docking protein 1, 62 kD (downstream of tyrosine kinase 1); centromere protein A (17 kD); catenin (cadherin-associated protein), beta 1 (88 kD); nuclear receptor subfamily 1, group I, member 2; v-rel avian reticuloendotheliosis viral oncogene homolog A; LGN Protein; CDC28 protein kinase 1; hypothetical protein; solute carrier family 17 (sodium phosphate), member 1; FOS-like antigen-1; nuclear matrix protein p84; LERK-6 (EPLG6); visinin-like 1; phosphodiesterase 10A; KH-type splicing regulatory protein (FUSE binding protein 2); Polyposis locus (DP1 gene) mRNA; microtubule-associated protein 2; CDC5 (cell division cycle 5, S pombe, homolog)-like; Centromere autoantigen C (CENPC) mRNA; RNA guanylyltransferase and 5'-phosphatase; Nijmegen breakage syndrome 1 (nibrin); ribonuclease, RNase A family, 4; keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris); basic helix-loop-helix domain containing, class B, 2; dual specificity phosphatase 1; annexin A11; putative receptor protein; Human endogenous retrovirus HERV-K(HML6); mitogen-activated protein kinase kinase kinase 12; TXK tyrosine kinase; kynureninase (L-kynurenine hydrolase); ubiquitin specific protease 4 (proto-oncogene); peroxisome biogenesis factor 13; olfactory receptor, family 2, subfamily F, member 1; membrane protein, palmitoylated 3 (MAGUK p55 subfamily member 3); origin recognition complex, subunit 1 (yeast homolog)-like; dTDP-D-glucose 4,6-dehydratase; cytochrome c oxidase subunit VIa polypeptide 2; gamma-tubulin complex protein 2; Monocyte chemotactic protein-3; myelin transcription factor 1; inhibitor of growth family, member 1-like; thyroid hormone receptor, alpha myosin-binding protein C, slow-type; fragile X mental retardation 2;

sonic hedgehog (Drosophila) homolog; 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2; SFRS protein kinase 2; excision repair cross-complementing rodent repair deficiency; cyclin-dependent kinase 5, regulatory subunit 1 (p35); poly(A)-specific ribonuclease (deadenylation nuclease); solute carrier family 12 (potassium/chloride transporters), member 4; Pseudogene for metallothionein; natriuretic peptide precursor A; intercellular adhesion molecule 2; apoptosis antagonizing transcription factor; similar to rat HREV107; major histocompatibility complex, class II, DP beta 1; MpV17 transgene, murine homolog, glomerulosclerosis; uroporphyrinogen decarboxylase; proteasome (prosome, macropain) 26S subunit, ATPase, 1; fms-related tyrosine kinase 3 ligand; actin, gamma 1; Protein Kinase Pitslre, Alpha, Alt. Splice 1-Feb; nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; pyruvate kinase, muscle; telomeric repeat binding factor 2; cell division cycle 2, G1 to S and G2 to M; ADP-ribosylation factor 3; NRF1 Protein; H factor (complement)-like 3; serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 6; mRNA of muscle specific gene M9; solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3; ribosomal protein L36a; suppressor of Ty (S. cerevisiae) 4 homolog 1; amino-terminal enhancer of split; ubiquitin A-52 residue ribosomal protein fusion product 1; hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase; chaperonin containing TCP1, subunit 2 (beta); tyrosine kinase with immunoglobulin and epidermal growth factor homology; domains; Fc fragment of IgG, receptor, transporter, alpha; NRD1 convertase; ADP-ribosylation factor 5; transcription elongation factor A (SII), 1; like mouse brain protein E46; titin; fibromodulin; and Abi-interactor 2 (Abi-2).

45. The array of claim 43, wherein the at least one polynucleotide includes at least one gene selected from the group consisting of CDKN1B, CDKN1C, CTGF, fibromodulin, and Abi-2.

46. The array of claim 43, wherein the at least one polynucleotide includes at least one gene selected from the group consisting of IL-11, IL-13, EGR1, EGR2, EGR3, CITED2, P300, E2F1, E2F2, E2F3, E2F4, E2F5, MCP3, CXCL5, CCL7, SMAD3, TYMS, GT198, SMAD7, NCOR2, TIMP-1, and ADAM17.

47. The array of claim 43, wherein the at least one polynucleotide includes at least one of those genes listed in Table 9.

48. The array of claim 43, wherein the at least one polynucleotide includes at least one gene selected from the group consisting of stanniocalcin 2, interleukin 11, disintegrin and

metalloproteinase domain 17, early growth response 3, fibromodulin, collagen type XVIII alpha 1, and interleukin 13.

49. The array of claim 43, wherein the at least one polynucleotide includes a plurality of genes comprising stanniocalcin 2, interleukin 11, disintegrin and metalloproteinase domain 17, early growth response 3, fibromodulin, collagen type XVIII alpha 1, and interleukin 13.

50. The array of claim 43, wherein the array further comprises a capture probe that can specifically bind at least one polynucleotide encoding a house-keeping gene as a control.

51. The array of claim 43, wherein each of said addresses comprises a well, and wherein each of said capture probes comprises a primer for amplifying RNA in a biological sample that is deposited in said well

52. The array of claim 43, wherein said capture probes bind said polynucleotides under stringent conditions.

53. The array of claim 43, wherein said polynucleotide bound by the capture probe of each address is unique among the plurality of addresses.

54. The array of claim 43, wherein said substrate has no more than 500 addresses.

55. The array of claim 43, wherein said substrate has 200 to 500 addresses.

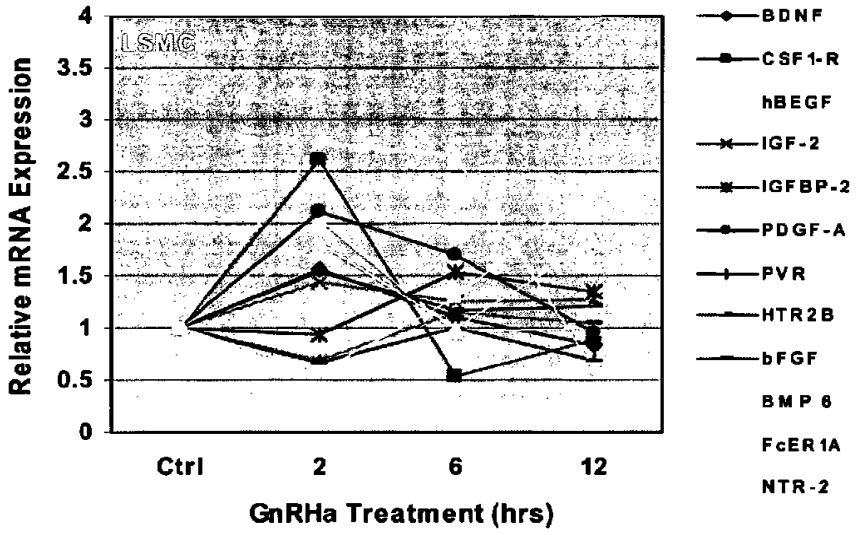

LSMC
Relative mRNA Expression
4
3.5
3
2.5
2
1.5
1
0.5
0
Ctrl
2
6
12
GnRHa Treatment (hrs)
BDNF
CSF1-R
hBEGF
IGF-2
IGFBP-2
PDGF-A
PVR
HTR2B
bFGF
BMP 6
FcER1A
NTR-2

FIG. 1A

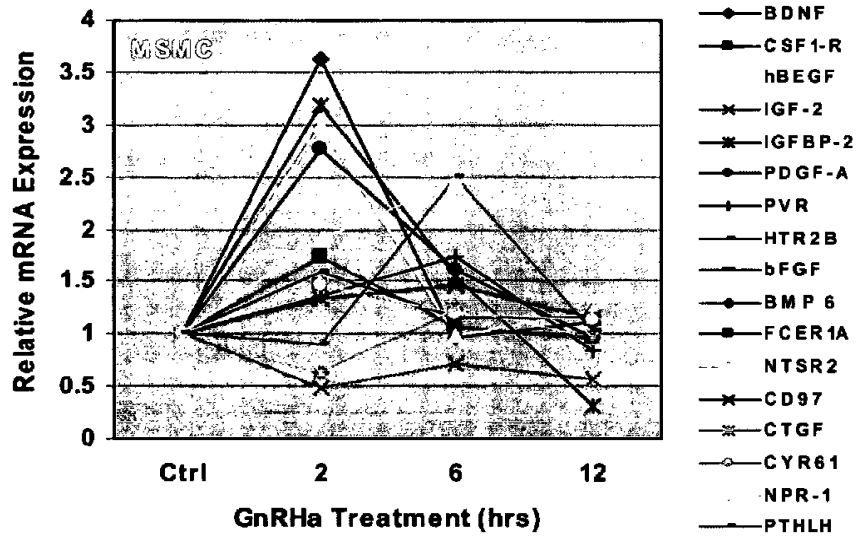

MSMC
Relative mRNA Expression
4
3.5
3
2.5
2
1.5
1
0.5
0
Ctrl
2
6
12
GnRHa Treatment (hrs)
BDNF
CSF1-R
hBEGF
IGF-2
IGFBP-2
PDGF-A
PVR
HTR2B
bFGF
BMP 6
FCER1A
NTSR2
CD97
CTGF
CYR61
NPR-1
PTHLH

FIG. 1B

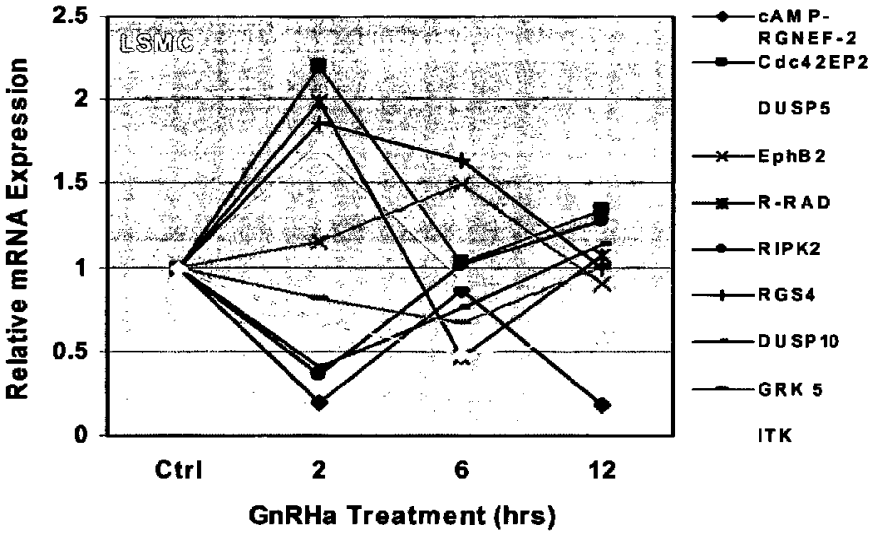

LSMC
Relative mRNA Expression
2.5
2
1.5
1
0.5
0
Ctrl
2
6
12
GnRHa Treatment (hrs)
cAMP-RGNEF-2
Cdc42EP2
DUSP5
EphB2
R-RAD
RIPK2
RGS4
DUSP10
GRK 5
ITK

FIG. 1C

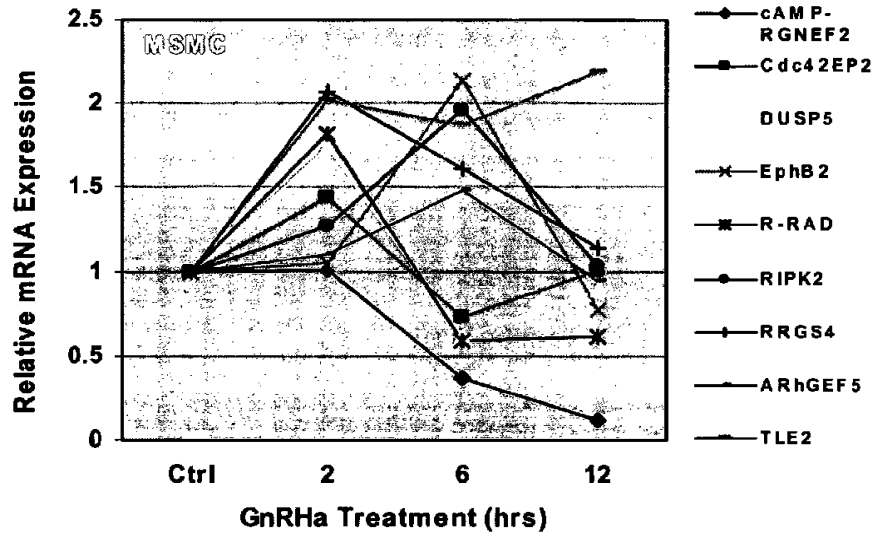

MSMC
Relative mRNA Expression
2.5
2
1.5
1
0.5
0
Ctrl
2
6
12
GnRHa Treatment (hrs)
cAMP-RGNEF2
Cdc42EP2
DUSP5
EphB2
R-RAD
RIPK2
RRGS4
ARhGEF5
TLE2

FIG. 1D

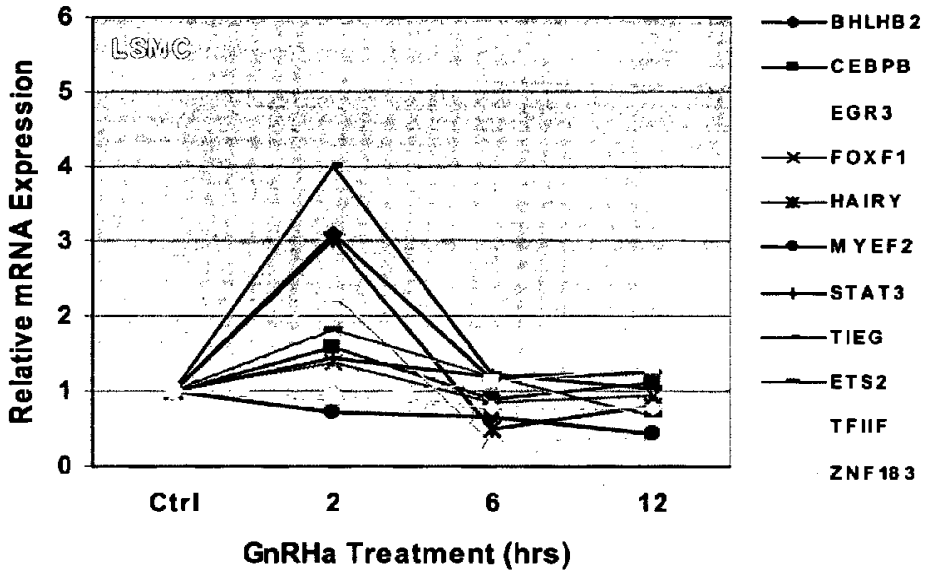

LSMC
Relative mRNA Expression
0
1
2
3
4
5
6
Ctrl
2
6
12
GnRHa Treatment (hrs)
BHLHB2
CEBPB
EGR3
FOXF1
HAIRY
MYEF2
STAT3
TIEG
ETS2
TFIIF
ZNF183

FIG. 1E

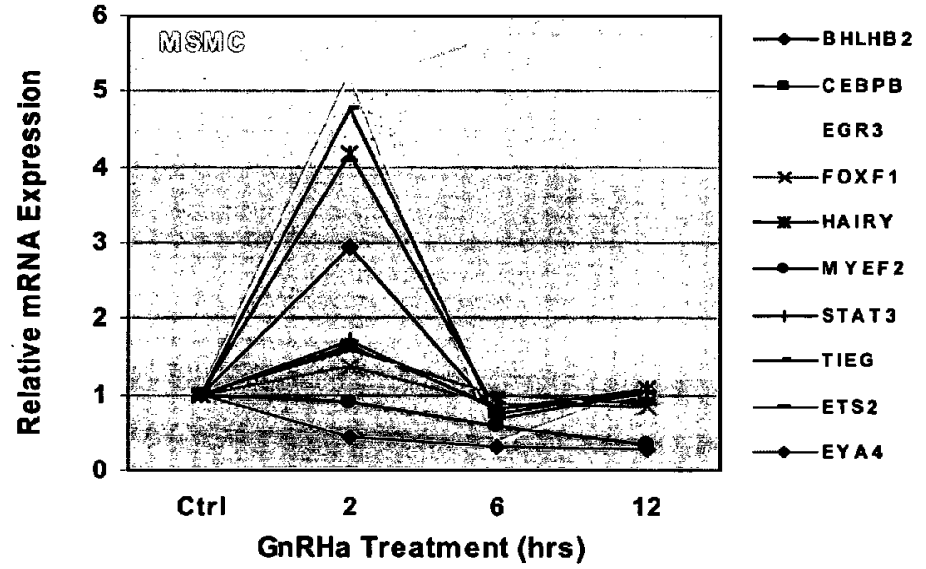

MSMC
Relative mRNA Expression
0
1
2
3
4
5
6
Ctrl
2
6
12
GnRHa Treatment (hrs)
BHLHB2
CEBPB
EGR3
FOXF1
HAIRY
MYEF2
STAT3
TIEG
ETS2
EYA4

FIG. 1F

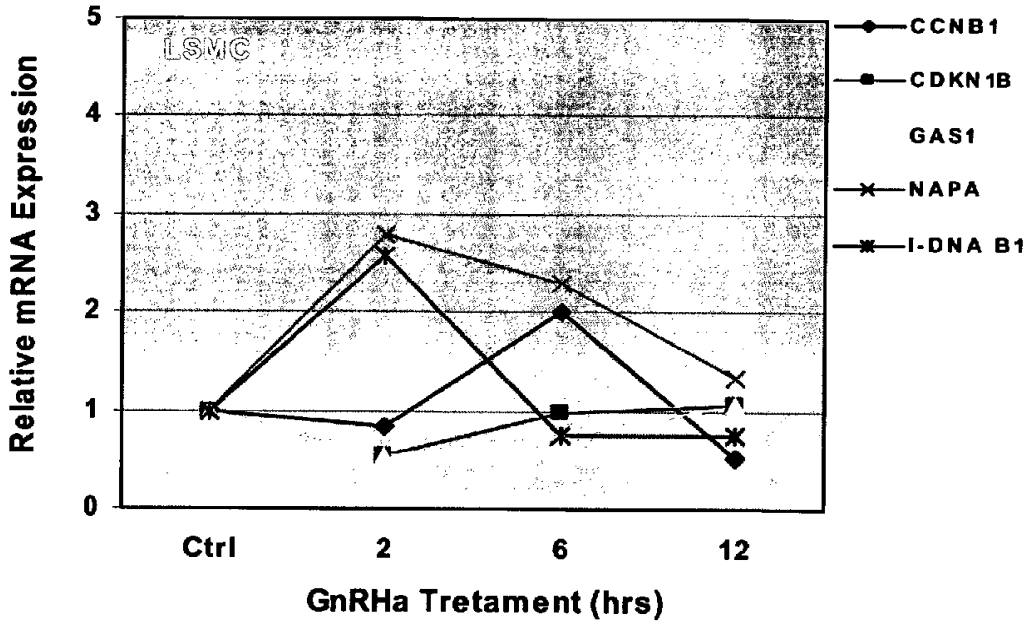

LSMC
Relative mRNA Expression
5
4
3
2
1
0
Ctrl
2
6
12
GnRHa Tretament (hrs)
CCNB1
CDKN1B
GAS1
NAPA
I-DNA B1

FIG. 1G

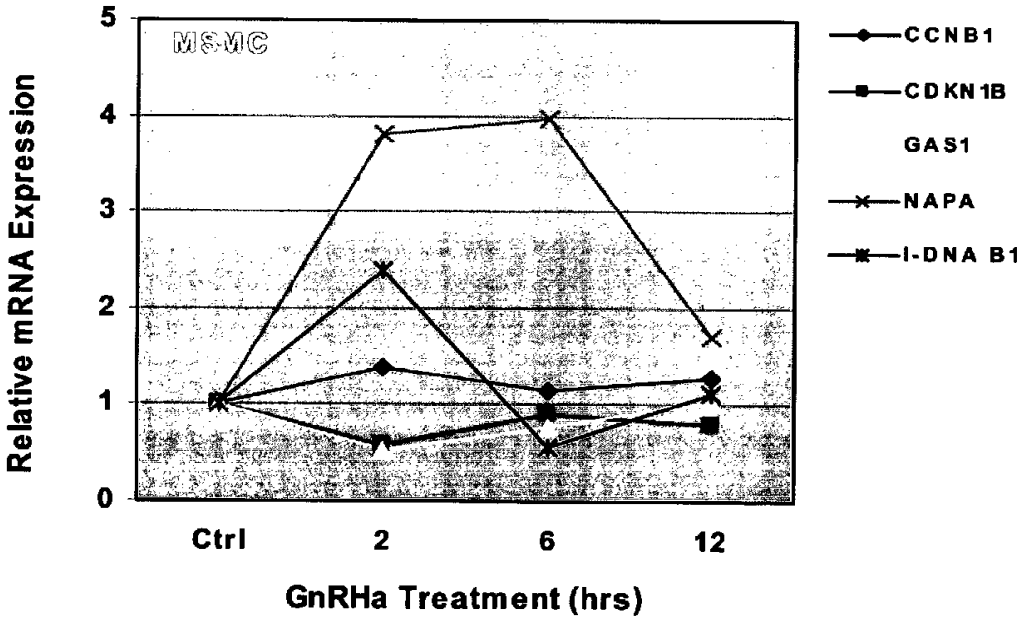

MS-MC
Relative mRNA Expression
5
4
3
2
1
0
Ctrl
2
6
12
GnRHa Treatment (hrs)
CCNB1
CDKN1B
GAS1
NAPA
I-DNA B1

FIG. 1H

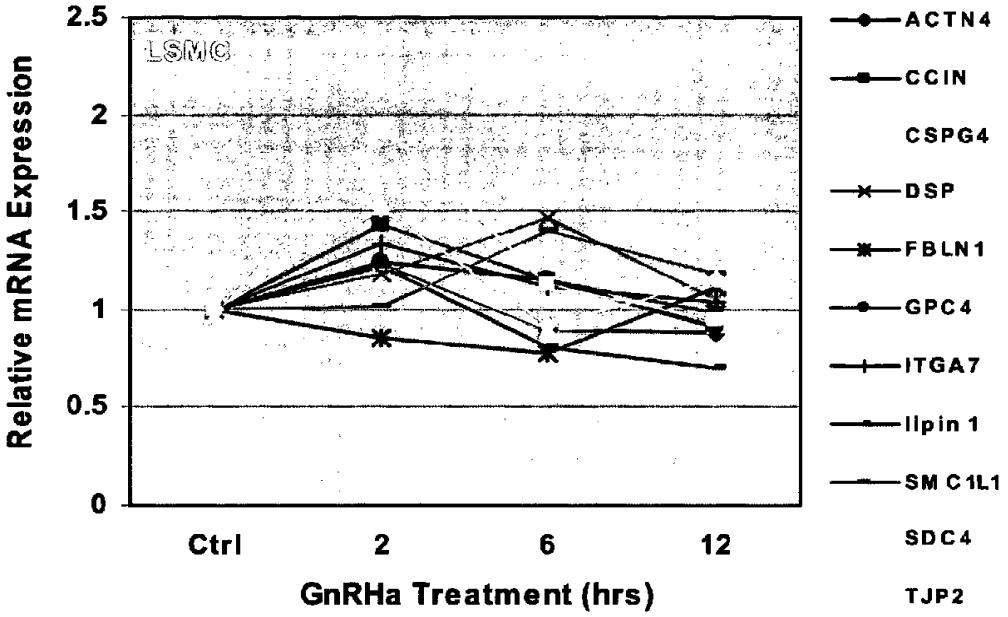

LSMC
Relative mRNA Expression
2.5
2
1.5
1
0.5
0
Ctrl
2
6
12
GnRHa Treatment (hrs)
ACTN4
CCIN
CSPG4
DSP
FBLN1
GPC4
ITGA7
Ilpin 1
SM C1L1
SDC4
TJP2

FIG. 1I

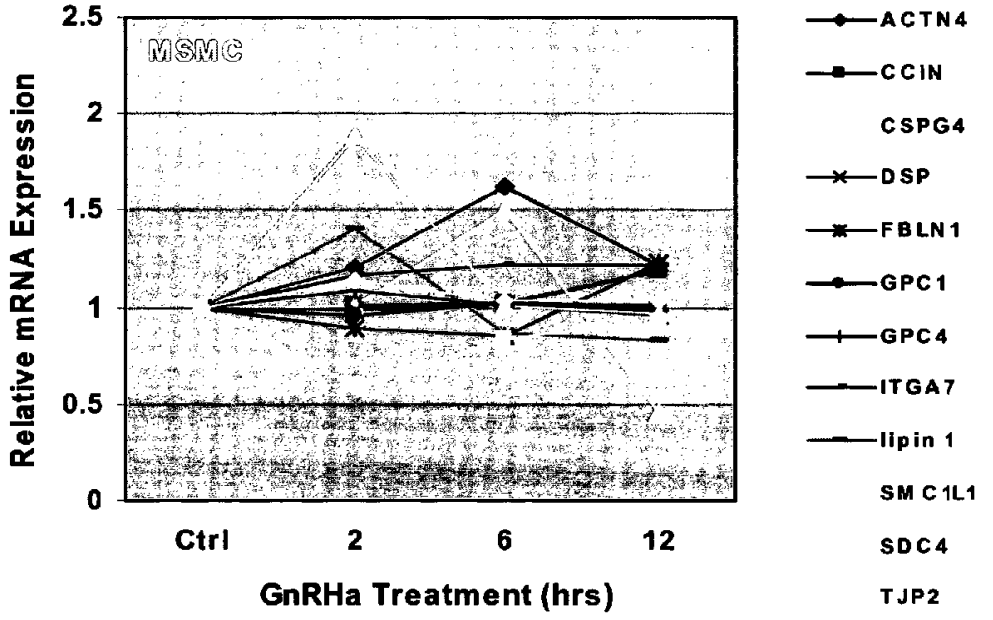

MSMC
Relative mRNA Expression
2.5
2
1.5
1
0.5
0
Ctrl
2
6
12
GnRHa Treatment (hrs)
ACTN4
CCIN
CSPG4
DSP
FBLN1
GPC1
GPC4
ITGA7
Ilpin 1
SM C1L1
SDC4
TJP2

FIG. 1J

FIG. 2A

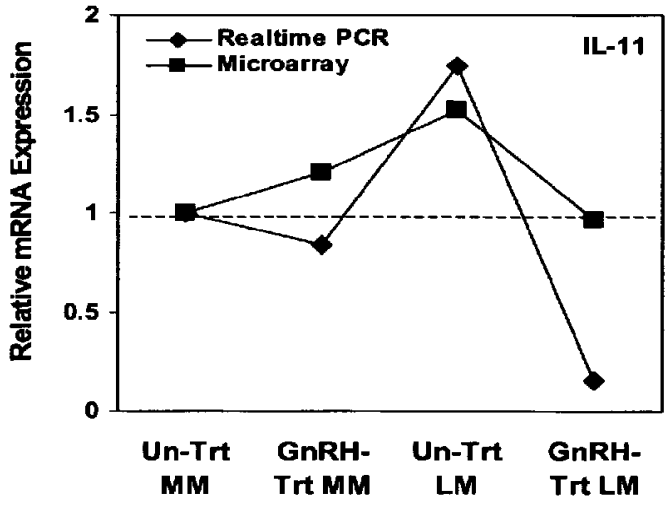


FIG. 2B

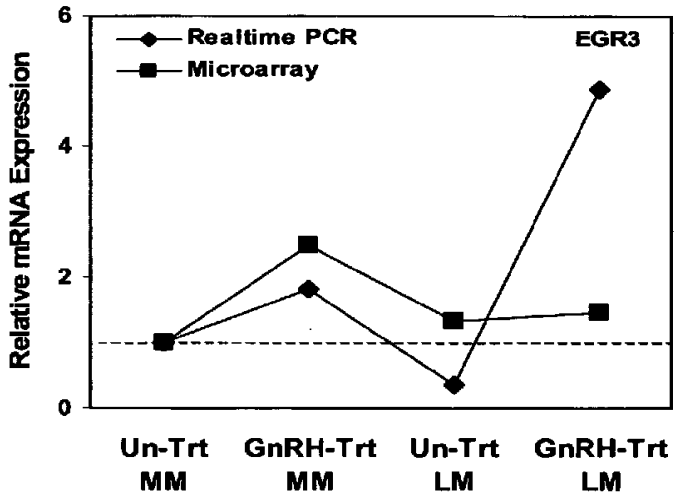


FIG. 2C

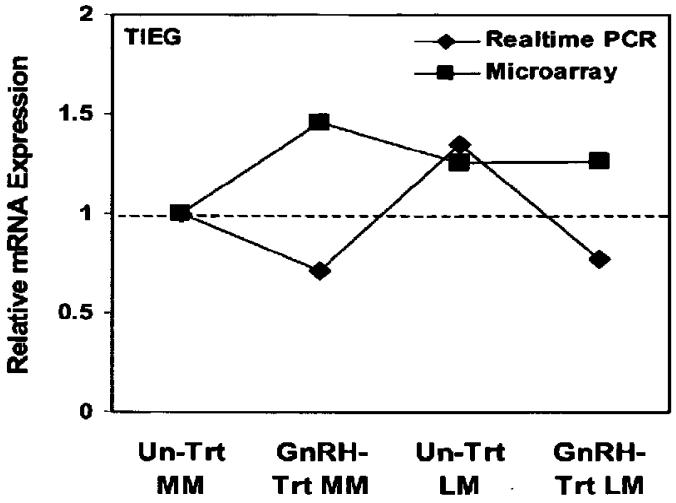


FIG. 2D

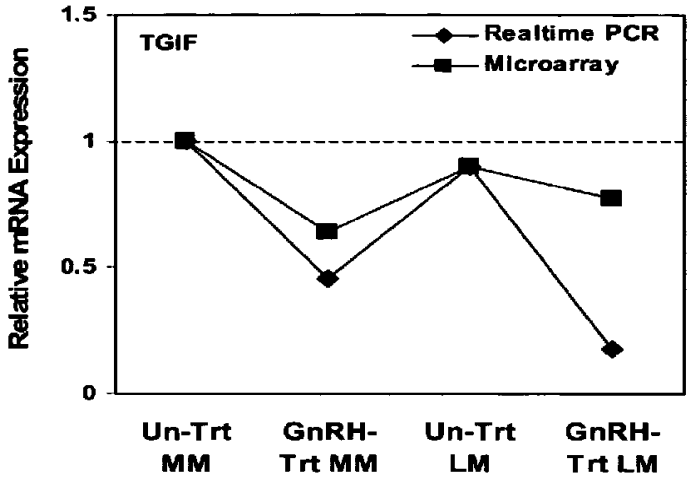


FIG. 2E

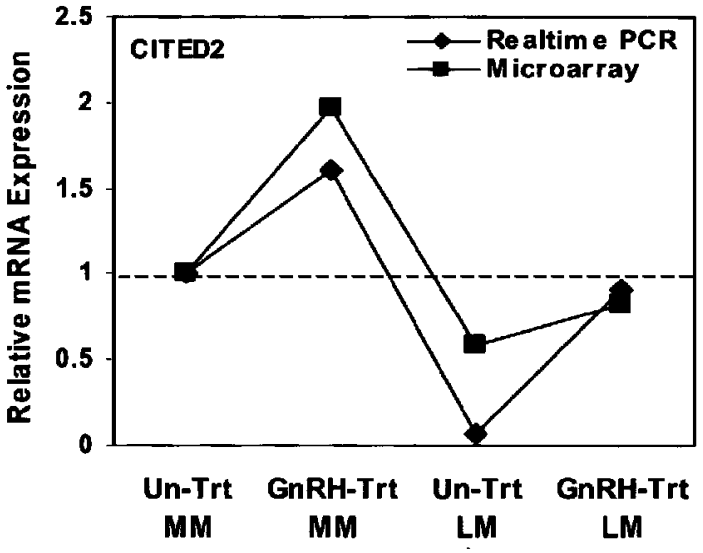


FIG. 2F

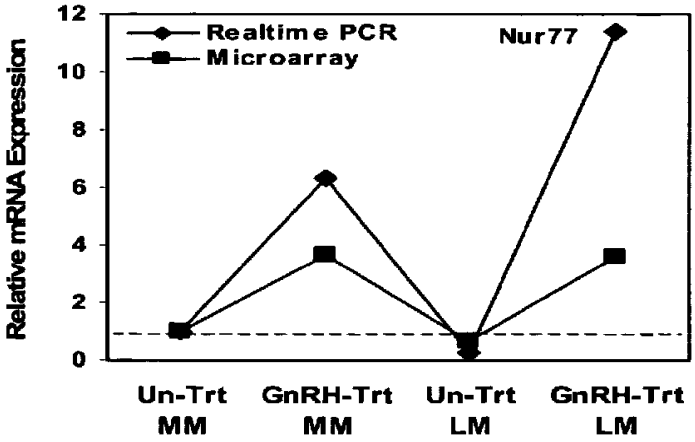


FIG. 2G

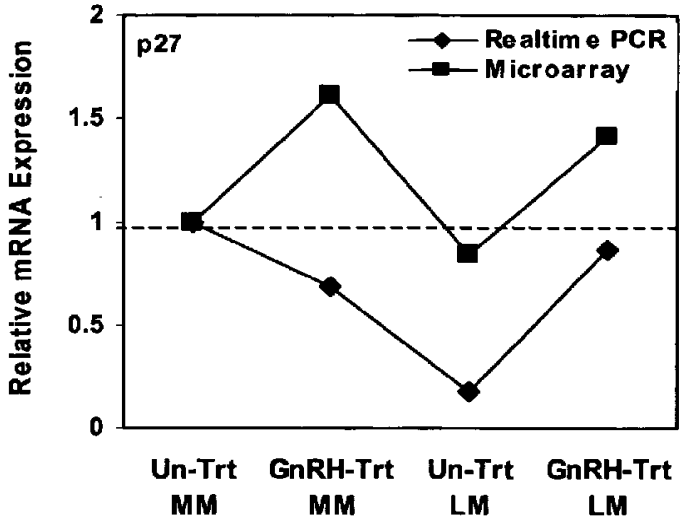


FIG. 2H

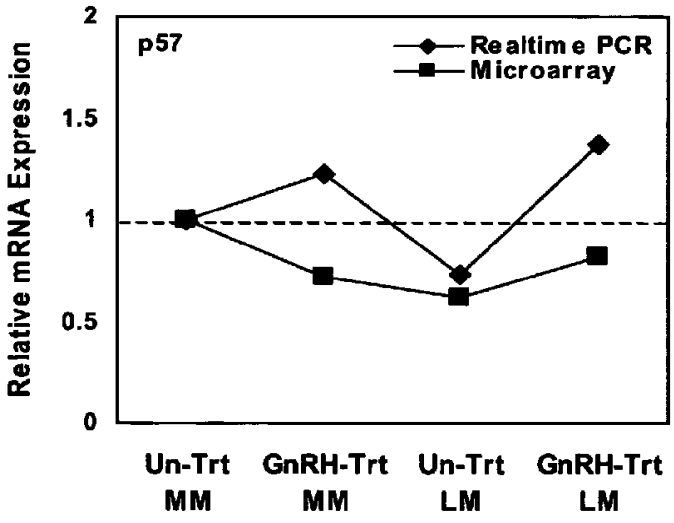


FIG. 2I

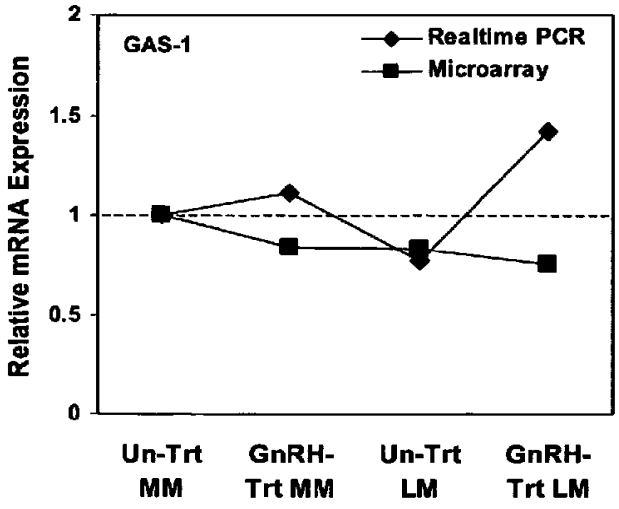


FIG. 2J

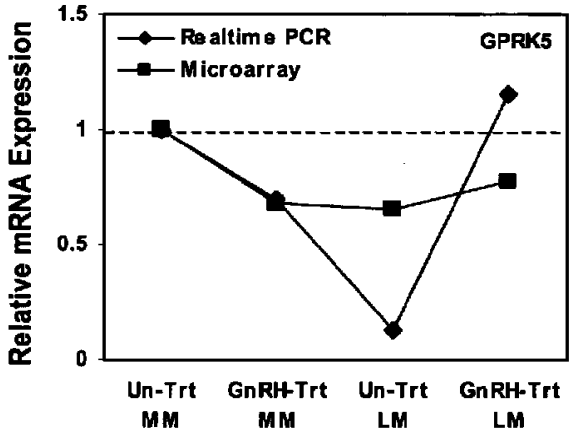


FIG. 3A

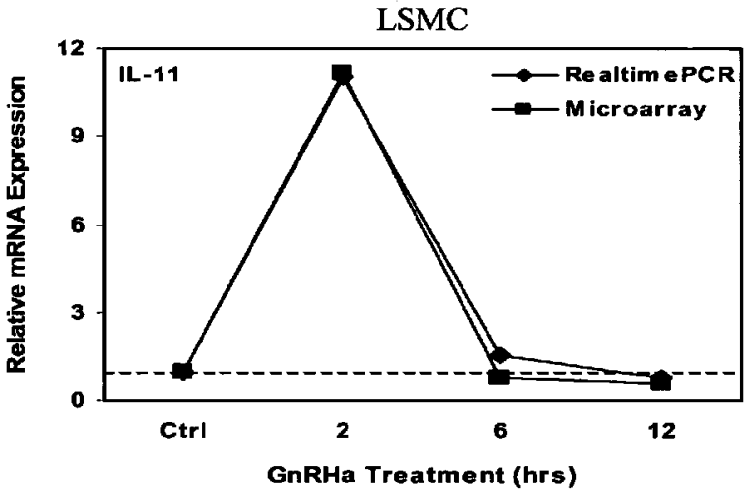


FIG. 3B

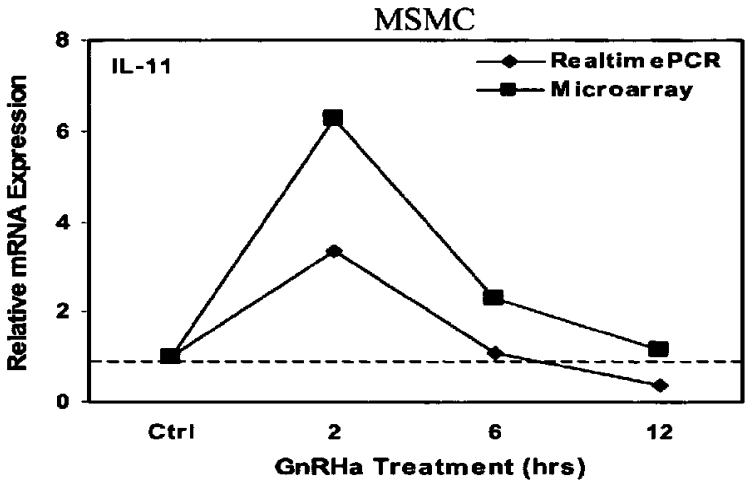


FIG. 3C

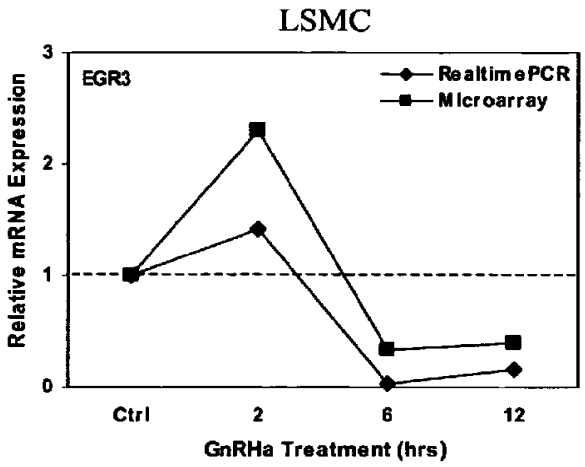


FIG. 3D

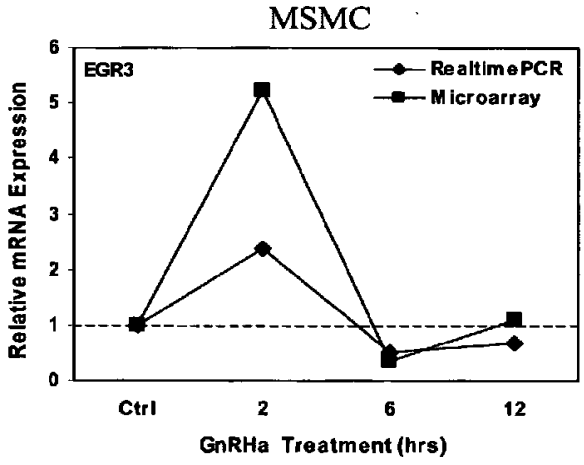


FIG. 3E

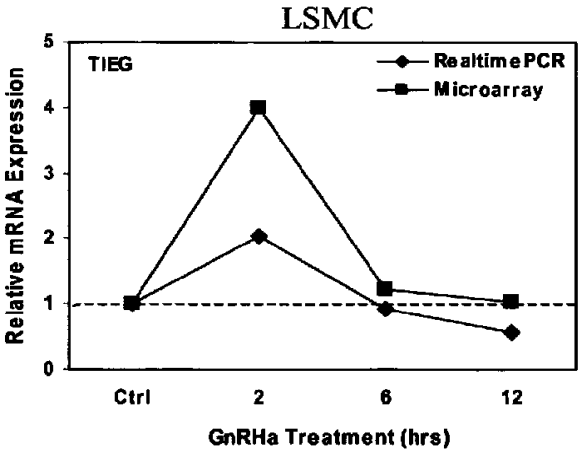


FIG. 3F

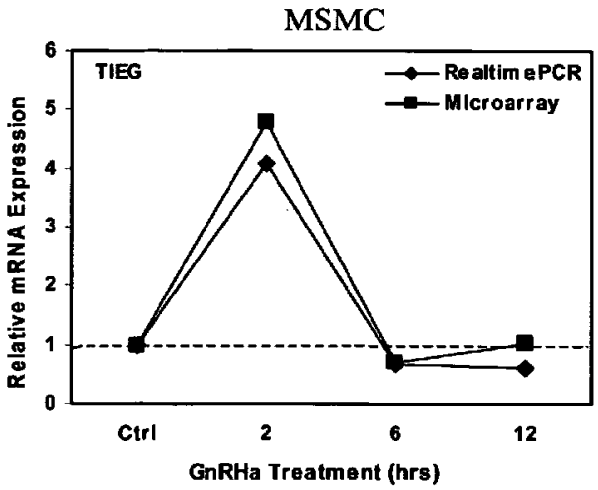


FIG. 3G

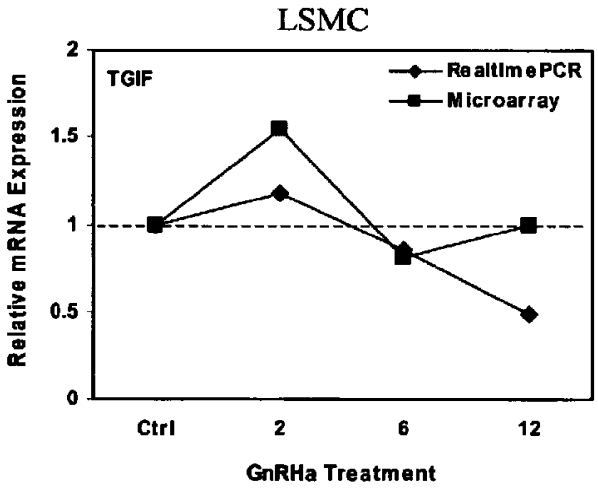


FIG. 3H

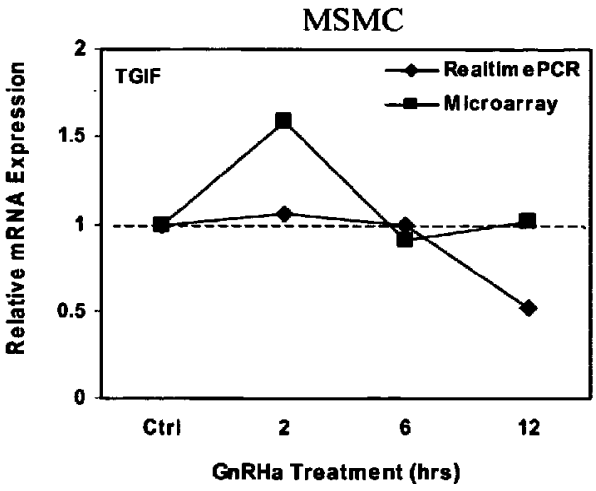


FIG. 3I

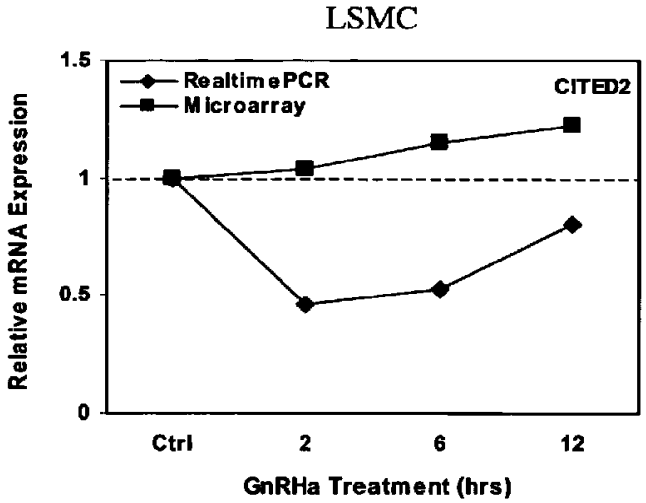


FIG. 3J

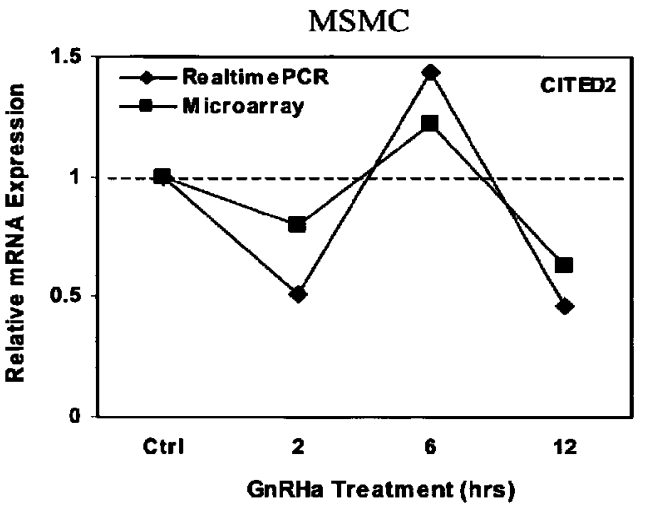


FIG. 3K

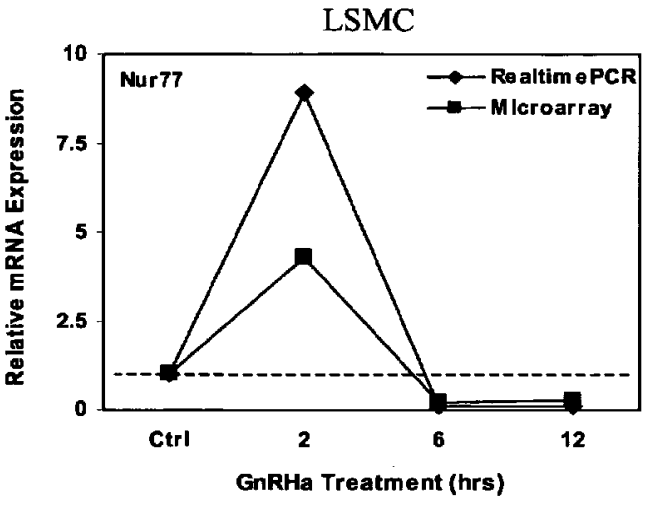


FIG. 3L

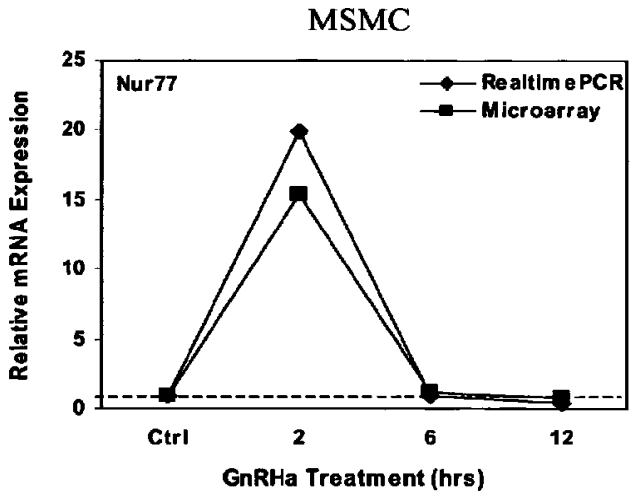


FIG. 3M

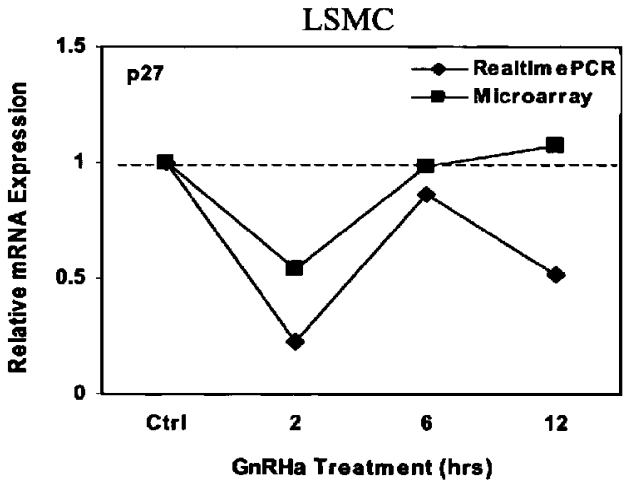


FIG. 3N

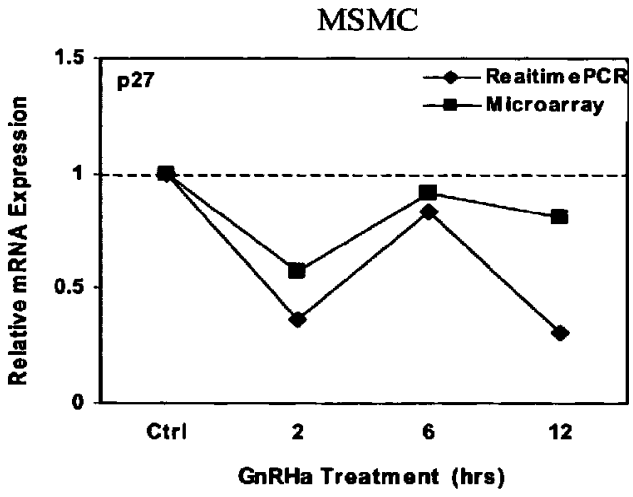


FIG. 3O

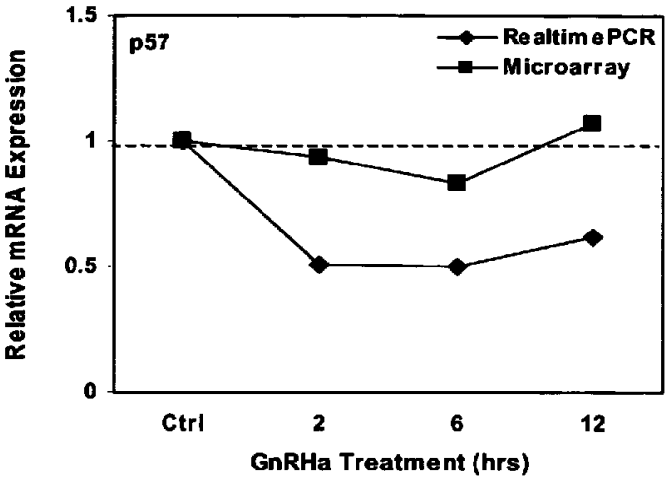


FIG. 3P

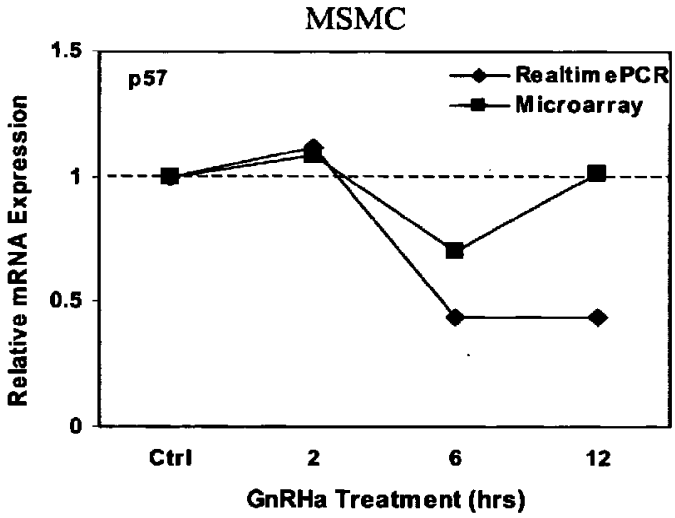


FIG. 3Q

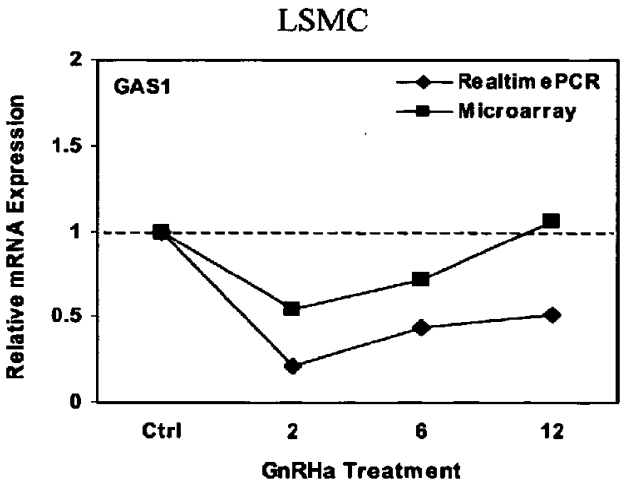


FIG. 3R

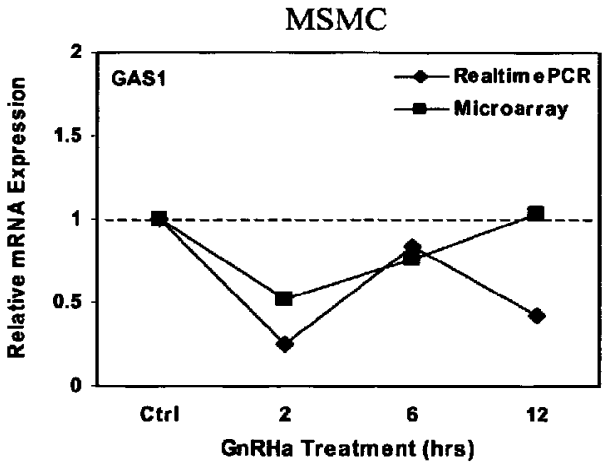


FIG. 3S

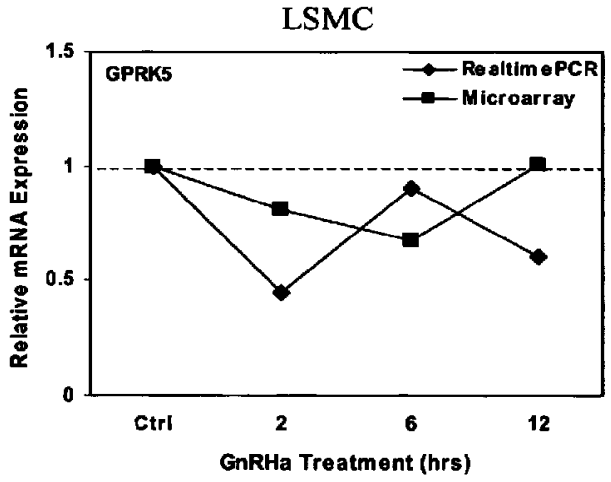
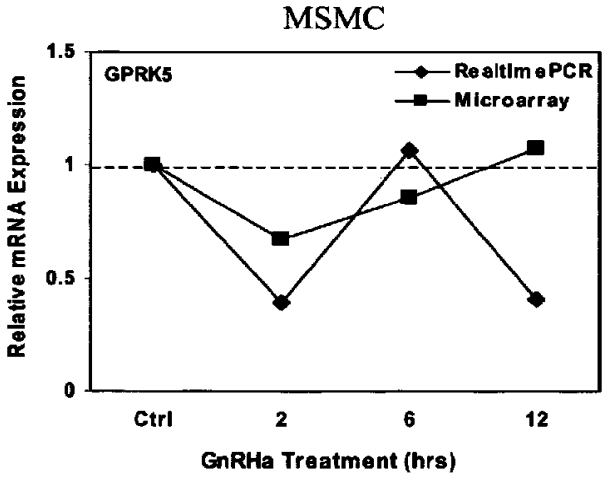


FIG. 3T



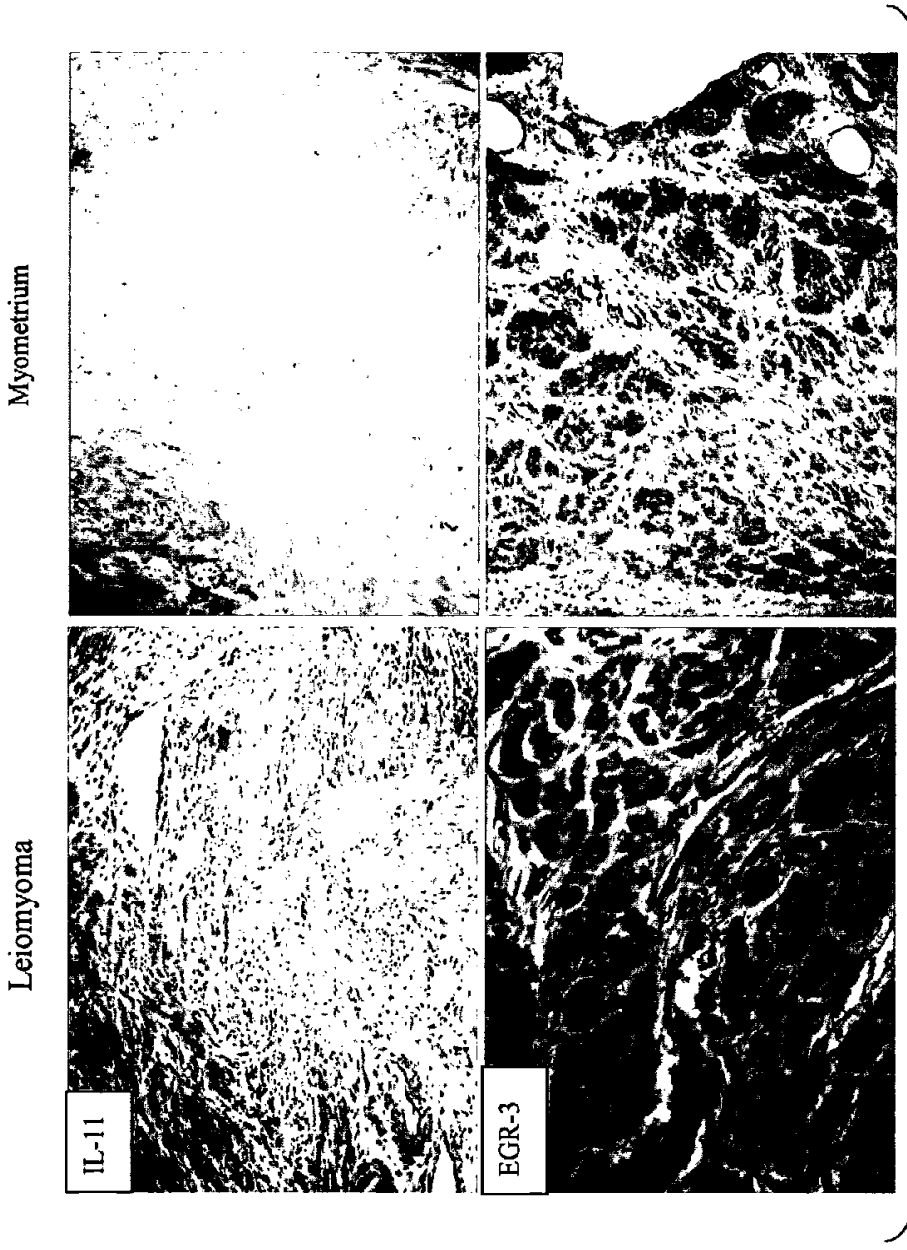


FIG. 4A

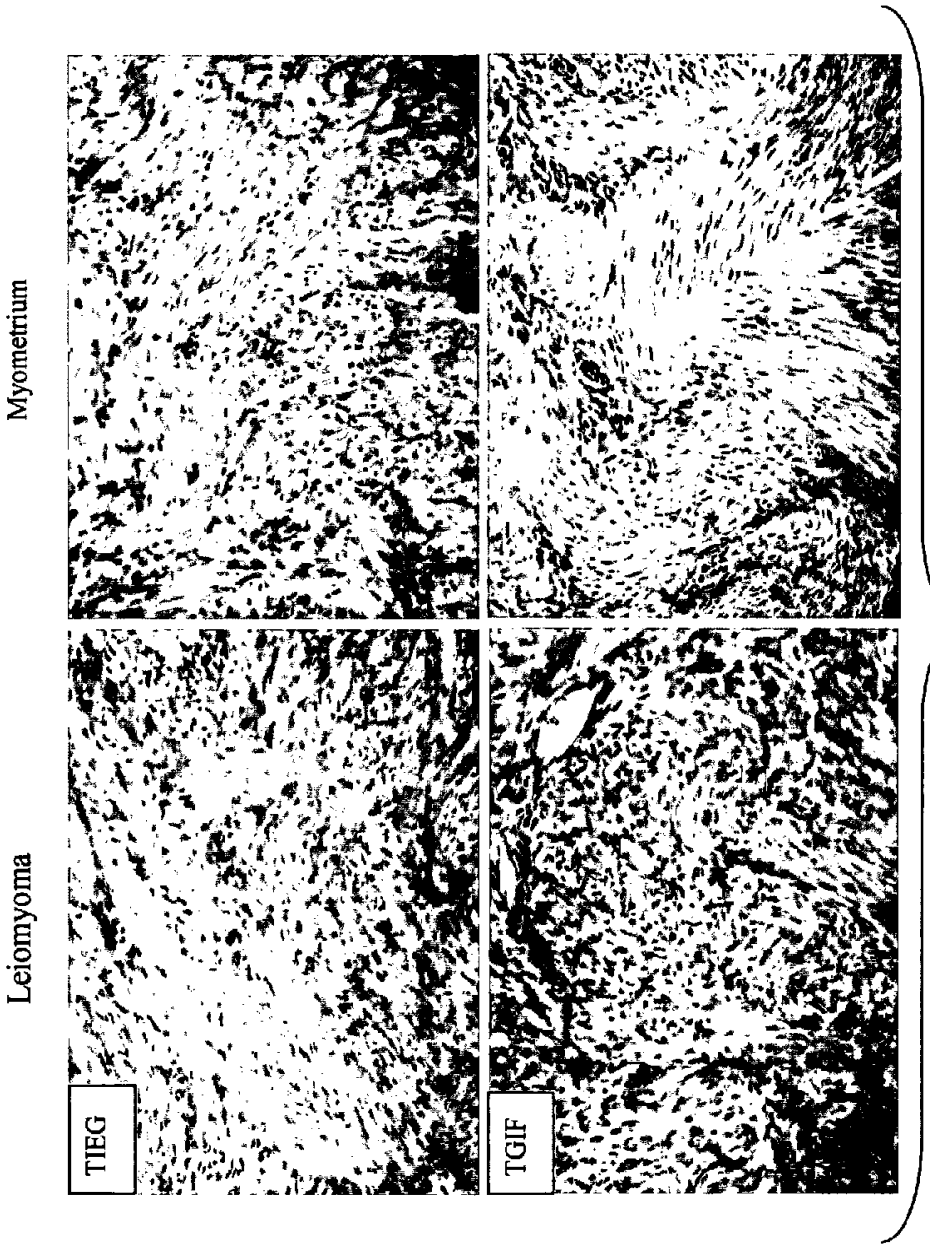


FIG. 4B

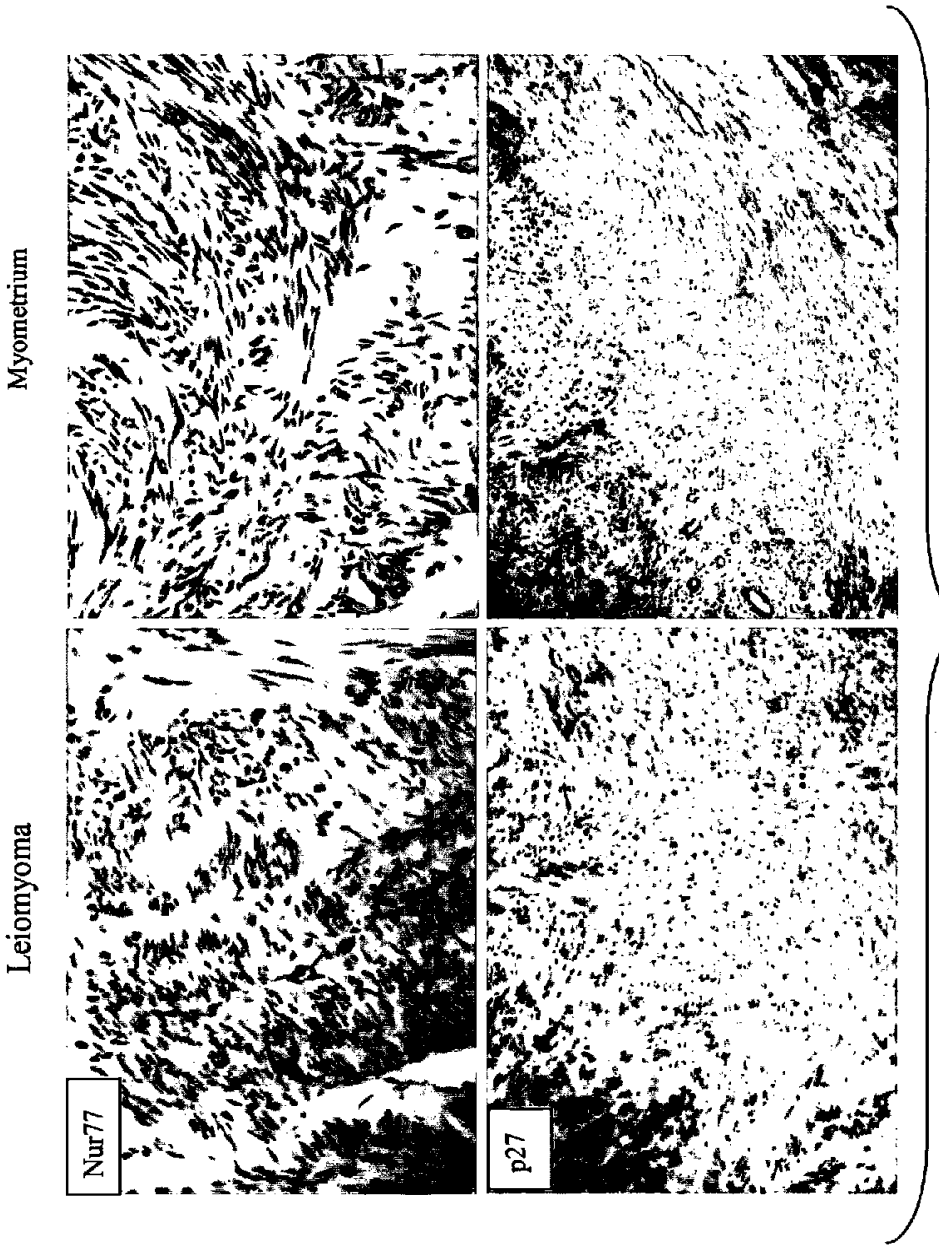


FIG. 4C

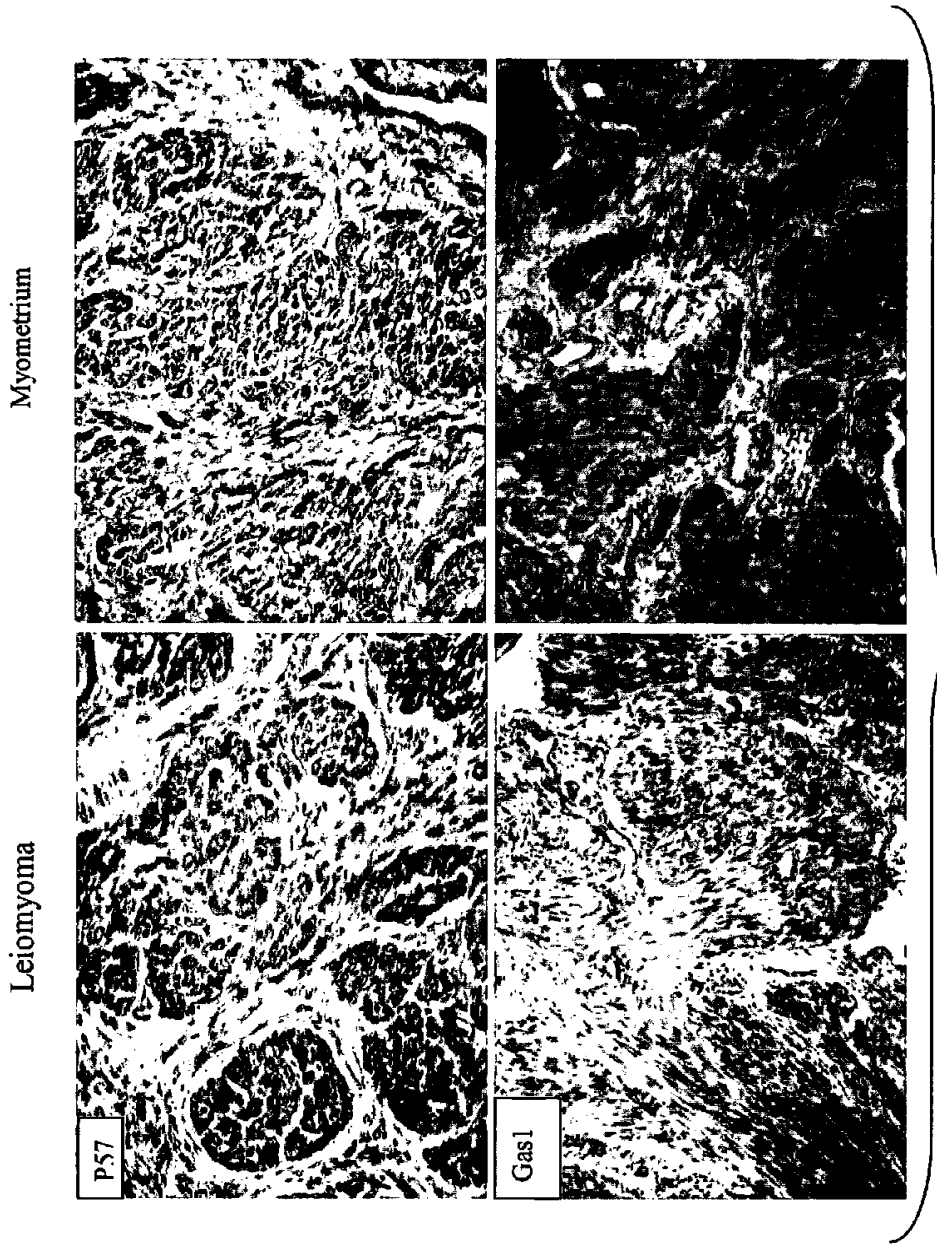


FIG. 4D

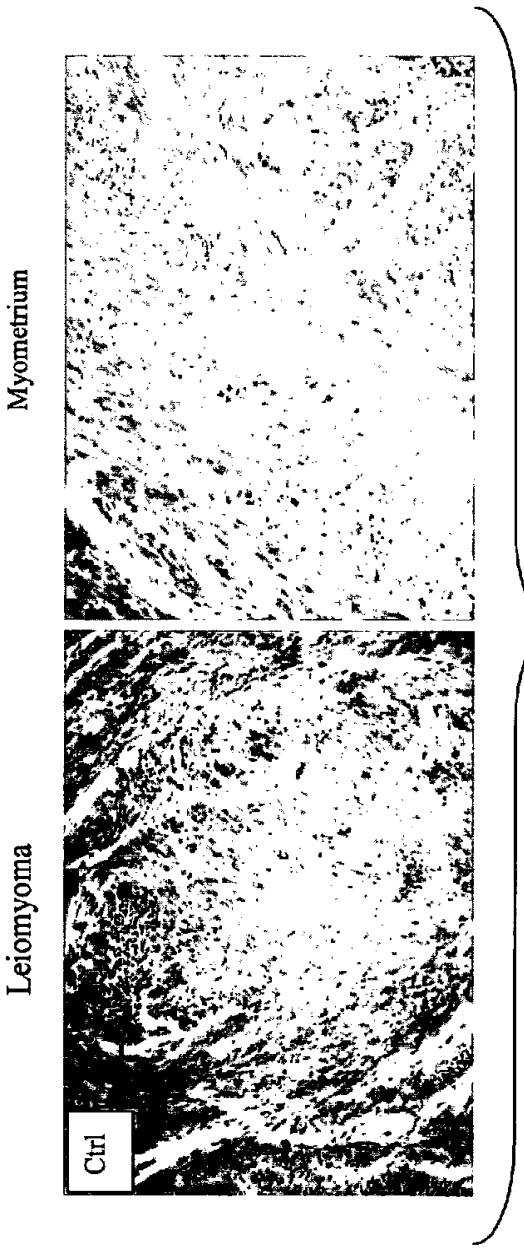


FIG. 4E

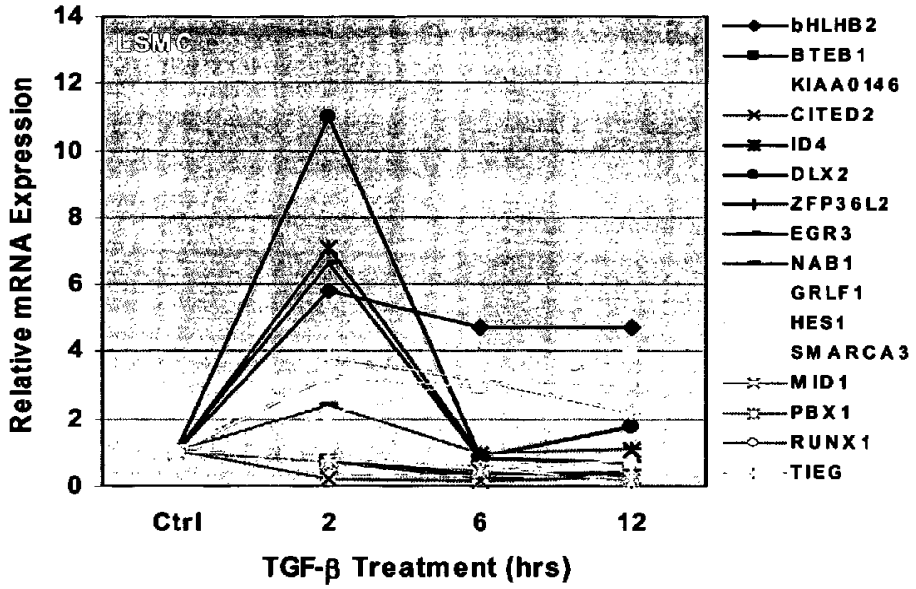

LSMC
Relative mRNA Expression
14
12
10
8
6
4
2
0
Ctrl
2
6
12
TGF-β Treatment (hrs)
bHLHB2
BTEB1
KIAA0146
CITED2
ID4
DLX2
ZFP36L2
EGR3
NAB1
GRLF1
HES1
SMARCA3
MID1
PBX1
RUNX1
TIEG

FIG. 5A

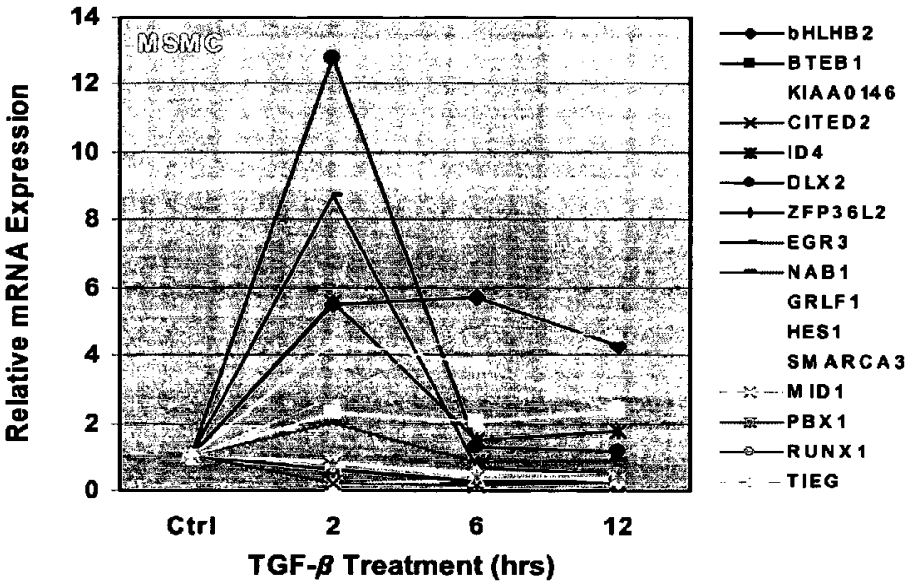

MSMC
Relative mRNA Expression
14
12
10
8
6
4
2
0
Ctrl
2
6
12
TGF-β Treatment (hrs)
bHLHB2
BTEB1
KIAA0146
CITED2
ID4
DLX2
ZFP36L2
EGR3
NAB1
GRLF1
HES1
SMARCA3
MID1
PBX1
RUNX1
TIEG

FIG. 5B

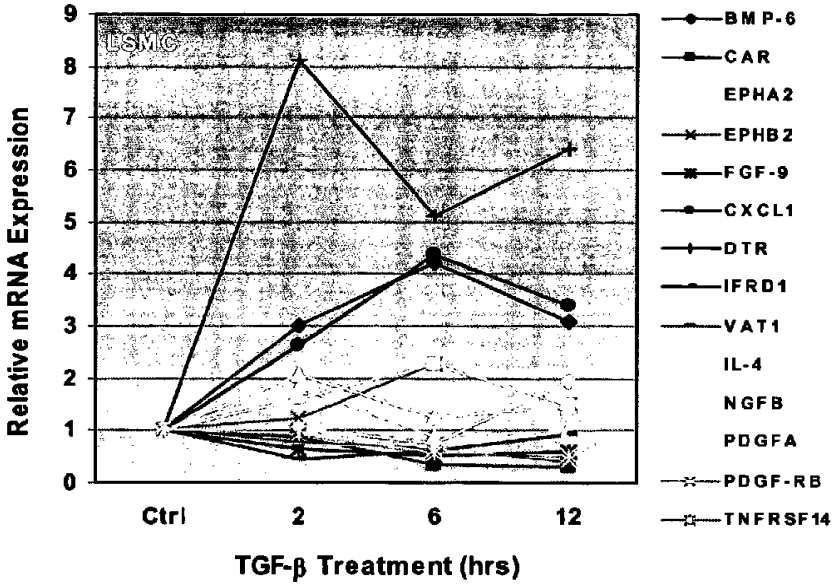

LSMC
Relative mRNA Expression
0
1
2
3
4
5
6
7
8
9
Ctrl
2
6
12
TGF-β Treatment (hrs)
BMP-6
CAR
EPHA2
EPHB2
FGF-9
CXCL1
DTR
IFRD1
VAT1
IL-4
NGFB
PDGFA
PDGF-RB
TNFRSF14

FIG. 5C

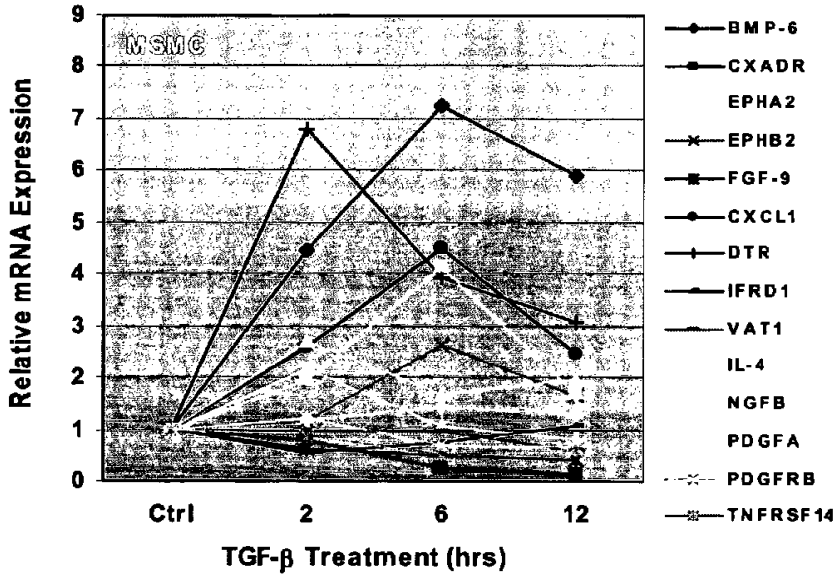

MSMC
Relative mRNA Expression
0
1
2
3
4
5
6
7
8
9
Ctrl
2
6
12
TGF-β Treatment (hrs)
BMP-6
CXADR
EPHA2
EPHB2
FGF-9
CXCL1
DTR
IFRD1
VAT1
IL-4
NGFB
PDGFA
PDGFRB
TNFRSF14

FIG. 5D

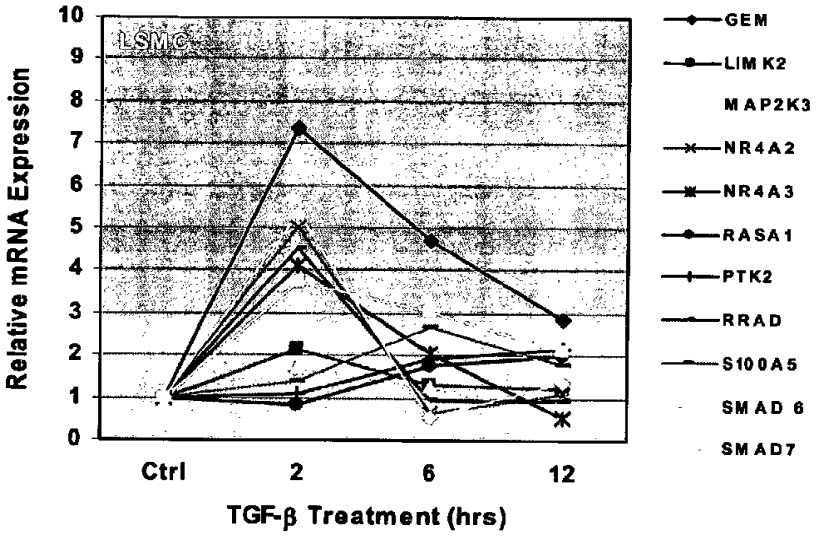

LSMC
Relative mRNA Expression
10
9
8
7
6
5
4
3
2
1
0
Ctrl
2
6
12
TGF-β Treatment (hrs)
GEM
LIMK2
MAP2K3
NR4A2
NR4A3
RASA1
PTK2
RRAD
S100A5
SMAD 6
SMAD7

FIG. 5E

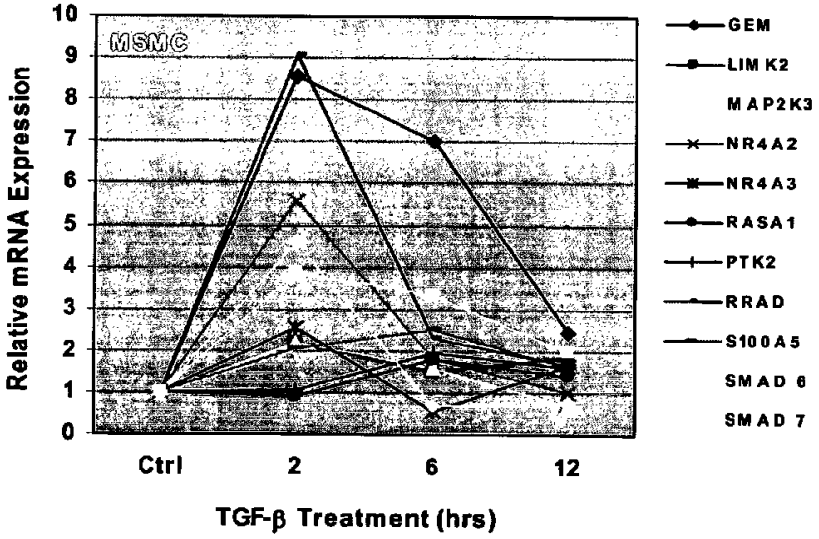

MSMC
Relative mRNA Expression
10
9
8
7
6
5
4
3
2
1
0
Ctrl
2
6
12
TGF-β Treatment (hrs)
GEM
LIMK2
MAP2K3
NR4A2
NR4A3
RASA1
PTK2
RRAD
S100A5
SMAD 6
SMAD 7

FIG. 5F

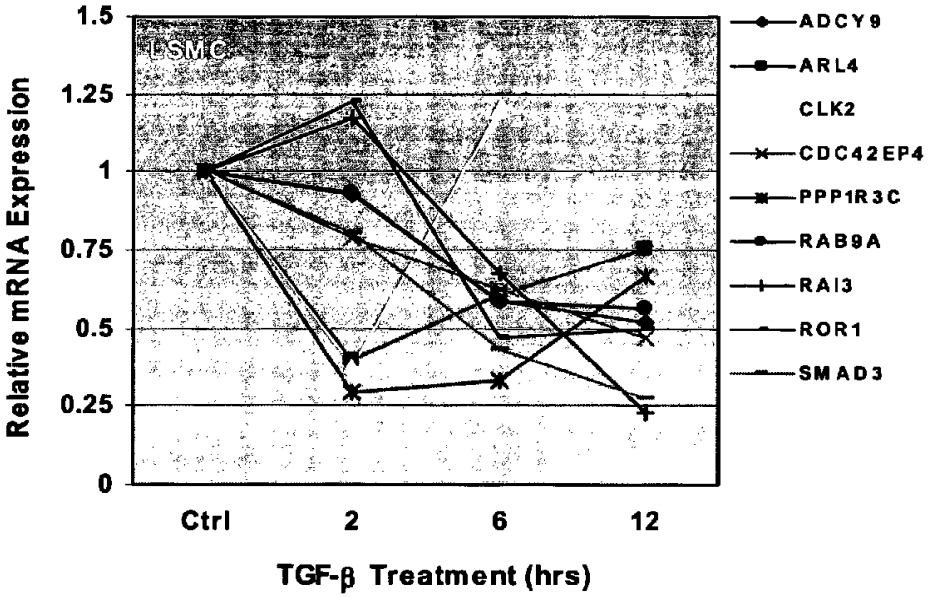

LSMC
Relative mRNA Expression
1.5
1.25
1
0.75
0.5
0.25
0
Ctrl
2
6
12
TGF-β Treatment (hrs)
ADCY9
ARL4
CLK2
CDC42EP4
PPP1R3C
RAB9A
RAI3
ROR1
SMAD3

FIG. 5G

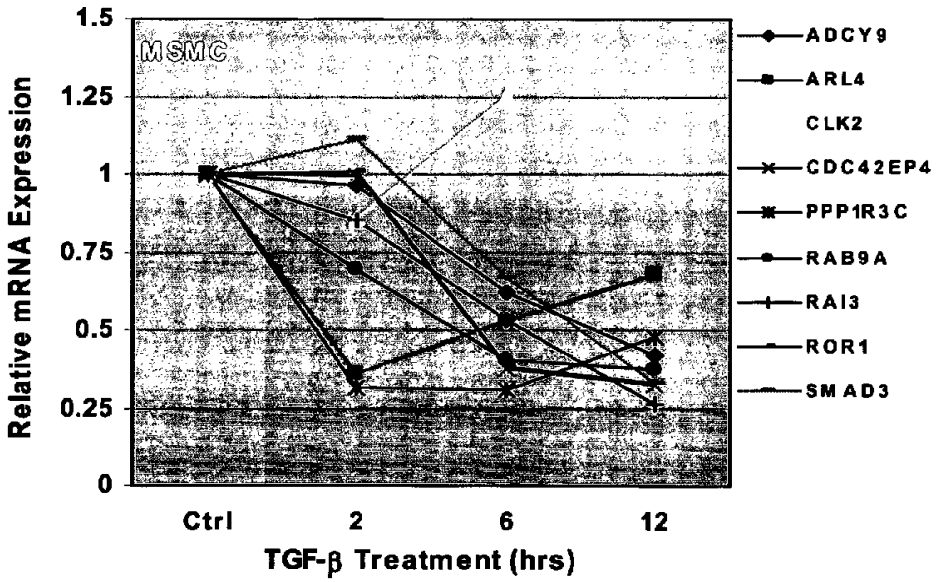

MSMC
Relative mRNA Expression
1.5
1.25
1
0.75
0.5
0.25
0
Ctrl
2
6
12
TGF-β Treatment (hrs)
ADCY9
ARL4
CLK2
CDC42EP4
PPP1R3C
RAB9A
RAI3
ROR1
SMAD3

FIG. 5H

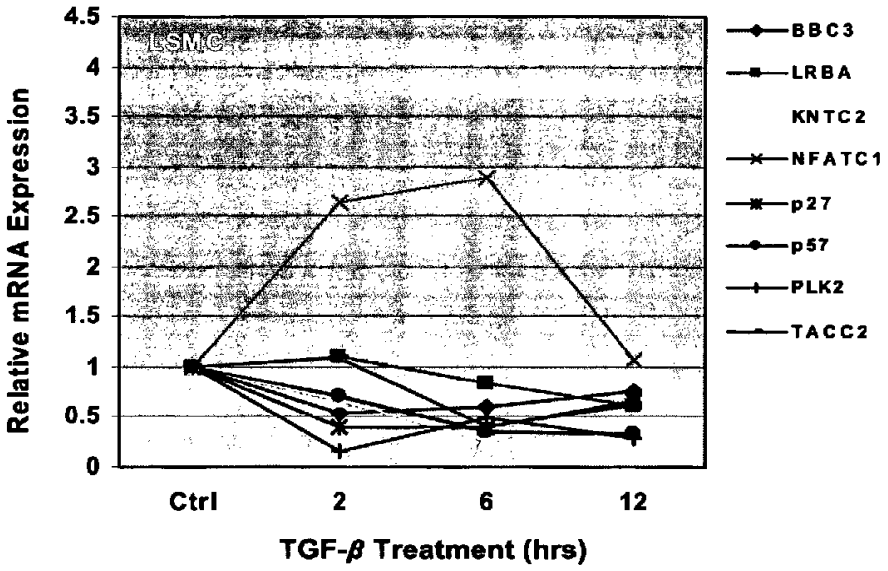

LSMC
Relative mRNA Expression
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Ctrl
2
6
12
TGF-β Treatment (hrs)
BBC3
LRBA
KNTC2
NFATC1
p27
p57
PLK2
TACC2

FIG. 5I

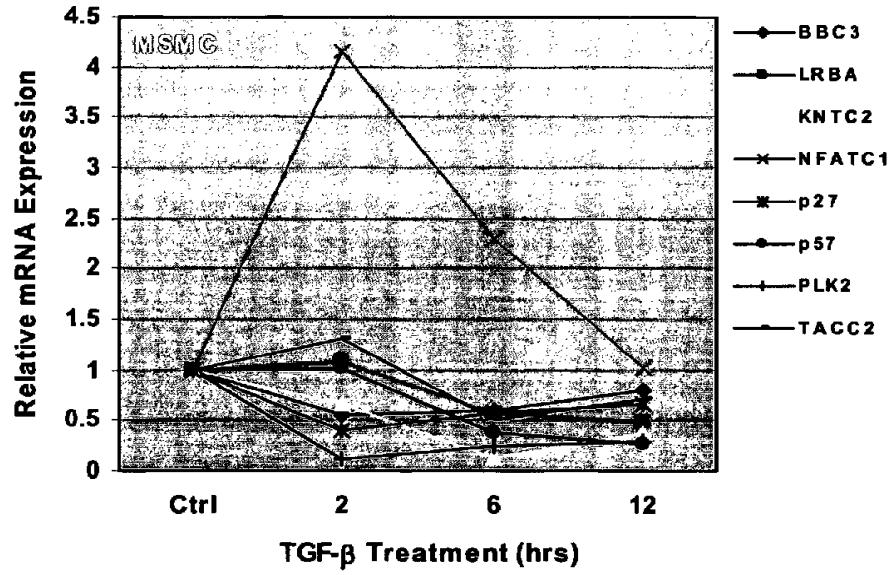

MSMC
Relative mRNA Expression
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Ctrl
2
6
12
TGF-β Treatment (hrs)
BBC3
LRBA
KNTC2
NFATC1
p27
p57
PLK2
TACC2

FIG. 5J

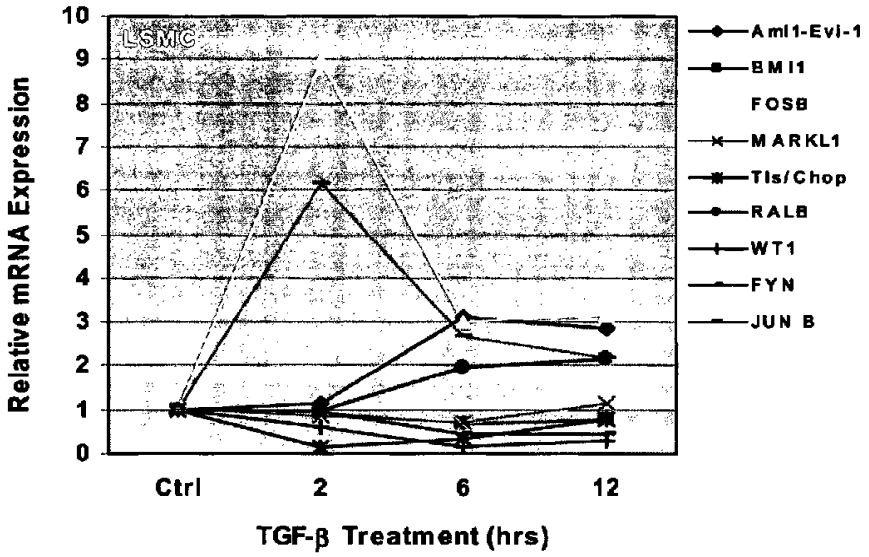

LSMC
Relative mRNA Expression
10
9
8
7
6
5
4
3
2
1
0
Ctrl
2
6
12
TGF-β Treatment (hrs)
Aml1-Evi-1
BMI1
FOSB
MARKL1
Tis/Chop
RALB
WT1
FYN
JUN B

FIG. 5K

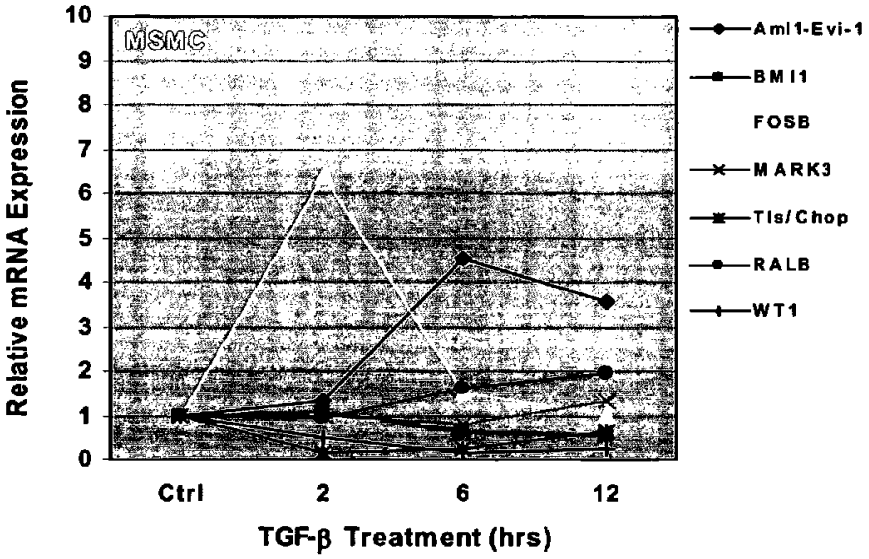

MSMC
Relative mRNA Expression
10
9
8
7
6
5
4
3
2
1
0
Ctrl
2
6
12
TGF-β Treatment (hrs)
Aml1-Evi-1
BMI1
FOSB
MARK3
Tis/Chop
RALB
WT1

FIG. 5L

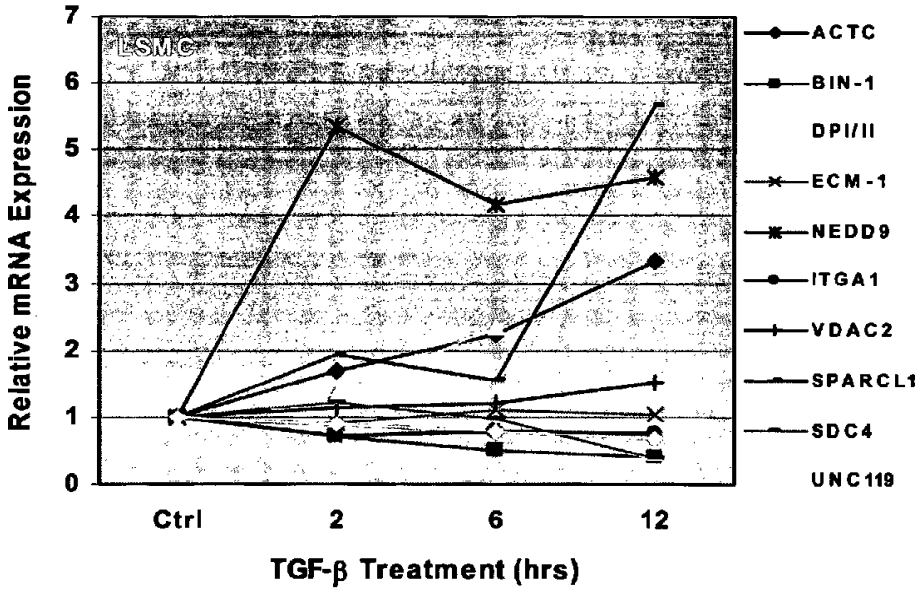

LSMC
Relative mRNA Expression
0
1
2
3
4
5
6
7
Ctrl
2
6
12
TGF-β Treatment (hrs)
ACTC
BIN-1
DPI/II
ECM-1
NEDD9
ITGA1
VDAC2
SPARCL1
SDC4
UNC119

FIG. 5M

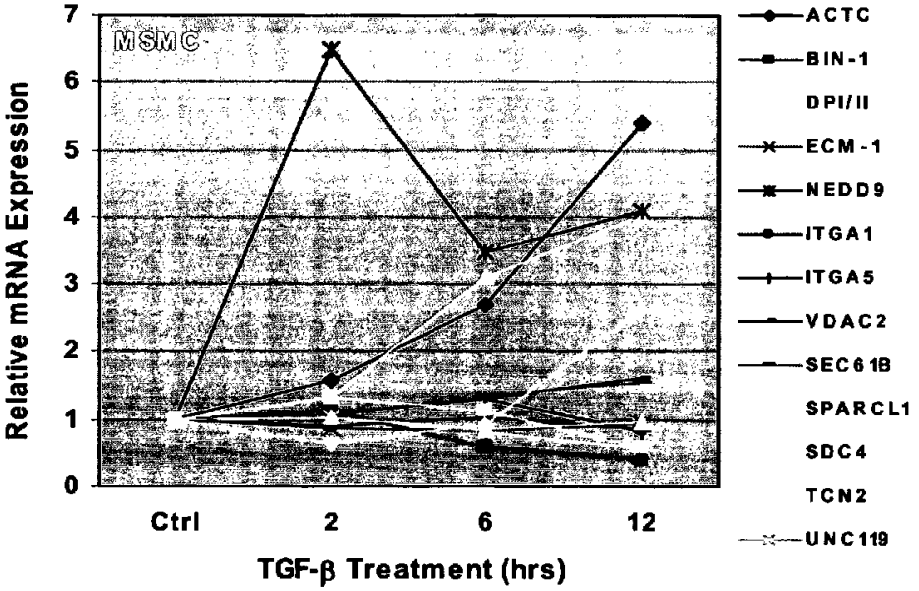

MSMC
Relative mRNA Expression
0
1
2
3
4
5
6
7
Ctrl
2
6
12
TGF-β Treatment (hrs)
ACTC
BIN-1
DPI/II
ECM-1
NEDD9
ITGA1
ITGA5
VDAC2
SEC61B
SPARCL1
SDC4
TCN2
UNC119

FIG. 5N

FIG. 6A

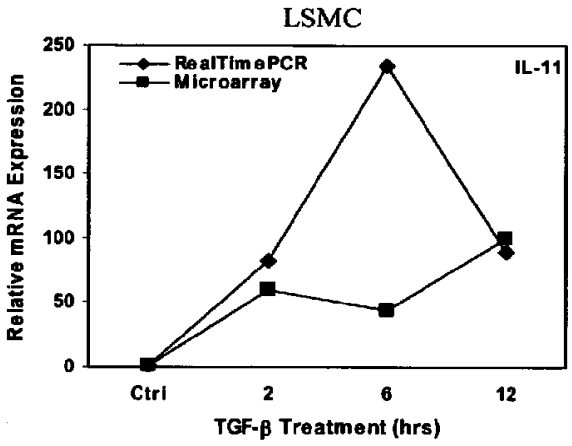


FIG. 6B

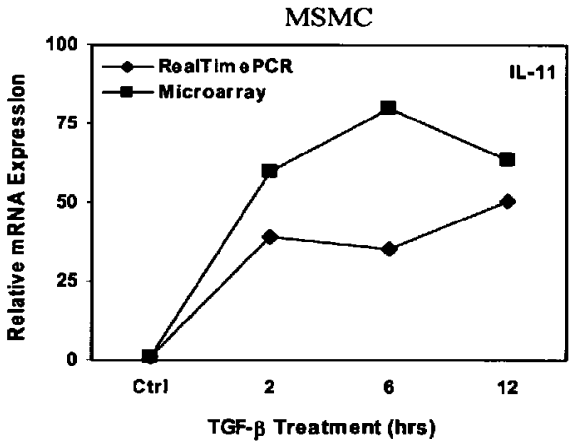


FIG. 6C

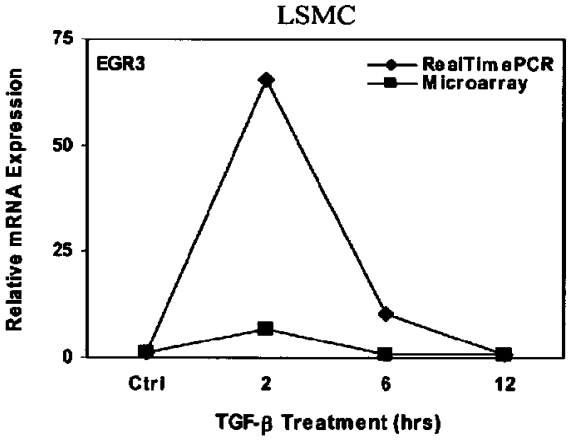


FIG. 6D

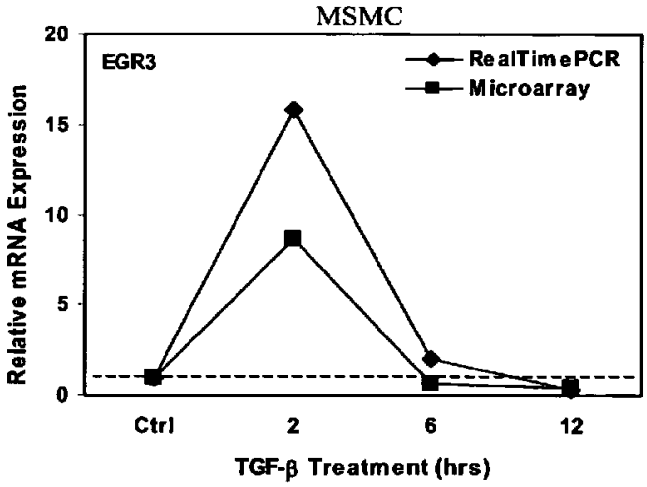


FIG. 6E

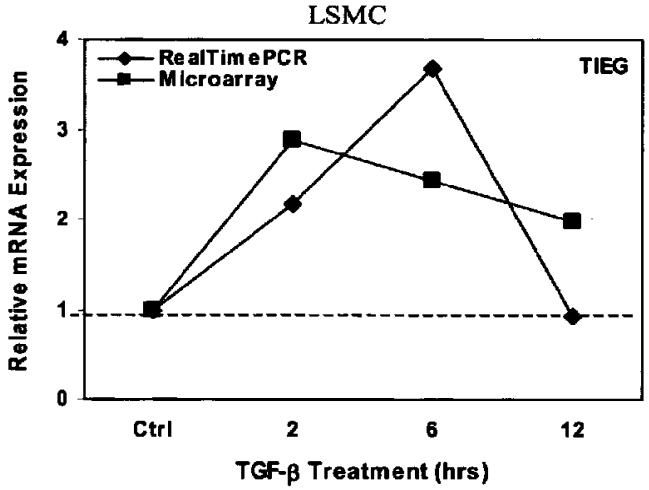


FIG. 6F

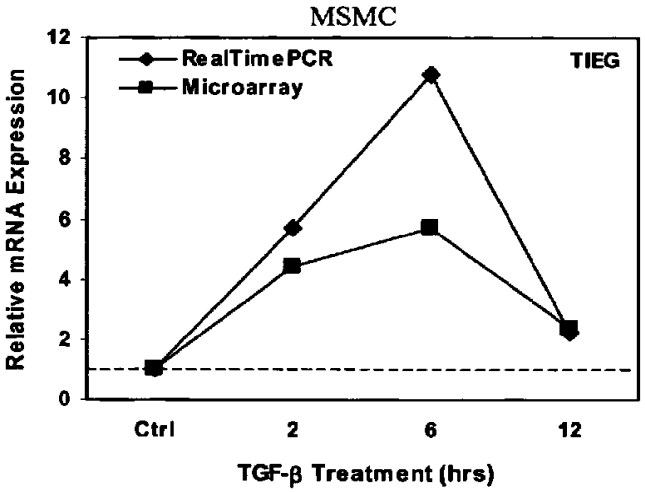


FIG. 6G

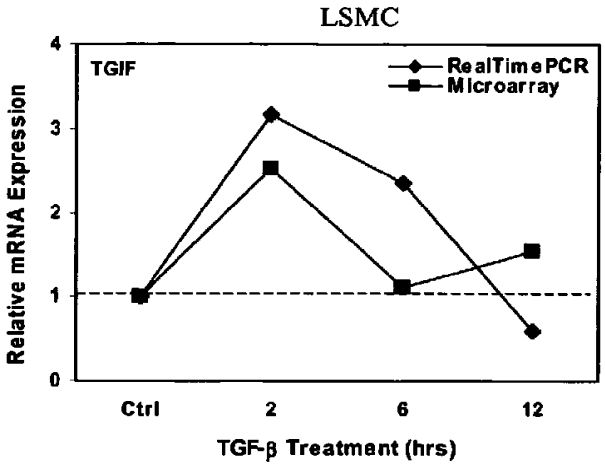


FIG. 6H

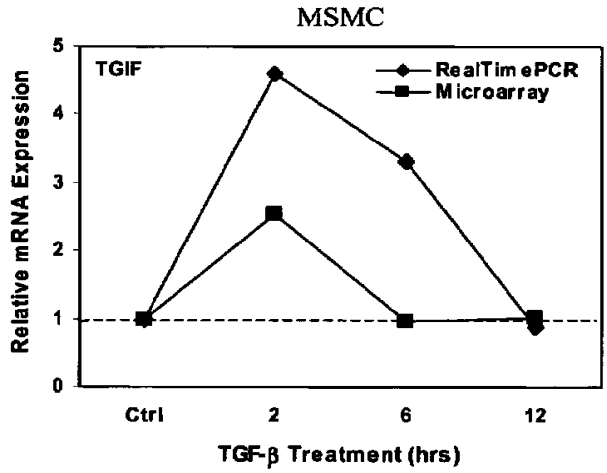


FIG. 6I

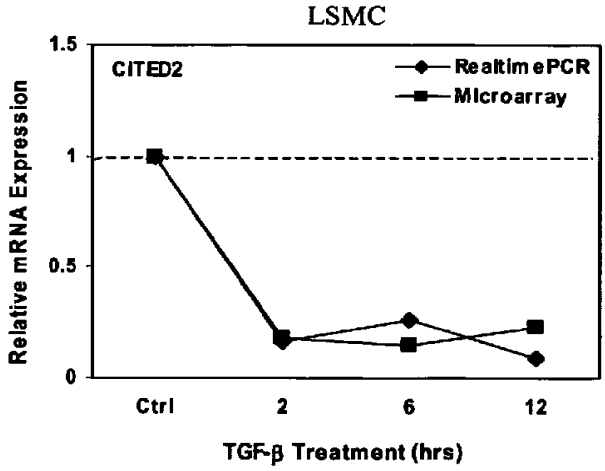


FIG. 6J

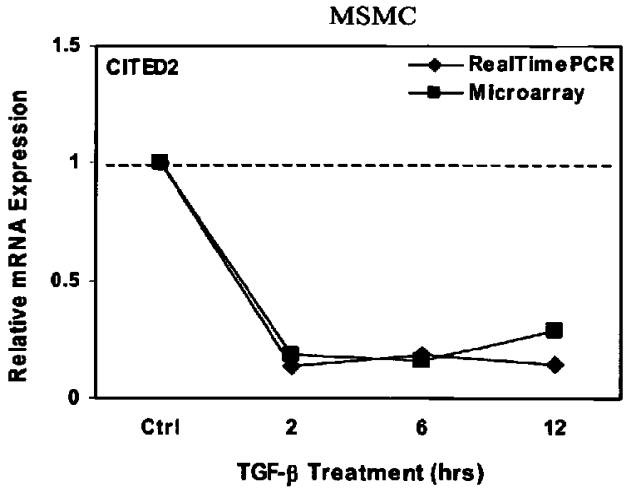


FIG. 6K

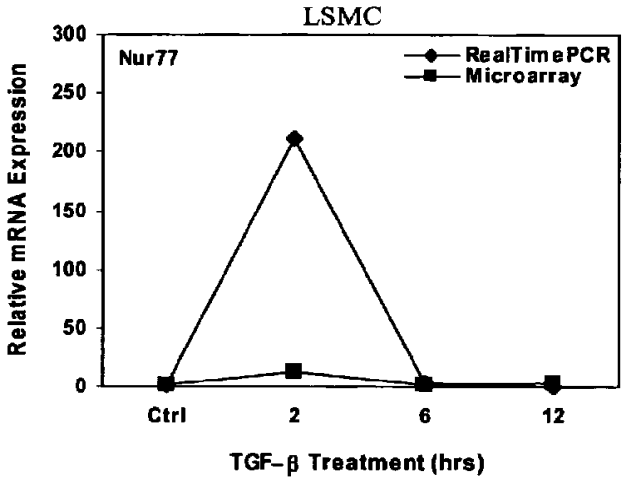


FIG. 6L

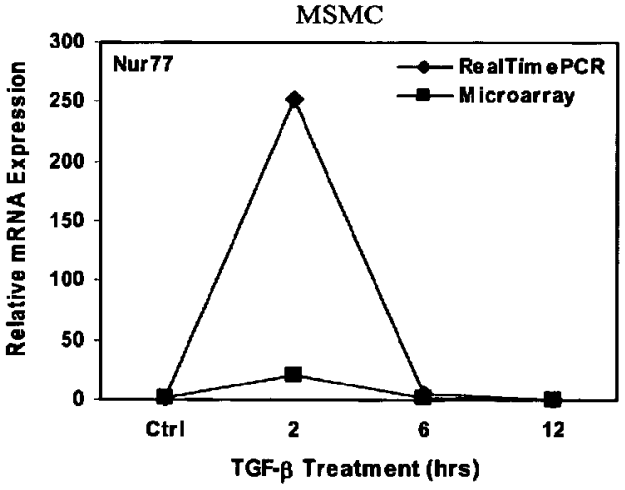


FIG. 6M

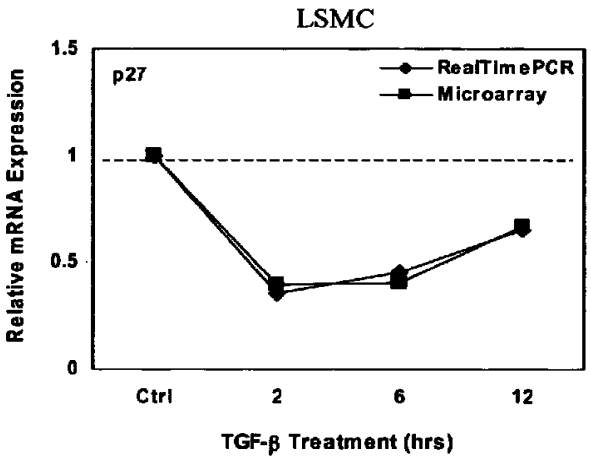


FIG. 6N

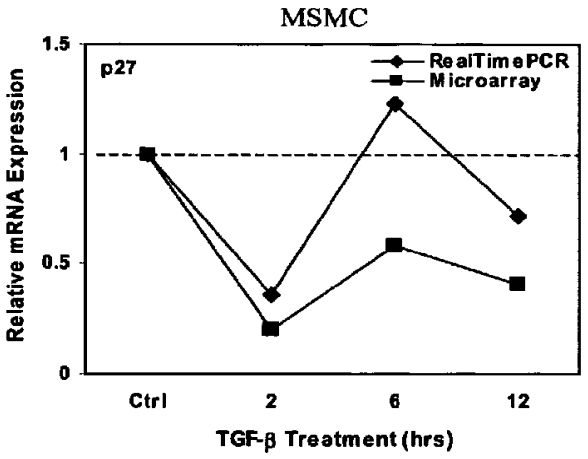


FIG. 6O

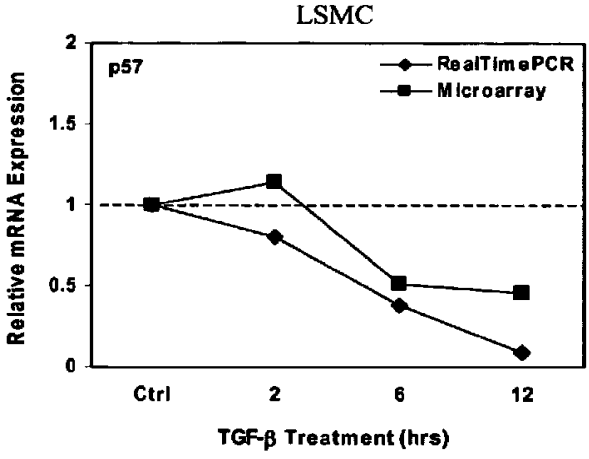


FIG. 6P

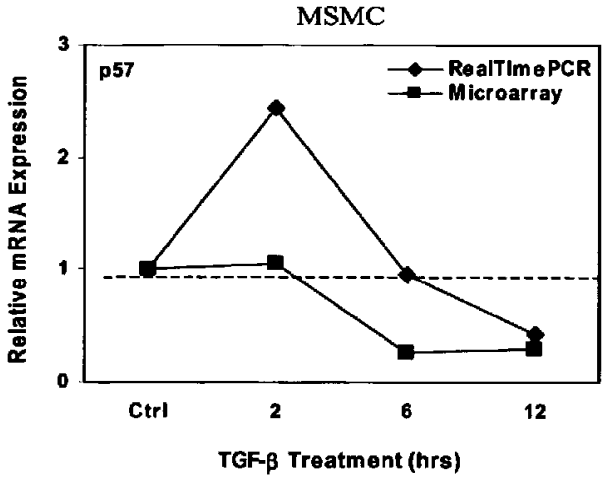


FIG. 6Q

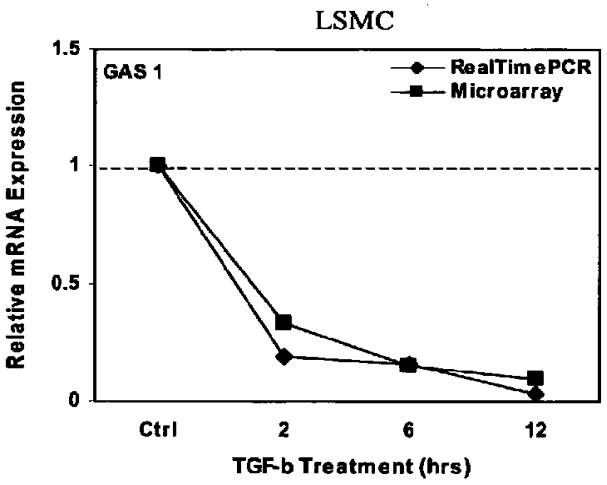


FIG. 6R

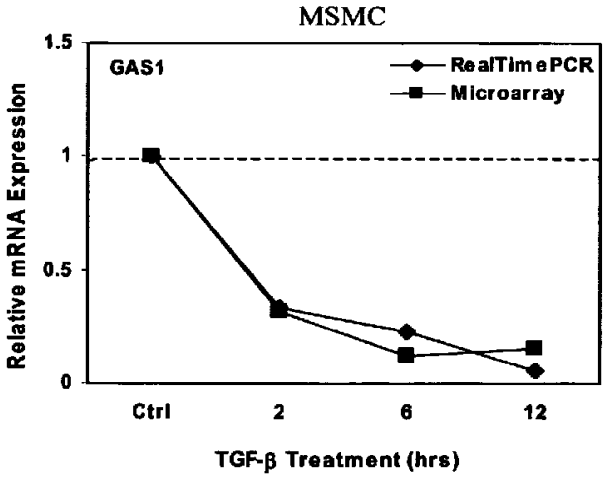


FIG. 7A

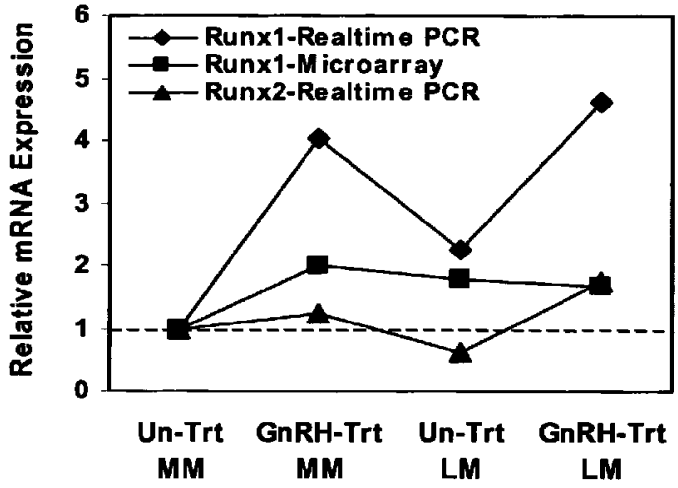


FIG. 7B

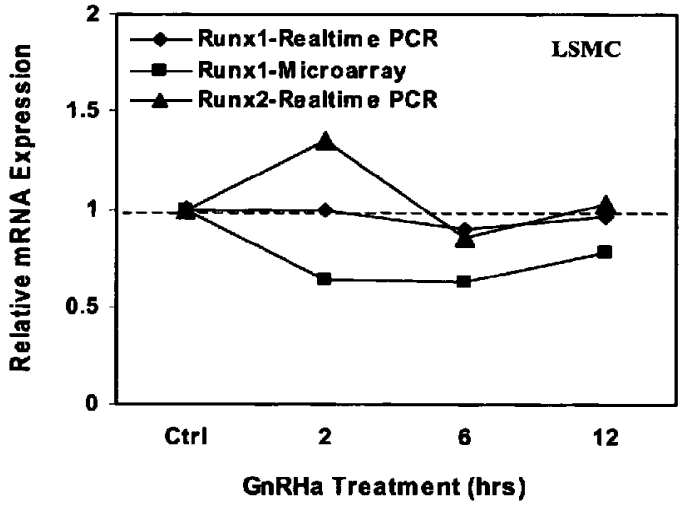


FIG. 7C

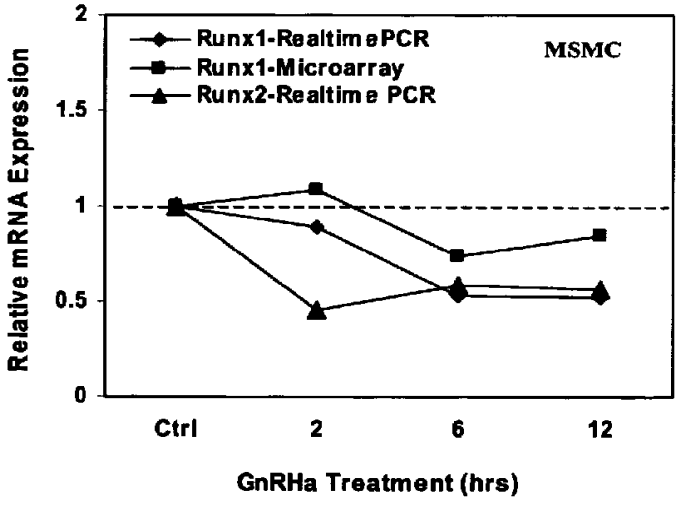


FIG. 7D

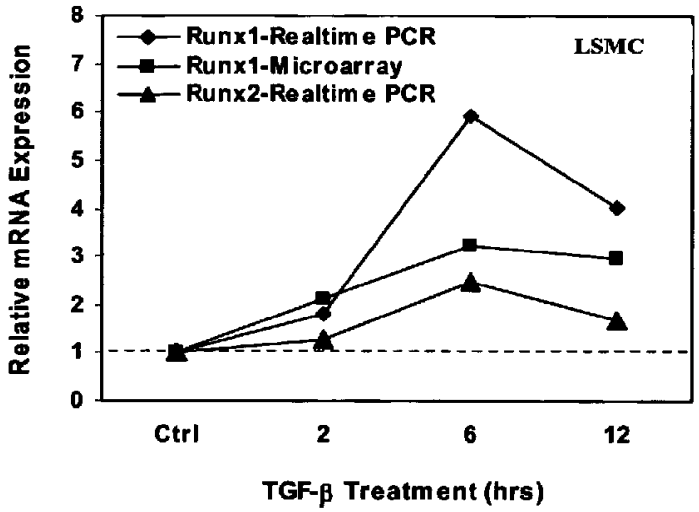
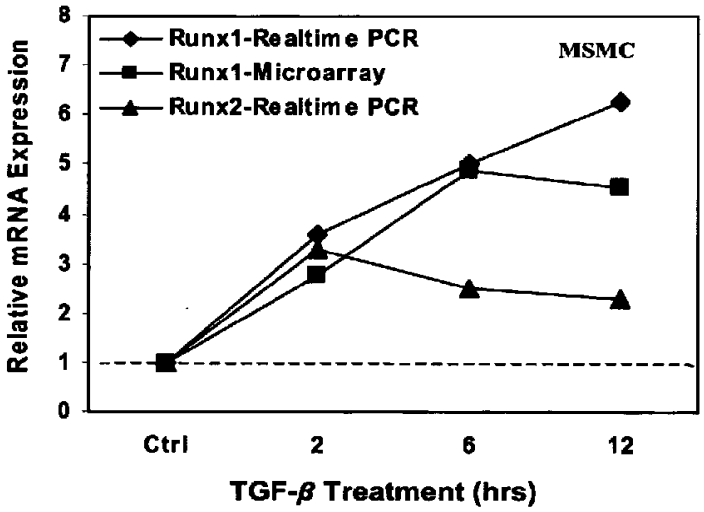

Runx1-Realtime PCR
Runx1-Microarray
Runx2-Realtime PCR
LSMC
Relative mRNA Expression
0
1
2
3
4
5
6
7
8
Ctrl
2
6
12
TGF-β Treatment (hrs)

FIG. 7E


Runx1-Realtime PCR
Runx1-Microarray
Runx2-Realtime PCR
MSMC
Relative mRNA Expression
0
1
2
3
4
5
6
7
8
Ctrl
2
6
12
TGF-β Treatment (hrs)

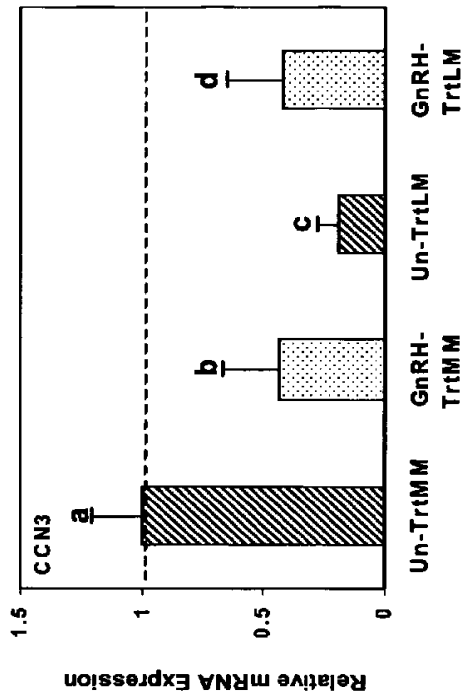

CCN3
Relative mRNA Expression
1.5
1
0.5
0
a
b
c
d
Un-TrtMM
GnRH-TrtMM
Un-TrtLM
GnRH-TrtLM

FIG. 8B

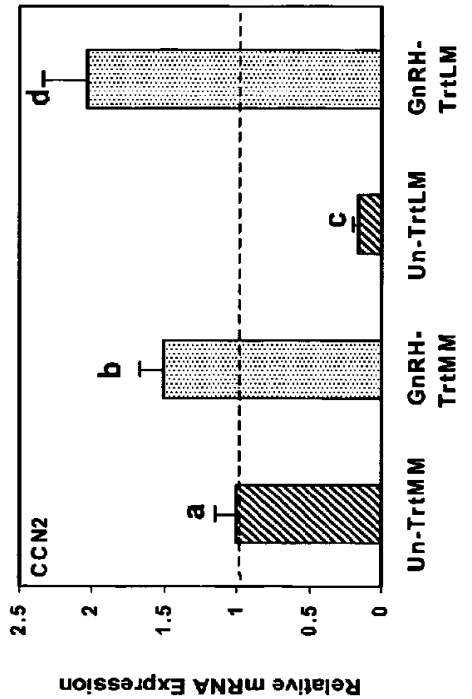

CCN2
Relative mRNA Expression
2.5
2
1.5
1
0.5
0
a
b
c
d
Un-TrtMM
GnRH-TrtMM
Un-TrtLM
GnRH-TrtLM

FIG. 8A

FIG. 8C

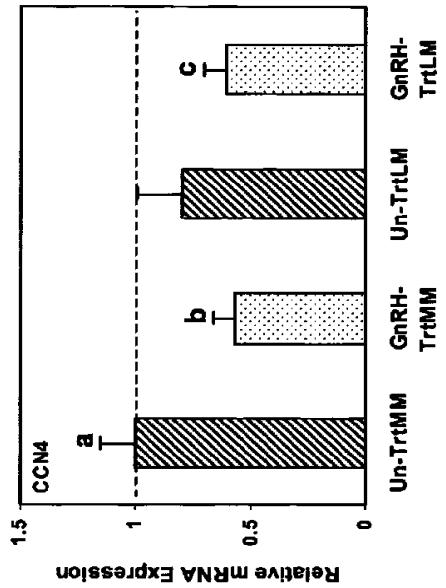


FIG. 8D

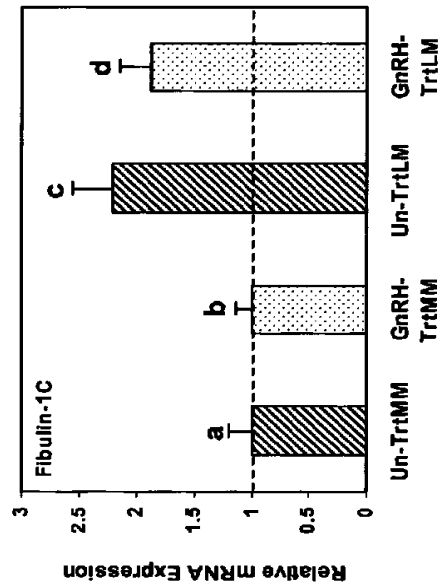
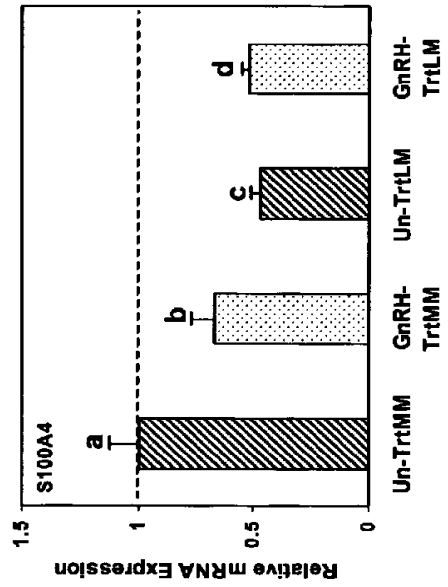


FIG. 8E



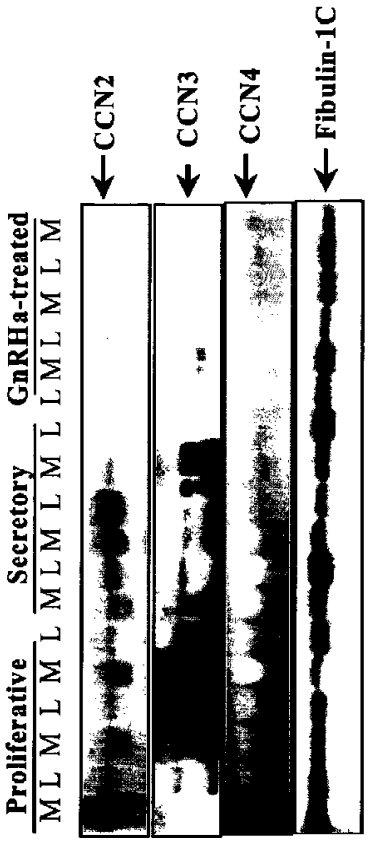

Proliferative
M L M L M L
Secretory
M L M L M L
GnRHa-treated
L M L M L M
CCN2
CCN3
CCN4
Fibulin-1C

FIG. 9

FIG. 10

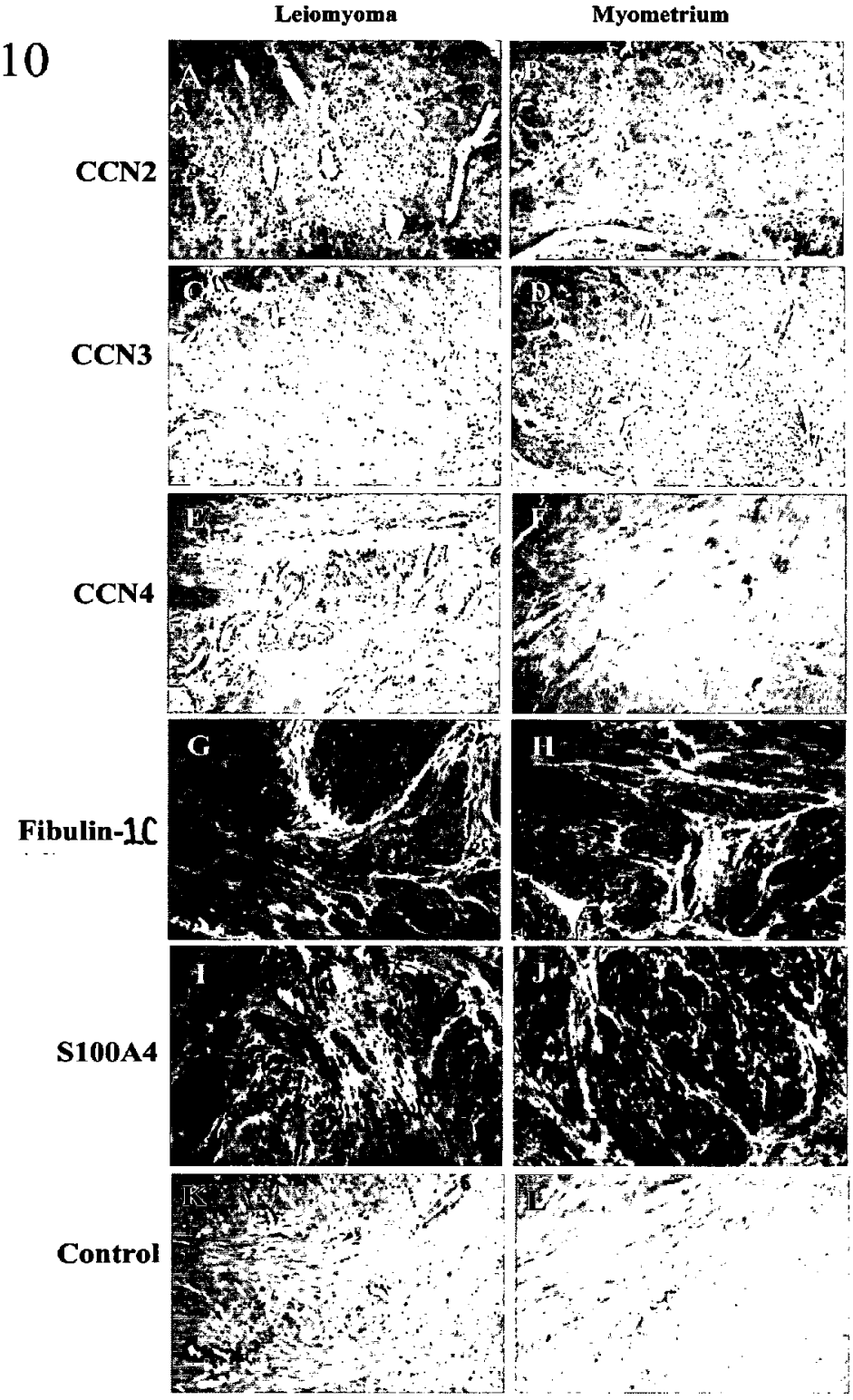

Leiomyoma
Myometrium
CCN2
CCN3
CCN4
Fibulin-1C
S100A4
Control
A
B
C
D
E
F
G
H
I
J
K
L

FIG. 11A

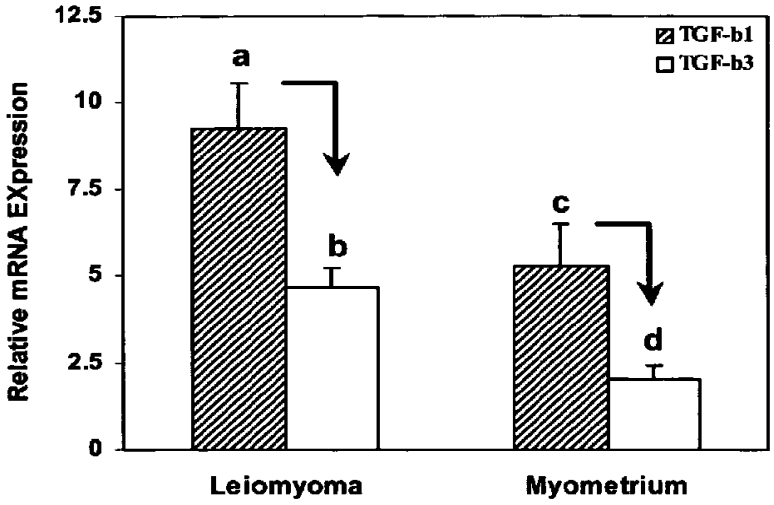
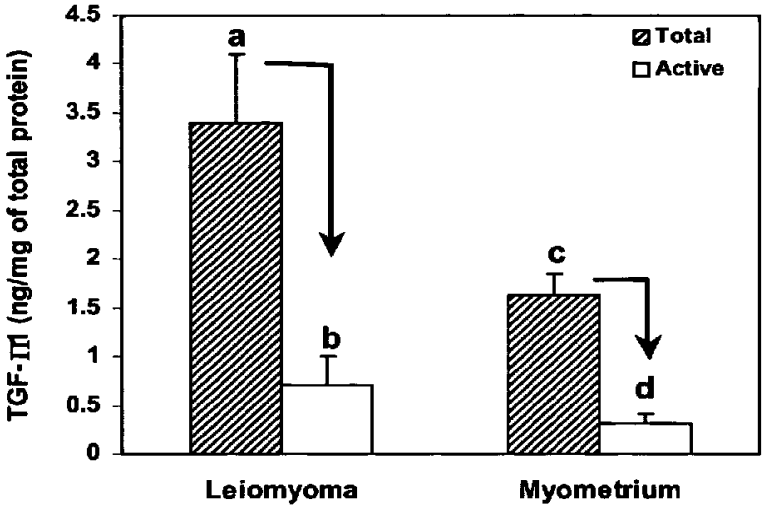


FIG. 11B



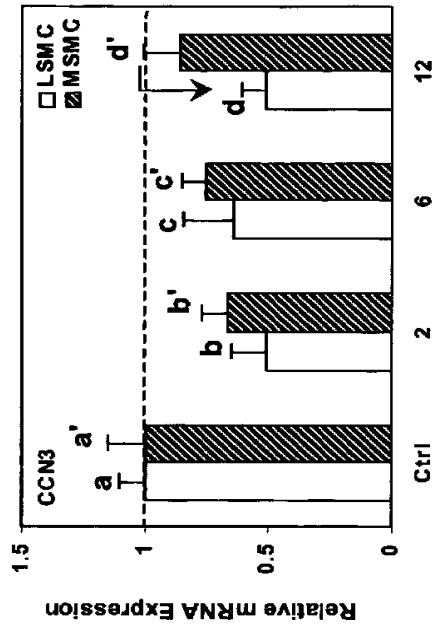


FIG. 12B

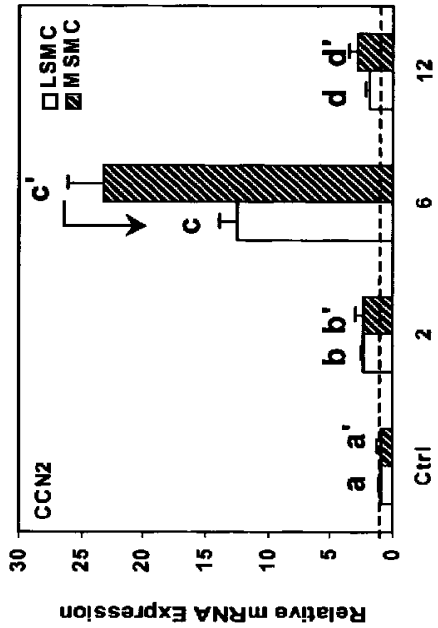


FIG. 12A

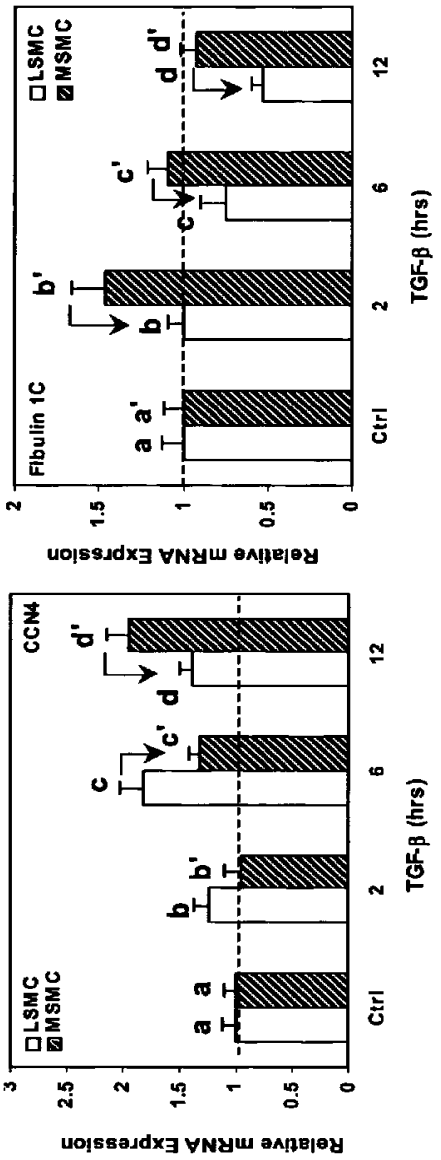


FIG. 12C

FIG. 12D

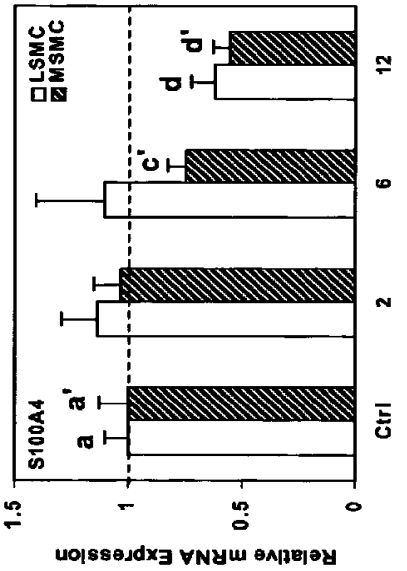


FIG. 12E

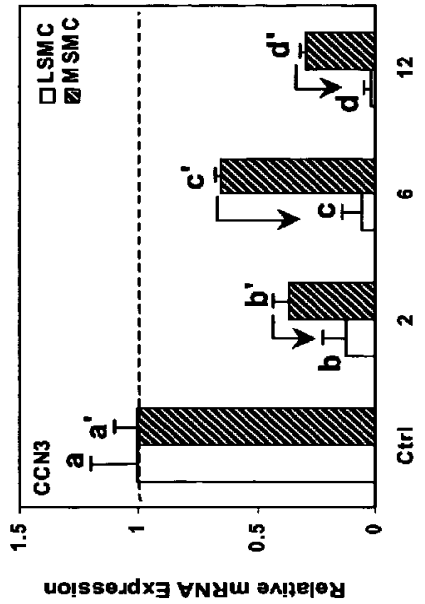


FIG. 13B

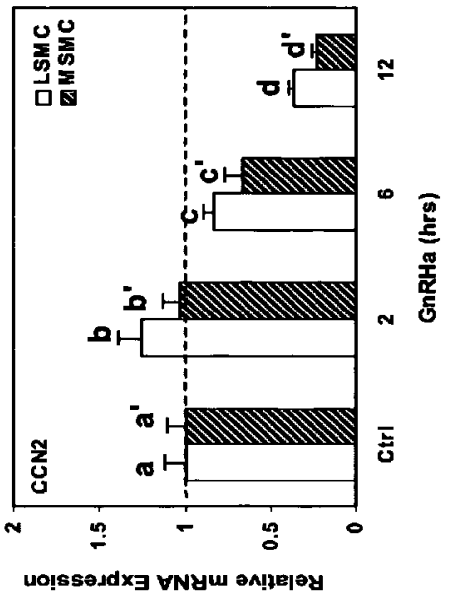


FIG. 13A

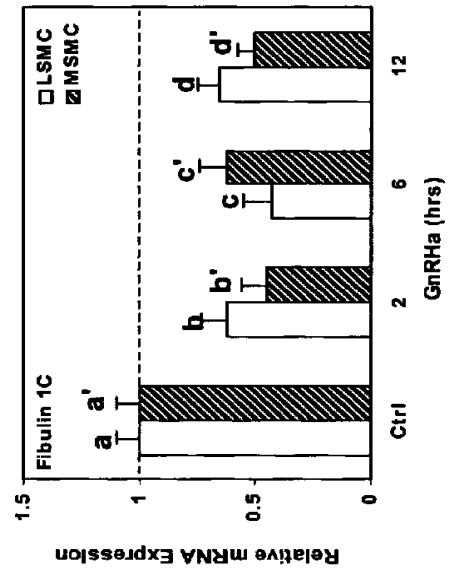


FIG. 13D

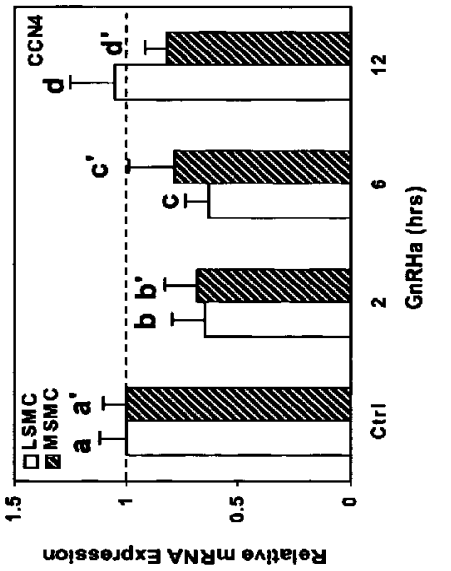


FIG. 13C

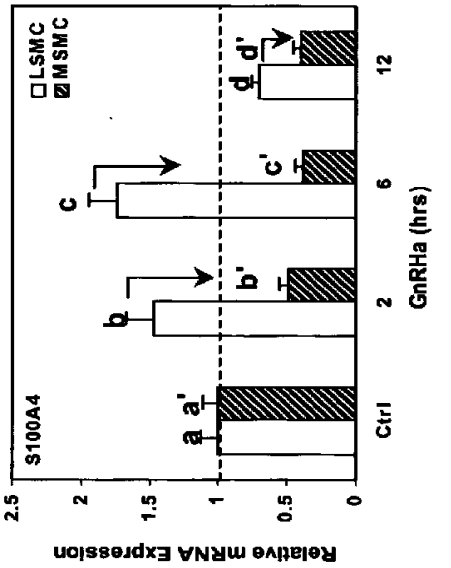


FIG. 13E

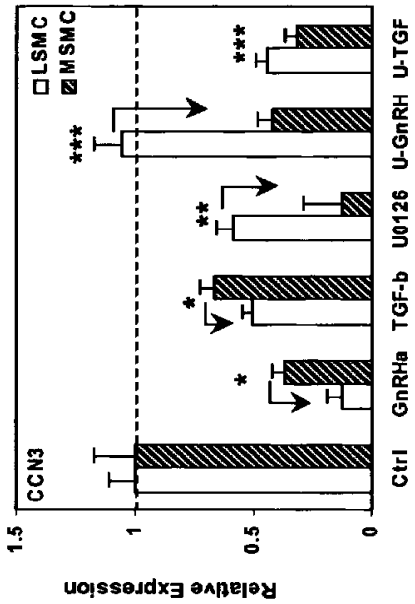


FIG. 14B

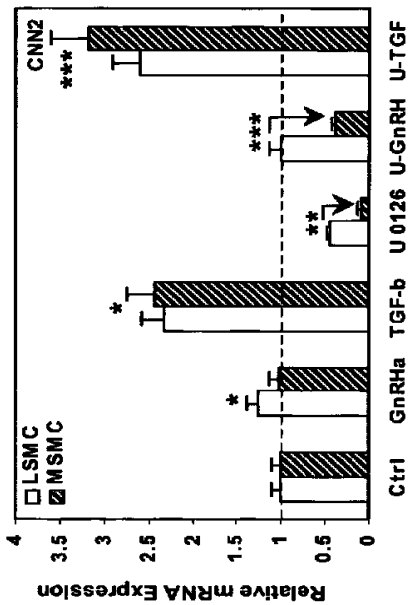


FIG. 14A

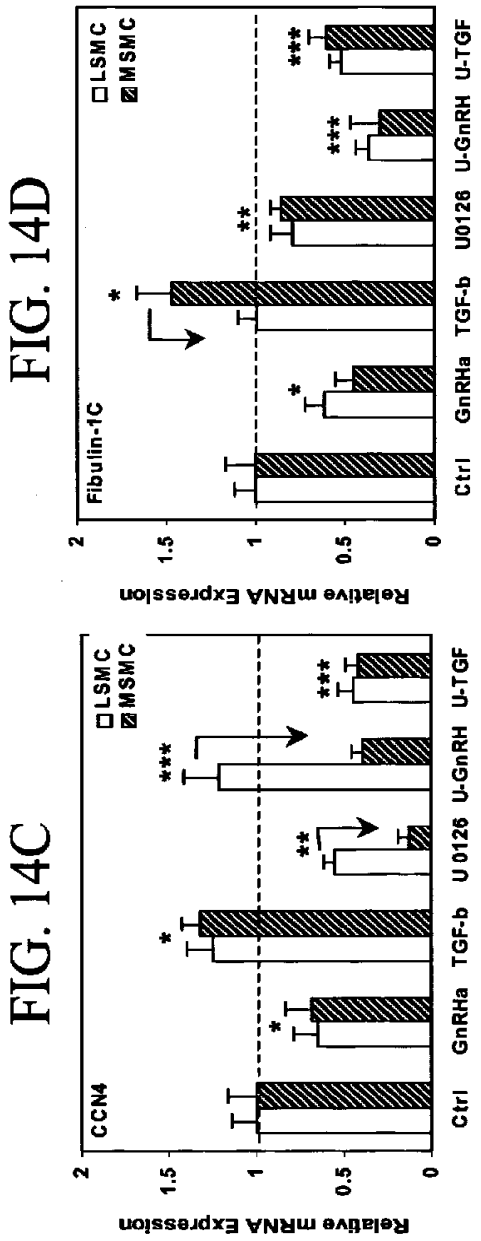


FIG. 14C

FIG. 14D

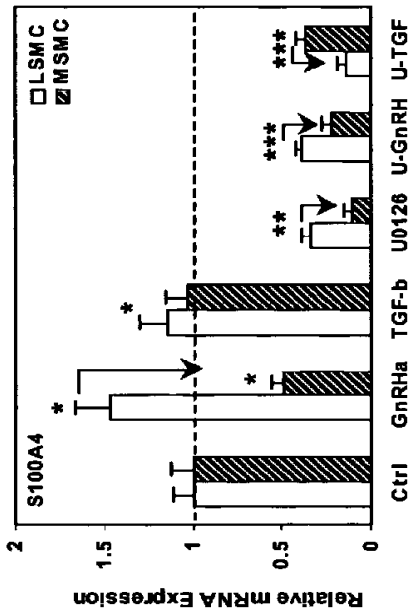


FIG. 14E

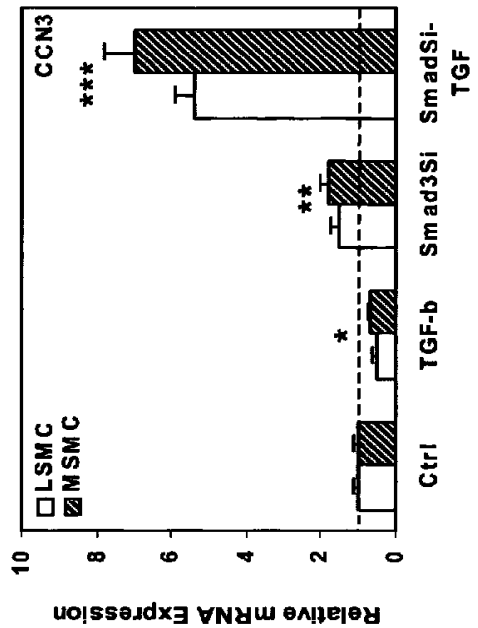


FIG. 15B

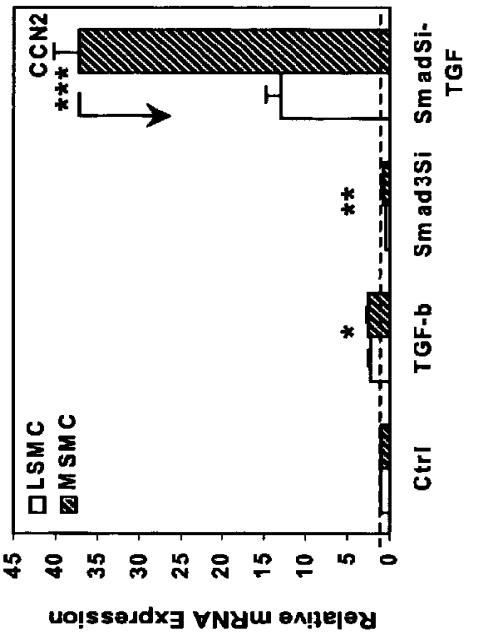


FIG. 15A

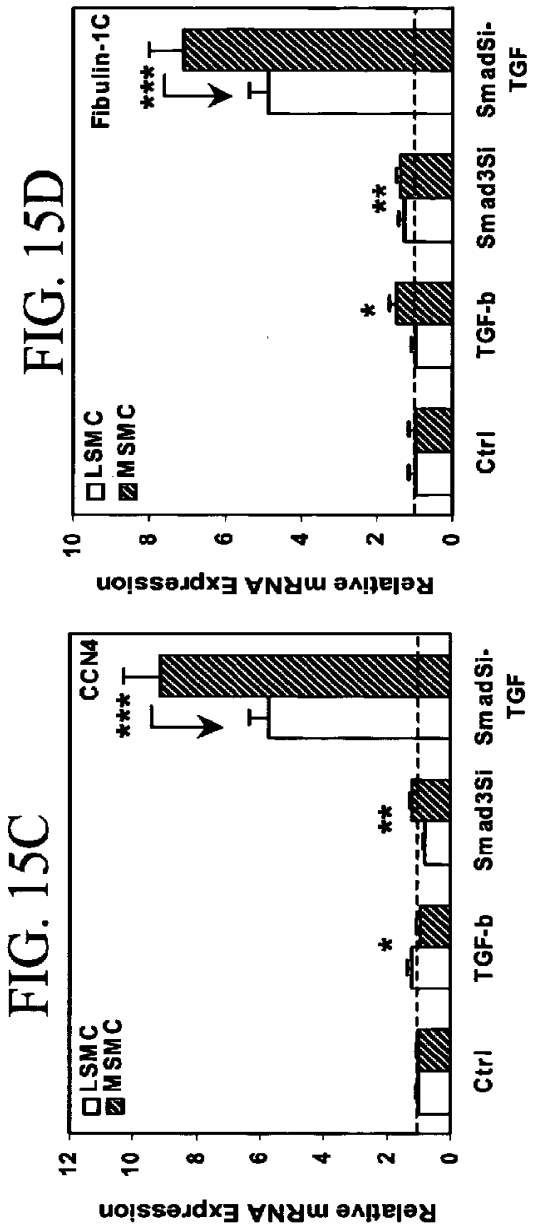


FIG. 15C
CCN4
LSMC
MSMC
Relative mRNA Expression
0
2
4
6
8
10
12
Ctrl
TGF-b
Smad3Si
SmadSi-TGF
*
**
***

FIG. 15D
Fibulin-1C
LSMC
MSMC
Relative mRNA Expression
0
2
4
6
8
10
Ctrl
TGF-b
Smad3Si
SmadSi-TGF
*
**
***

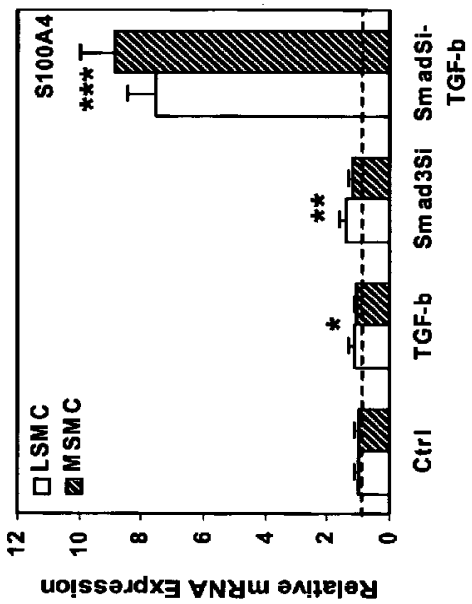


FIG. 15E
S100A4
LSMC
MSMC
Relative mRNA Expression
0
2
4
6
8
10
12
Ctrl
TGF-b
Smad3Si
SmadSi-TGF-b
*
**
***

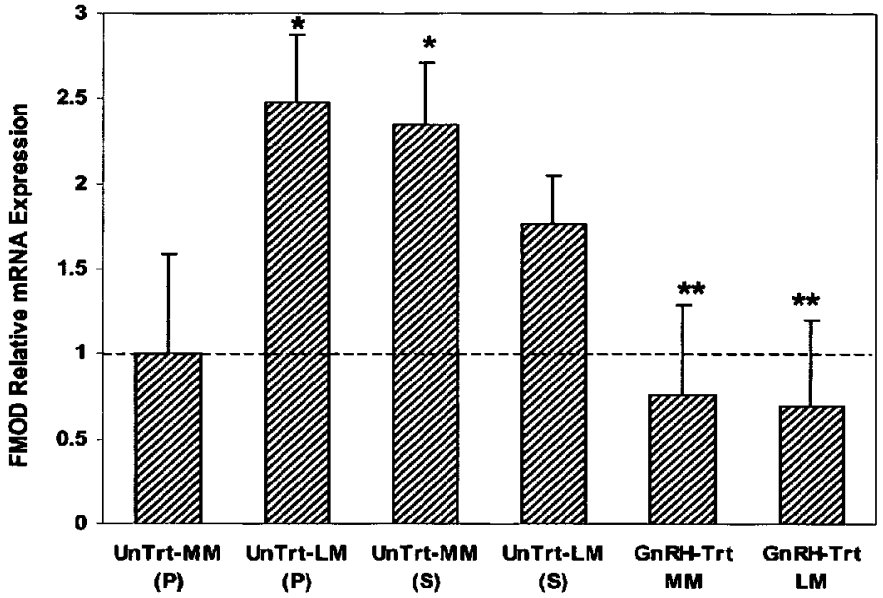
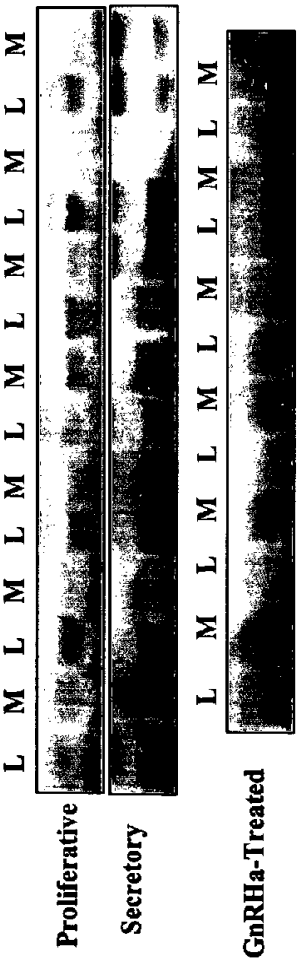

FMOD Relative mRNA Expression
3
2.5
2
1.5
1
0.5
0
*
*
**
**
UnTrt-MM (P)
UnTrt-LM (P)
UnTrt-MM (S)
UnTrt-LM (S)
GnRH-Trt MM
GnRH-Trt LM

FIG. 16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Proliferative
Secretory
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GnRHa-Treated

FIG. 17

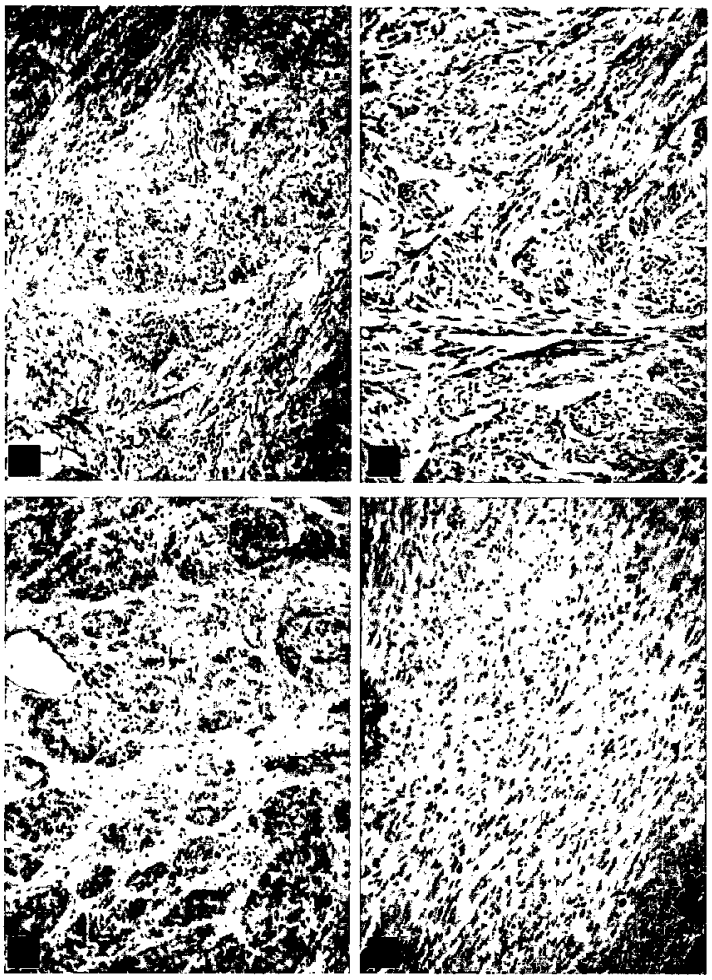

FIG. 18A FIG. 18B

FIG. 18C FIG. 18D

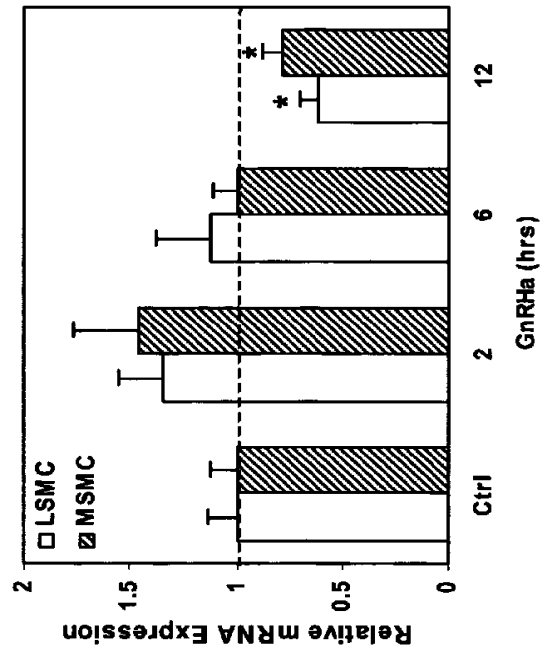

FIG. 19B

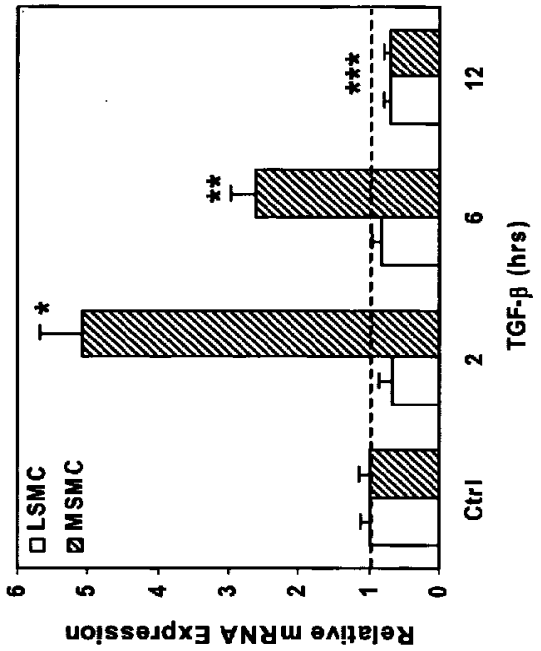

FIG. 19A

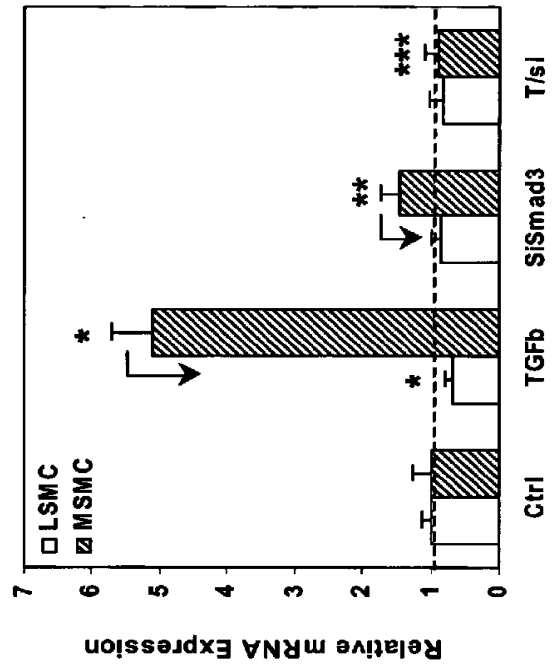

FIG. 19D

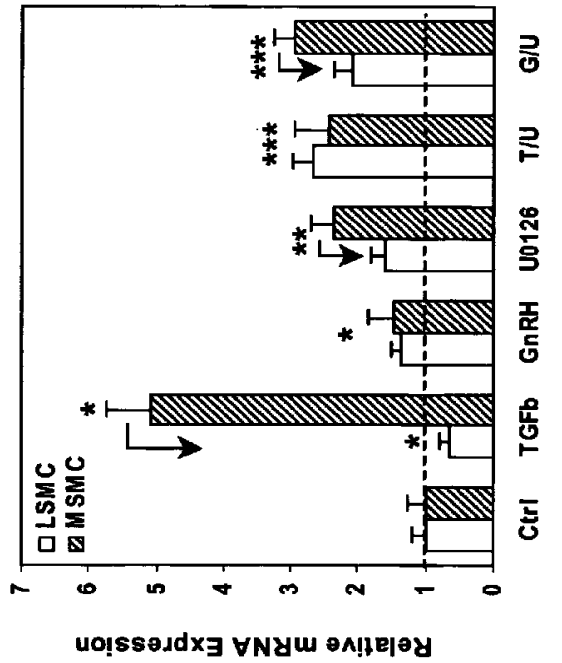

FIG. 19C

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(54) Title: CYTOKINE MUTEINS

(57) Abstract: The present invention relates generally to the treatment of an interleukin-11 (IL-11)-mediated condition. More particularly, the present invention provides the use of modified forms of IL-11 which modulate IL-11 signaling in the treatment of IL-11-mediated conditions.

CYTOKINE MUTEINS

APPLICATION DATA

[0001] This application is associated with and claims priority from U.S. Provisional Patent Application No. 61/000,576, filed on 26 October, 2007, the entire contents of which are incorporated herein by reference.

FIELD

[0002] The present invention relates generally to the treatment of an interleukin-11 (IL-11)-mediated condition. More particularly, the present invention provides the use of modified forms of IL-11 which modulate IL-11 signaling in the treatment of IL-11-mediated conditions.

BACKGROUND

[0003] Bibliographic details of references provided in the subject specification are listed at the end of the specification.

[0004] Reference to any prior art is not, and should not be taken as an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in any country.

[0005] Interleukin-11 (IL-11) is a member of the IL-6 family of cytokines which includes IL-6, viral IL-6 (vIL-6), leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neutrophic factor (CNTF), cardiotrophin-1 (CT-1), cardiotrophin-like cytokine/cytokine-like factor-1 (CLC/CLF), IL-27 and neuropoietin (NP). IL-11 is able to stimulate the growth and differentiation of various lineages of hematopoietic cells, either alone or in synergy with other cytokines. IL-11 is also able to stimulate megakaryopoiesis and

platelet production, and is used clinically to prevent chemotherapy-induced thrombocytopenia (Tepler *et al*, *Blood 87(9)*:3607-3614, 1996) and is currently being assessed as a new approach to the treatment of chemotherapy-induced gastrointestinal mucositis (Herrlinger *et al*, *Am J Gastroenterol 101(4)*:793-797, 2006). IL-11 has also been suggested as being of benefit in arthritis and inflammatory bowel disease.

[0006] IL-11 also exerts a variety of biological activities outside the hematopoietic system. It is a regulator of osteoclast development and believed to be a regulator of bone metabolism (Girasole *et al*, *J clin Invest 93*:1516-1524, 1994; Hughes *et al*, *Calcif tissue Int* 53:362-364, 1993; Heymann and Rousselle, *Cytokine 12(10)*:1455-1468, 2000). IL-11 is expressed at high levels in cells of the CNS (Du *et al*, *J Cell Physiol 168*:362-372, 1996) and stimulates the survival and proliferation of neuronal progenitor cells (Mehler *et al*, *nature* 362:62-65, 1993). In female mice, IL-11 is essential for successful embryo implantation (Dimitriadeis *et al*, *Mol Hum Reprod. 6(10)*:907-914, 2000; Robb *et al*, *Nat Med* 4:303-308, 1998; Bilinski *et al*, *Genes Dev 12*:2234-2243, 1998) and the expression pattern of IL-11 and its receptors during the menstrual cycle suggests a similar role in humans. Other non-hematopoietic activities of IL-11 include inhibition of adipogenesis (Ohsumi *et al*, *FEBS Lett 288*:13-16, 1991; Ohsumi *et al*, *Biochem Mol Biol Int 32*:705-712, 1994), induction of a febrile response (Lopez-Valpuesta *et al*, *Neruopharmacology 33*:989-994, 1994), modulation of extracellular matrix metabolism (Maier *et al*, *J Biol chem.* 268:21527-21532, 1993), stimulation of acute-phase reactants (Baumann and Schendel, *J Biol Chem 266*:20424020427 1991), and proposed pro- and anti-inflammatory roles (Trepicchio *et al*, *J Clin Invest 104*:1527-1537, 1999; Redlich *et al*, *J Immunol 157*:1705-1710, 1996).

[0007] IL-11 has also been suggested as a potential therapeutic agent in various other inflammatory disorders including radiation-induced lung damage (Redlich *et al*, *supra* 1996), sepsis (Chang *et al*, *Blood Cells Mol Dis 22(1)*:57-67, 1996) and psoriasis (Trepicchio *et al*, *supra* 1999). US Patent No. 6,270,759 suggests that IL-11 may be therapeutically useful for a variety of inflammatory conditions including asthma and rhinitis.

[0008] Indicative of the therapeutic interest in IL-11, US Patent Application No. 2007/0190024 describes modified forms of IL-11 with mutations at His 182 (H182) and Asp 186 (D186) which act as agonists and hyperagonists of IL-11.

[0009] IL-11 exerts its effects *via* association with a specific cell surface receptor (IL-11Rα) as well as the shared receptor subunit gp130. While all IL-6 family cytokines signal through receptor complexes involving one or more gp130 molecules, the IL-11 signaling complex is most similar to that of IL-6 in that it comprises two molecules each of the cytokine, specific α-chain receptor and gp130 (Barton *et al*, *J Biol Chem (2000) 275*:36197-36203, 2000).

[0010] While neutralizing antibodies and soluble receptor proteins are a common strategy for inhibiting cytokines, a third class of antagonist molecules are referred to as "cytokine muteins" which prevent signaling by binding to only one of the two receptor chains. A number of these muteins has previously been described and one, an antagonistic variant of growth hormone, is used clinically to treat acromegaly (Cunningham and Wells, *Science 244*:1081-1085, 1989). Within the IL-6 family of cytokines, cytokine muteins have been described for IL-6, CNTF, LIF and IL-11 (Ehlers *et al*, *J Biol Chem 270*:8158-8163, 1995; Brakenhoff *et al*, *J Biol Chem 269*:86-93, 1994; Savino *et al*, *Embo J 13*:5863-5870, 1994; Hudson *et al*, *J Biol Chem 271*:11971-11978, 1996; Saggio *et al*, *Embo J 14*; 3045-3054, 1995; Underhill-Day *et al*, *Endocrinology 144*; 3406-3414, 2003). In each case, these cytokine mutein contain specific mutations which prevent binding of cytokine to gp130. In the case of IL-11, a single point mutation, W147A (a tryptophan to alanine substitution at amino acid residue 147), is sufficient to convert IL-11 from an agonist into an antagonist of IL-11 signaling with the affinity for IL-11Rα unchanged (Underhill-Day *et al*, *supra* 2003).

[0011] In addition, structure-function studies have identified various regions of murine and human IL-11 which are important for IL-11Rα binding (Czupryn *et al*, *J. Biol. Chem. 270 (2)*: 978-985, 1995; Miyadai *et al*, *Biosci. Biotechnol. Biochem. 60.3*:541-542, 1996;

Czupryn *et al*, *Ann. N.Y. Acad. Sci. 762*:152-164, 1995; Tacken *et al*, *Eur. J. Biochem. 265.2*:645-655, 1999; Harmegnies *et al*, *Biochem J. 375(1)*:23-32, 2003). In particular, residues D165, W166, R169, L172 and L173 at the C-terminal end of the D-helix, and M58, L64 and L67 in the A-B loop were found to contribute to IL-11Rα binding.

[0012] US Patent Application No. 2007/0190024 describes IL-11 muteins with mutations at His 182 (H182) and Asp 186 (D186) of IL-11 as agonists and hyperagonists of IL-11, but does not suggest antagonists. The IL-11 mutein, W147A IL-11, is an antagonistic variant of IL-11 that prevents the recruitment of gp130 to the IL-11 receptor complex (Underhill-Day *et al*, *supra* 2003) thereby preventing IL-11 signaling. However, W147A IL-11 has the same affinity for IL-11Rα as wild-type IL-11.

[0013] There is a role for IL-11 modulators in therapy. The identification of further IL-11 modulators is required.

SUMMARY

[0014] Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

[0015] Nucleotide and amino acid sequences are referred to by a sequence identifier number (SEQ ID NO:). The SEQ ID NOs: correspond numerically to the sequence identifiers <400>1 (SEQ ID NO:1), <400>2 (SEQ ID NO:2), etc. A summary of the sequence identifiers is provided in Table 1. A sequence listing is provided after the claims.

[0016] The present invention relates generally to modified forms of mammalian interleukin-11 (referred to hereinafter as "IL-11 muteins") which exhibit enhanced binding to the IL-11 receptor alpha chain (IL-11Rα). More particularly, the IL-11 muteins of the present invention modulate IL-11 signaling and are therefore useful as therapeutics in the treatment or prophylaxis of IL-11-mediated conditions. By "modulate" means up-regulate ("agonize") or down-regulate ("antagonize").

[0017] Accordingly, the present invention provides an IL-11 mutein comprising an amino acid sequence wherein the amino acid sequence AMSAG (using single letter amino acid code) [SEQ ID NO:23] at position 58 to 62 of wild-type mammalian IL-11 is replaced with the amino acid sequence PAIDY (SEQ ID NO:24) or FMQIQ (SEQ ID NO:25). In one embodiment, the IL-11 mutein is in isolated form although the present invention is not to be so limited.

[0018] In another aspect, the IL-11 mutein has, in addition to the mutation at amino acid position 58 to 62 of wild-type mammalian IL-11, a mutation that inhibits its binding to gp130.

[0019] In another aspect, the IL-11 mutein has, in addition to the mutation at amino acid

position 58 to 62 of wild-type mammalian IL-11, a tryptophan at amino acid position 147 of wild-type IL-11 mutated to inhibit its binding to gp130. Reference to "mutated" in this context includes an amino acid substitution, addition and/or deletion.

[0020] Specific IL-11 muteins of the present invention include an IL-11 mutein comprising SEQ ID NO:4, amino acids 10 to 178 of SEQ ID NO:4, amino acids 10 to 175 of SEQ ID NO:4, SEQ ID NO:5, amino acids 10 to 178 of SEQ ID NO:5, amino acids 10 to 175 of SEQ ID NO:5, SEQ ID NO:6, amino acids 10 to 178 of SEQ ID NO:6, amino acids 10 to 175 of SEQ ID NO:6, SEQ ID NO:7, amino acids 10 to 178 of SEQ ID NO:7, amino acids 10 to 175 of SEQ ID NO:7, SEQ ID NO:8, amino acids 10 to 178 of SEQ ID NO:8, amino acids 10 to 175 of SEQ ID NO:8, SEQ ID NO:13, amino acids 10 to 178 of SEQ ID NO:13 or amino acids 10 to 175 of SEQ ID NO:13.

[0021] Other specific IL-11 muteins of the present invention include an IL-11 mutein comprising SEQ ID NO:9, amino acids 10 to 178 of SEQ ID NO:9, amino acids 10 to 175 of SEQ ID NO:9, SEQ ID NO:10, amino acids 10 to 178 of SEQ ID NO:10, amino acids 10 to 175 of SEQ ID NO:10, SEQ ID NO:11, amino acids 10 to 178 of SEQ ID NO:11, amino acids 10 to 175 of SEQ ID NO:11, SEQ ID NO:12, amino acids 10 to 178 of SEQ ID NO:12, amino acids 10 to 175 of SEQ ID NO:12, SEQ ID NO:14, amino acids 10 to 178 of SEQ ID NO:14, amino acids 10 to 175 of SEQ ID NO:14, SEQ ID NO:15, amino acids 10 to 178 of SEQ ID NO:15, amino acids 10 to 175 of SEQ ID NO:15, SEQ ID NO:16, amino acids 10 to 178 of SEQ ID NO:16, amino acids 10 to 175 of SEQ ID NO:16, SEQ ID NO:17, amino acids 10 to 178 of SEQ ID NO:17, amino acids 10 to 175 of SEQ ID NO:17, SEQ ID NO:18, amino acids 10 to 178 of SEQ ID NO:18, amino acids 10 to 175 of SEQ ID NO:18, SEQ ID NO:19, amino acids 10 to 178 of SEQ ID NO:19, amino acids 10 to 175 of SEQ ID NO:19, SEQ ID NO:20, amino acids 10 to 178 of SEQ ID NO:20, amino acids 10 to 175 of SEQ ID NO:20, SEQ ID NO:21, amino acids 10 to 178 of SEQ ID NO:21 or amino acids 10 to 175 of SEQ ID NO:21.

[0022] The present invention also provides a nucleic acid sequence encoding an IL-11 mutein described herein.

[0023] In another aspect the present invention provides an IL-11 mutein which is PEGylated.

[0024] In another aspect the present invention contemplates a method for the treatment of an IL-11-mediated condition, the method comprising administering to said subject an effective amount of an IL-11 mutein of the present invention.

[0025] The present invention is further directed to the use of an IL-11 mutein of the present invention in the manufacture of a medicament for the treatment of an IL-11-mediated condition.

[0026] An IL-11-mediated condition includes (a) any condition which benefits or might benefit from increasing treatment with exogenous IL-11 or an IL-11 agonist, for example thrombocytopenia, rheumatoid arthritis, inflammatory bowel disease, infertility, and mucosal damage from chemotherapy and/or radiation therapy; and (b) any condition which benefits or might benefit from treatment with an IL-11 antagonist to reduce or block the activity of endogenous IL-11, for example conditions that result in diminished total bone mass, including metastatic bone cancer, myeloma, Paget's disease of the bone and osteoporosis, and fertility (i.e. an IL-11 antagonist may be used for contraception). In one embodiment, the IL-11 mutein antagonist comprises a substitution of AMSAG at amino acid position 58 to 62 together with a mutation that disrupts binding to gp130. An example of the latter is a mutation at amino acid 147 (e.g. a W147 mutation or a W147A or W147C substitution).

[0027] The present invention extends to compositions comprising IL-11 muteins of the present invention and one or more pharmaceutically acceptable carriers and/or diluents and/or excipient.

TABLE 1

Summary of sequence identifiers

SEQUENCE ID NO:	DESCRIPTION
1	Human mature IL-11 amino acid sequence
2	Murine mature IL-11 amino acid sequence
3	Macaque Mature IL-11 amino acid sequence
4	Macaque PAIDY IL-11 mutein amino acid sequence
5	Human PAIDY IL-11 mutein amino acid sequence
6	Murine PAIDY IL-11 mutein amino acid sequence
7	Human FMQIQ IL-11 mutein amino acid sequence
8	Murine FMQIQ IL-11 mutein amino acid sequence
9	Human PAIDY and W147A IL-11 mutein amino acid sequence
10	Murine PAIDY and W147A IL-11 mutein amino acid sequence
11	Human PAIDY and W147C IL-11 mutein amino acid sequence
12	Murine PAIDY and W147C IL-11 mutein amino acid sequence
13	Macaque FMQIQ IL-11 mutein amino acid sequence
14	Macaque PAIDY and W147A IL-11 mutein amino acid sequence
15	Macaque PAIDY and W147C IL-11 mutein amino acid sequence
16	Human FMQIQ and W147A IL-11 mutein amino acid sequence
17	Murine FMQIQ and W147A IL-11 mutein amino acid sequence
18	Macaque FMQIQ and W147A IL-11 mutein amino acid sequence
19	Human FMQIQ and W147C IL-11 mutein amino acid sequence
20	Murine FMQIQ and W147C IL-11 mutein amino acid sequence
21	Macaque FMQIQ and W147C IL-11 mutein amino acid sequence
22	N-terminally tagged Human PAIDY and W147C IL-11 mutein amino acid sequence
23	Amino acids at position 58 to 62 of wild-type IL-11
24	Substitution amino acids at position 58 to 62 of wild-type IL-11
25	Substitution amino acids at position 58 to 62 of wild-type IL-11

[0028] A summary of amino acid single and three letter codes in provided in Table 2.

TABLE 2

Amino Acid Abbreviations

Amino Acid	Three-letter Abbreviation	One-letter Symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalamine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

BRIEF DESCRIPTION OF THE FIGURES

[0029] Figures 1a to 1g disclose the amino acid sequences of human, murine and monkey IL-11 muteins of the present invention.

DETAILED DESCRIPTION

[0030] As used herein, the singular forms "a", "an" and "the" include plural aspects unless the context clearly dictates otherwise. Thus, for example, reference to "a mutein" includes a single mutein, as well as two or more muteins; reference to "an agent" includes a single agent, as well as two or more agent; reference to "the invention" includes single and multiple aspects of the invention; and so forth.

[0031] The term "IL-11" or its full name "interleukin-11" as used herein includes all mature forms of wild-type mammalian IL-11, including murine, macaque and human, and all truncated forms of such IL-11 that retain IL-11 activity, i.e. the ability to bind with IL-11Rα and form a functional receptor complex with gp130. Mature human IL-11 (SEQ ID NO:1) is a 178 amino acid protein (i.e. lacking the 21 amino acid leader sequence of NP_000632, NCBI protein database Accession Number), mature murine IL-11 (SEQ ID NO:2) is a 178 amino acid protein (i.e. lacking the 21 amino acid leader sequence of NP_032376, NCBI protein database Accession Number) and mature macaque IL-11 (SEQ ID NO:3) is a 178 amino acid protein (i.e. lacking the 21 amino acid leader sequence of P20808, NCBI protein database Accession Number).

[0032] The term "IL-11 mutein" as used herein refers to an IL-11 in which the amino acid sequence of the wild-type protein has been altered by amino acid substitutions, additions and/or deletions to provide enhanced binding to the IL-11Rα chain to generate an IL-11 mutein agonist or, in the case of an IL-11 mutein antagonist, the amino acid sequence has been further altered by amino acid substitutions, additions and/or deletions to antagonize IL-11 signaling by inhibiting the formation of an IL-11 receptor complex with gp130 while retaining enhanced binding to the IL-11Rα chain. Particularly, the IL-11 mutein is based on a human, macaque or murine IL-11, and more particularly human IL-11. The IL-11 muteins may be further modified, for example to increase their *in vivo* half life, including for example by the attachment of other elements such as a PEG group. Methods for the

PEGylation of peptides are well known in the art. IL-11 muteins may sometimes be referred to as IL-11 mutant proteins or as IL-11 mutants.

[0033] The expression "enhanced binding to the IL-11 receptor alpha (IL-11Rα) chain" when used in relation to the IL-11 muteins of the present invention means that the IL-11 mutein exhibits a greater affinity for the IL-11Rα chain than does the corresponding wild-type IL-11 as determined by competition ELISA.

[0034] The terms "antagonist", "agonist" and "compound" may each be used herein to refer to the IL-11 muteins described throughout the specification. The terms also encompass pharmaceutically acceptable and pharmacologically active forms thereof, including salts.

[0035] The term "effective amount" as used herein means a sufficient amount of an IL-11 mutein to provide the desired physiological and/or therapeutic effect such as to antagonize IL-11 signaling. In addition, the effect may be an amelioration of the symptoms of an IL-11-mediated condition. Undesirable effects, e.g. side effects, are sometimes manifested along with the desired physiological and/or therapeutic effect; hence, a practitioner balances the potential benefits against the potential risks when determining what is an appropriate "effective amount". The exact amount required will vary from subject to subject, depending on the species, age and general condition of the subject, mode of administration and the like. Thus, it may not be possible to specify an exact "effective amount". However, an appropriate "effective amount" in any individual case may be determined by one of ordinary skill in the art using routine experimentation. One of ordinary skill in the art would be able to determine the required amounts based on such factors as the subject's size, the severity of the subject's symptoms, and the particular composition or route of administration selected.

[0036] Insofar as one embodiment of the present invention relates to the use of an IL-11 mutein, the effective amount includes from about 10μg/kg body weight to 20mg/kg body weight of antibody such as 10, 20, 30, 40, 50, 60, 70, 80, 90, 100μg/kg body weight, 100,

200, 300, 400, 500, 600, 700, 800, 900, 1000µg/kg body weight or 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20mg/kg body weight. Similar amounts are provided for single or combination therapy.

[0037] Reference to "a condition mediated by IL-11" or an "IL-11-mediated condition" includes (a) any condition which benefits or might benefit from increasing treatment with exogenous IL-11 or an IL-11 agonist, for example thrombocytopenia, rheumatoid arthritis, inflammatory bowel disease, infertility, and mucosal damage from chemotherapy and/or radiation therapy; and (b) any condition which benefits or might benefit from treatment with an IL-11 antagonist to reduce or block the activity of endogenous IL-11, for example conditions that result in diminished total bone mass, including metastatic bone cancer, myeloma, Paget's disease of the bone and osteoporosis, and fertility (i.e. an IL-11 antagonist may be used for contraception).

[0038] A "pharmaceutically acceptable" carrier and/or diluent is a pharmaceutical vehicle comprised of a material that is not biologically or otherwise undesirable, i.e. the material may be administered to a subject along with the selected mutein without causing any or a substantial adverse reaction. Carriers and diluents may include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, agents used for adjusting tonicity, buffers, chelating agents, and absorption delaying agents and the like.

[0039] Similarly, a "pharmacologically acceptable" salt of a compound as provided herein is a salt that is not biologically or otherwise undesirable.

[0040] The terms "treating" and "treatment" as used herein refer to therapeutic treatment. For example, treatment may result in a reduction in severity and/or the frequency of symptoms of the condition, the elimination of symptoms and/or underlying cause of the condition, the prevention of the occurrence of symptoms of the condition and/or their underlying cause and improvement or remediation or amelioration of damage. Hence, the treatment may not result in a "cure" but rather an amelioration of symptoms. In addition, treatment may not commence until an exacerbated event occurs. In this context, the term

"prophylaxis" also applies to the prevention or treatment of a likelihood of an event associated with the condition occurring.

[0041] The terms "treating" and "treatment" as used herein also refer to the reduction of one or more symptoms or characteristics associated with the conditions.

[0042] A "subject" as used herein refers to an animal, preferably a mammal and more preferably a human who can benefit from the pharmaceutical compositions and methods of the present invention. Other preferred mammals are laboratory test animals, examples of which include mice, rats, rabbits, guinea pigs, hamsters, cats and dogs. There is no limitation on the type of animal that could benefit from the presently described pharmaceutical compositions and methods. A subject regardless of whether a human or non-human animal may be referred to as an individual, patient, animal or recipient as well as subject. The methods of the present invention have applications in human medicine and veterinary medicine.

[0043] It is shown herein that IL-11 muteins with enhanced binding to the IL-11Rα chain are produced when the amino acid sequence AMSAG (using single letter amino acid code) [SEQ ID NO:23] at positions 58 to 62 of wild-type mammalian IL-11 is replaced with the amino acid sequence PAIDY (SEQ ID NO:24) or FMQIQ (SEQ ID NO:25).

[0044] Accordingly, the present invention provides an IL-11 mutein wherein the amino acid sequence AMSAG (SEQ ID NO:23) at positions 58 to 62 of wild-type mammalian IL-11 is replaced with the amino acid sequence PAIDY (SEQ ID NO:24) or FMQIQ (SEQ ID NO:25).

[0045] One aspect of present invention provides an IL-11 mutein wherein the amino acid sequence AMSAG at positions 58 to 62 of wild-type human IL-11 (SEQ ID NO:1), murine IL-11 (SEQ ID NO:2) or macaque IL-11 (SEQ ID NO:3) is replaced with the amino acid sequence PAIDY (SEQ ID NO:24).

[0046] Another aspect of the present invention provides an IL-11 mutein wherein the amino acid sequence AMSAG at positions 58 to 62 of wild-type human IL-11 (SEQ ID NO:1), murine IL-11 (SEQ ID NO:2) or macaque IL-11 (SEQ ID NO:3) is replaced with the amino acid sequence FMQIQ (SEQ ID NO:25).

[0047] In one aspect, an IL-11 mutein of the present invention has a 10-fold, more particularly a 15-fold, and even more particularly a 20-fold higher binding affinity for the IL-11Rα chain than the binding affinity of the corresponding wild-type IL-11.

[0048] The inclusion of additional mutations that retain enhanced binding to the IL-11Rα chain but inhibit binding to gp130 provides IL-11 mutein antagonists which compete with IL-11 for binding to the IL-11Rα chain but do not form an IL-11 receptor complex with gp130.

[0049] Accordingly, in another aspect the IL-11 mutein may have, in addition to the mutations at amino acid positions 58 to 62 of wild-type mammalian IL-11, an additional mutation that inhibits binding to gp130.

[0050] A mutation of the tryptophan (W) residue at amino acid position 147 of wild-type murine IL-11 to alanine (A) is known to inhibit binding of the resulting IL-11 mutant to gp130. It is shown herein that a substitution mutation of the tryptophan residue at amino acid position 147 of wild-type murine IL-11 to cysteine inhibits binding of the resulting IL-11 mutant to gp130. Reference to "mutation" includes an amino acid substitution, addition and/or deletion. A substitution mutation is conveniently described herein as W147A or W147C to denote a change from a tryptophan (W) to an alanine (A) or cysteine (C).

[0051] Accordingly, in another aspect the IL-11 mutein may, in addition to the mutation at amino acid positions 58 to 62 of wild-type mammalian IL-11, have the tryptophan at amino acid position 147 of wild-type IL-11 mutated to inhibit its binding to gp130.

[0052] In another aspect, the tryptophan at amino acid position 147 of wild-type mammalian IL-11 is mutated to an alanine or a cysteine.

[0053] In another aspect, the IL-11 mutein may, in addition to the mutation at amino acid positions 58 to 62 of wild-type human IL-11 (SEQ ID NO:1), murine IL-11 (SEQ ID NO:2) or macaque IL-11 (SEQ ID NO:3) have an additional mutation which is inhibit its binding to gp130.

[0054] In another aspect, the IL-11 mutein may, in addition to the mutation at amino acid positions 58 to 62 of wild-type human IL-11 (SEQ ID NO:1), murine IL-11 (SEQ ID NO:2) or macaque IL-11 (SEQ ID NO:3) have the tryptophan residue at amino acid position 147 of wild-type mammalian IL-11 mutated to inhibit binding to gp130. In another aspect the tryptophan residue at amino acid position 147 of wild-type mammalian IL-11 is mutated to an alanine or a cysteine.

[0055] The present inventors have observed that up to nine N-terminal amino acid residues can be removed from murine IL-11 without loss of activity. Wang *et al*, *Eur J Biochem. 269(1)*:61-68, 2002, removed ten N-terminal amino acid residues from human IL-11. Barton *et al*, *J Biol Chem. 274(9)*:5755-61, 1999, report that amino acid residue thirteen of murine IL-11 forms part of the site II gp130 binding site and that substitution causes a loss in activity, suggesting that up to the first twelve N-terminal amino acid residues may be removed without significant loss of activity. US Patent Application No. 20070190024 which describes IL-11 muteins that are IL-11 agonists suggests that the first thirteen N-terminal amino acid residues may be removed without loss of activity.

[0056] It has also been reported that up to the last three, but not four, amino acid residues may be removed from the C-terminus of human IL-11 without a loss of activity (Czupryn *et al*, *supra* 1995).

[0057] In another aspect the IL-11 muteins of the present invention may, in addition to the mutations at amino acid positions 58 to 62 of wild-type IL-11, have up to the first thirteen,

preferably only up to the first twelve, N-terminal amino acid residues of the corresponding wild-type IL-11 deleted and/or have up to the last three C-terminal amino acid residues of the corresponding wild-type IL-11 deleted.

[0058] Specific IL-11 muteins of the present invention include an IL-11 mutein comprising SEQ ID NO:4, amino acids 10 to 178 of SEQ ID NO:4, amino acids 10 to 175 of SEQ ID NO:4, SEQ ID NO:5, amino acids 10 to 178 of SEQ ID NO:5, amino acids 10 to 175 of SEQ ID NO:5, SEQ ID NO:6, amino acids 10 to 178 of SEQ ID NO:6, amino acids 10 to 175 of SEQ ID NO:6, SEQ ID NO:7, amino acids 10 to 178 of SEQ ID NO:7, amino acids 10 to 175 of SEQ ID NO:7, SEQ ID NO:8, amino acids 10 to 178 of SEQ ID NO:8, amino acids 10 to 175 of SEQ ID NO:8, SEQ ID NO:13, amino acids 10 to 178 of SEQ ID NO:13 or amino acids 10 to 175 of SEQ ID NO:13.

[0059] Other specific IL-11 muteins of the invention include an IL-11 mutein comprising SEQ ID NO:9, amino acids 10 to 178 of SEQ ID NO:9, amino acids 10 to 175 of SEQ ID NO:9, SEQ ID NO:10, amino acids 10 to 178 of SEQ ID NO:10, amino acids 10 to 175 of SEQ ID NO:10, SEQ ID NO:11, amino acids 10 to 178 of SEQ ID NO:11, amino acids 10 to 175 of SEQ ID NO:11, SEQ ID NO:12, amino acids 10 to 178 of SEQ ID NO:12, amino acids 10 to 175 of SEQ ID NO:12, SEQ ID NO:14, amino acids 10 to 178 of SEQ ID NO:14, amino acids 10 to 175 of SEQ ID NO:14, SEQ ID NO:15, amino acids 10 to 178 of SEQ ID NO:15, amino acids 10 to 175 of SEQ ID NO:15, SEQ ID NO:16, amino acids 10 to 178 of SEQ ID NO:16, amino acids 10 to 175 of SEQ ID NO:16, SEQ ID NO:17, amino acids 10 to 178 of SEQ ID NO:17, amino acids 10 to 175 of SEQ ID NO:17, SEQ ID NO:18, amino acids 10 to 178 of SEQ ID NO:18, amino acids 10 to 175 of SEQ ID NO:18, SEQ ID NO:19, amino acids 10 to 178 of SEQ ID NO:19, amino acids 10 to 175 of SEQ ID NO:19, SEQ ID NO:20, amino acids 10 to 178 of SEQ ID NO:20, amino acids 10 to 175 of SEQ ID NO:20, SEQ ID NO:21, amino acids 10 to 178 of SEQ ID NO:21 or amino acids 10 to 175 of SEQ ID NO:21.

[0060] IL-11 from monkey (SEQ ID NO:3) and mouse (SEQ ID NO:2) demonstrate considerable identity with the human sequence (SEQ ID NO:1, ~94% and ~87% identity

respectively). All these proteins have full cross-species reactivity indicating that their tertiary structures are similar and other functionally critical residues are probably conserved. (Czupryn *et al*, *supra* 1995). In view of the cross reactivity of IL-11 from different species it is clear that IL-11 can tolerate some level of amino acid variation and retain IL-11 activity. Accordingly, the IL-11 muteins of the present invention include IL-11 muteins comprising sequences that are at least 85% identical, preferably at least 90% identical and more preferably at least 94% identical to any one of those of SEQ ID NO:4, amino acids 10 to 178 of SEQ ID NO:4, amino acids 10 to 175 of SEQ ID NO:4, SEQ ID NO:5, amino acids 10 to 178 of SEQ ID NO:5, amino acids 10 to 175 of SEQ ID NO:5, SEQ ID NO:6, amino acids 10 to 178 of SEQ ID NO:6, amino acids 10 to 175 of SEQ ID NO:6, SEQ ID NO:7, amino acids 10 to 178 of SEQ ID NO:7, amino acids 10 to 175 of SEQ ID NO:7, SEQ ID NO:8, amino acids 10 to 178 of SEQ ID NO:8, amino acids 10 to 175 of SEQ ID NO:8, SEQ ID NO:9, amino acids 10 to 178 of SEQ ID NO:9, amino acids 10 to 175 of SEQ ID NO:9, SEQ ID NO:10, amino acids 10 to 178 of SEQ ID NO:10, amino acids 10 to 175 of SEQ ID NO:10, SEQ ID NO:11, amino acids 10 to 178 of SEQ ID NO:11, amino acids 10 to 175 of SEQ ID NO:11, SEQ ID NO:12, amino acids 10 to 178 of SEQ ID NO:12, amino acids 10 to 175 of SEQ ID NO:12, SEQ ID NO:13, amino acids 10 to 178 of SEQ ID NO:13, amino acids 10 to 175 of SEQ ID NO:13, SEQ ID NO:14, amino acids 10 to 178 of SEQ ID NO:14, amino acids 10 to 175 of SEQ ID NO:14, SEQ ID NO:15, amino acids 10 to 178 of SEQ ID NO:15, amino acids 10 to 175 of SEQ ID NO:15, SEQ ID NO:16, amino acids 10 to 178 of SEQ ID NO:16, amino acids 10 to 175 of SEQ ID NO:16, SEQ ID NO:17, amino acids 10 to 178 of SEQ ID NO:17, amino acids 10 to 175 of SEQ ID NO:17, SEQ ID NO:18, amino acids 10 to 178 of SEQ ID NO:18, amino acids 10 to 175 of SEQ ID NO:18, SEQ ID NO:19, amino acids 10 to 178 of SEQ ID NO:19, amino acids 10 to 175 of SEQ ID NO:19, SEQ ID NO:20, amino acids 10 to 178 of SEQ ID NO:20, amino acids 10 to 175 of SEQ ID NO:20, SEQ ID NO:21, amino acids 10 to 178 of SEQ ID NO:21 or amino acids 10 to 175 of SEQ ID NO:21.

[0061] The present invention also provides nucleic acid sequences encoding the IL-11 muteins described herein. A nucleic acid sequence or nucleic acid includes a polynucleotide or nucleic acid molecule.

[0062] The present invention employs conventional molecular biology, microbiology, and recombinant DNA techniques to modify wild-type IL-11 nucleic acid sequences to produce the IL-11 muteins of the present invention. The techniques are well known in the art and are described in various publications, such as Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y, 1989.; *DNA Cloning: A Practical Approach*, Volumes I and II (D. N. Glover ed. 1985), Ausubel, *et al.* (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc., 1994, Sidhu *et al*, *Methods Enzymol 328:*333-363, 2000) and Kunkel *et al*, *Methods Enzymol 204*: 1991.

[0063] The terms "polynucleotide", "nucleic acid" or "nucleic acid molecule" refer to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules"), or any phosphoester analogs thereof, such as phosphorothioates and thioesters, in single stranded form, double-stranded form or otherwise.

[0064] The terms "polynucleotide sequence", "nucleic acid sequence" or "nucleotide sequence" refer to a series of nucleotide bases (also referred to as "nucleotides") in a nucleic acid, such as DNA or RNA, and means any chain of two or more nucleotides.

[0065] The terms "coding sequence" or a sequence "encoding" an expression product, such as a RNA, polypeptide, protein, or enzyme, is a nucleotide sequence that, when expressed, results in the production of the product.

[0066] The term "gene" means a DNA sequence that codes for or corresponds to a particular sequence of ribonucleotides or amino acids which comprise all or part of one or

more RNA molecules, proteins or enzymes, and may or may not include regulatory DNA sequences, such as promoter sequences, which determine, for example, the conditions under which the gene is expressed. Genes may be transcribed from DNA to RNA which may or may not be translated into an amino acid sequence.

[0067] The term "amplification" of nucleotide sequence as used herein may denote the use of the polymerase chain reaction (PCR) to increase the concentration of a particular nucleotide sequence within a mixture of nucleotide sequence sequences. Saiki, *et al*, *Science 239*:487, 1988 provide a description of PCR.

[0068] The term "oligonucleotide" refers to a nucleic acid, generally of at least 10, particularly at least 15, and more particularly at least 20 nucleotides, particularly no more than 100 nucleotides that may be hybridizable to a genomic DNA molecule, a cDNA molecule, or an mRNA molecule encoding a gene, mRNA, cDNA, or other nucleic acid of interest. Oligonucleotides can be labeled for example, by incorporation of 32^{P}-nucleotides, 3^{H}-nucleotides, 14^{C}-nucleotides, 35^{S}-nucleotides or nucleotides to which a label, such as biotin, has been covalently conjugated. In one embodiment, a labeled oligonucleotide can be used as a probe to detect the presence of a nucleic acid. In another embodiment, oligonucleotides (one or both of which may be labeled) can be used as PCR primers, either for cloning full length or a fragment of the gene, or to detect the presence of nucleic acids. Generally, oligonucleotides are prepared synthetically, preferably on a nucleic acid synthesizer.

[0069] The sequence of any nucleic acid (for example, a nucleic acid encoding a wild-type IL-11 protein or an IL-11 mutein) may be sequenced by any method known in the art such as by chemical sequencing or enzymatic sequencing. "Chemical sequencing" of DNA may be done by the method of Maxam and Gilbert (*Proc. Natl. Acad. Sci. USA 74(2)*: 560-564, 1977), in which DNA is randomly cleaved using individual base-specific reactions. "Enzymatic sequencing" of DNA may be done by the method of Sanger (Sanger *et al*, *Proc. Natl. Acad. Sci. USA 74(12)*:5463 5467, 1977).

[0070] Nucleic acids of the present invention may be flanked by natural regulatory (expression control) sequences, or may be associated with heterologous sequences, including promoters, internal ribosome entry sites (IRES) and other ribosome binding site sequences, enhancers, response elements, suppressors, signal sequences, polyadenylation sequences, introns, 5'-and 3'-non-coding regions, and the like.

[0071] A "promoter" or "promoter sequence" is a DNA regulatory region capable of binding an RNA polymerase in a cell and initiating transcription of a coding sequence. A promoter sequence is generally bounded at its 3' terminus by the transcription initiation site and extends upstream in the 5' direction to include the minimum number of bases or elements necessary to initiate transcription at any level. A transcription initiation site as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase may be found within the promoter sequence. The promoter may be operably associated with other expression control sequences, including enhancer and repressor sequences or with a nucleic acid of the invention. Promoters which may be used to control gene expression include, but are not limited to, the cytomegalovirus (CMV) promoter (US Patent Nos. 5,385,839 and 5,168,062) and the SV40 early promoter region (Benoist, *et al*, *Nature 290*:304-310, 1981).

[0072] A coding sequence is "under the control of", "functionally associated with" or "operably associated with" transcriptional and translational control sequences in a cell when the sequences direct RNA polymerase mediated transcription of the coding sequence into RNA, preferably mRNA, which then may be trans-RNA spliced (if it contains introns) and, optionally, translated into a protein encoded by the coding sequence.

[0073] The terms "express" and "expression" mean allowing or causing the information in a gene, RNA or DNA sequence to be converted into a product; for example, producing a protein by activating the cellular functions involved in transcription and translation of a nucleotide sequence. A DNA sequence is expressed in or by a cell to form an "expression product" such as RNA (such as mRNA) or a protein (such as an IL-11 mutein). The expression product itself may also be said to be "expressed" by the cell.

[0074] The terms "vector", "cloning vector" and "expression vector" mean the vehicle (such as a plasmid) by which a DNA or RNA sequence can be introduced into a host cell, so as to transform the host and, optionally, promote expression and/or replication of the introduced sequence.

[0075] The term "transfection" or "transformation" means the introduction of a nucleic acid into a cell. These terms may refer to the introduction of a nucleic acid encoding an IL-11 mutein into a cell. The introduced gene or sequence may be called a "clone". A host cell that receives the introduced DNA or RNA has been "transformed" and is a "transformant" or a "clone". The DNA or RNA introduced to a host cell can come from any source, including cells of the same genus or species as the host cell, or cells of a different genus or species.

[0076] The term "host cell" means any cell of any organism that is selected, modified, transfected, transformed, grown, or used or manipulated in any way, for the production of a substance by the cell, for example the expression or replication, by the cell, of a gene, a DNA or RNA sequence, a protein or an enzyme.

[0077] The term "expression system" means a host cell and compatible vector which, under suitable conditions, can express a protein or nucleic acid which is carried by the vector and introduced to the host cell. Common expression systems include *E.coli* host cells and plasmid vectors, insect host cells and Baculovirus vectors, and mammalian host cells and vectors.

[0078] The present invention contemplates any slight modifications of the amino acid or nucleotide sequences which corresponds to or encodes the IL-11 muteins of the sequences described herein excluding modifications that would change the amino acid segment corresponding to amino acids 58 to 62 of wild-type mammalian IL-11 which segment will have the sequence PAIDY (SEQ ID NO:24) or FMQIQ (SEQ ID NO:25). In particular, the present invention contemplates sequence conservative variants of the nucleic acids which

encode the IL-11 muteins of the invention. "Sequence-conservative variants" of a polynucleotide sequence are those in which a change of one or more nucleotides in a given codon results in no alteration in the amino acid encoded at that position. Function-conservative variants of the IL-11 muteins of the invention are also contemplated by the present invention. "Function-conservative variants" are those in which one or more amino acid residues in a protein have been changed without altering the overall conformation and function of the protein, including, but, by no means, limited to, replacement of an amino acid with one having similar properties. Amino acids with similar properties are well known in the art. For example, polar/hydrophilic amino acids which may be interchangeable include asparagine, glutamine, serine, cysteine, threonine, lysine, arginine, histidine, aspartic acid and glutamic acid; nonpolar/hydrophobic amino acids which may be interchangeable include glycine, alanine, valine, leucine, isoleucine, proline, tyrosine, phenylalanine, tryptophan and methionine; acidic amino acids which may be interchangeable include aspartic acid and glutamic acid and basic amino acids which may be interchangeable include histidine, lysine and arginine. Preferably, function-conservative variants of the IL-11 muteins of the invention have less than 20, more preferably less than 15, more preferably less than 10 amino acid changes.

[0079] Also included in the present invention are IL-11 muteins wherein the amino acid sequence AMSAG (SEQ ID NO:23) at positions 58 to 62 of wild-type mammalian IL-11 is replaced with the amino acid sequence PAIDY (SEQ ID NO:24) or FMQIQ (SEQ ID NO:25) and comprising amino acid sequences which are at least 85% identical, particularly at least 90% identical, more particularly at least 94% identical (e.g. 94%, 95%, 96%, 97%, 98%, 99%) to the amino acid sequences described herein when the comparison is performed by a BLAST algorithm wherein the parameters of the algorithm are selected to give the largest match between the respective sequences over the entire length of the respective reference sequences.

[0080] Sequence identity refers to exact matches between the amino acids of two sequences which are being compared.

[0081] Descriptions for BLAST algorithms can be found in the following references which herein incorporated by reference: BLAST ALGORITHMS: Altschul *et al*, *J. Mol. Biol. 215*:403-410, 1990; Altschul *et al*, *Nucleic Acids Res. 25*:3389-3402, 1997; Altschul, *J. Mol. Biol. 219*:555-565, 1991.

[0082] The IL-11 muteins of the present invention may be produced recombinantly, for example, in an *E.coli* expression system. Transformation can be by any known method for introducing polynucleotides into a host cell. Methods for introduction of heterologous polynucleotides into mammalian cells are well known in the art and include dextran-mediated transfection, calcium phosphate precipitation, polybrene-mediated transfection, protoplast fusion, electroporation and encapsulation of the polynucleotide(s) in liposomes, biolistic injection and direct microinjection of the DNA into nuclei. In addition, nucleic acid molecules may be introduced into mammalian cells by viral vectors. Methods of transforming cells are well known in the art. See, for example, US Patent Nos. 4,399,216; 4,912,040; 4,740,461 and 4,959,455.

[0083] In one aspect, the present invention provides a method for the production of an IL-11 mutein of the invention, said method comprising cloning a nucleic acid sequence encoding an IL-11 mutein into an appropriate vector, transforming a host cell line with the vector, and culturing the transformed host cell line under conditions suitable for the expression of the antibodies of the present invention.

[0084] Vectors available for cloning and expression in host cell lines are well known in the art, and include but are not limited to vectors for cloning and expression in mammalian cell lines, vectors for cloning and expression in bacterial cell lines and vectors for cloning and expression insect cell lines. The IL-11 muteins can be recovered using standard protein purification methods.

[0085] In another aspect, the present invention provides nucleic acid sequences encoding IL-11 muteins having the amino acid sequences shown in SEQ ID NOs:5 to 12.

[0086] In still a further aspect, the present invention provides host cell lines transformed with the vectors of the present invention. Host cell lines include, but are not limited to, bacterial cells, such as *E.coli* and mammalian cell lines.

[0087] Mammalian cell lines available as hosts for expression are well known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC). These include, inter alia, Chinese hamster ovary (CHO) cells, NSO, SP2 cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human hepatocellular carcinoma cells (e.g., Hep G2), A549 cells, 3T3 cells, and a number of other cell lines. Mammalian host cells include human, mouse, rat, dog, monkey, pig, goat, bovine, horse and hamster cells. Cell lines of particular preference are selected through determining which cell lines have high expression levels. Other cell lines that may be used are insect cell lines, such as Sf9. cells, amphibian cells, bacterial cells, plant cells and fungal cells. When recombinant expression vectors encoding the heavy chain or antigen-binding portion thereof, the light chain and/or antigen-binding portion thereof are introduced into mammalian host cells, the antibodies are produced by culturing the host cells for a period of time sufficient to allow for expression of the antibody in the host cells or, more preferably, secretion of the antibody into the culture medium in which the host cells are grown.

[0088] The IL-11 muteins can be recovered from the culture medium using standard protein purification methods. Further, expression of IL-11 muteins of the invention from production cell lines can be enhanced using a number of known techniques. For example, the glutamine synthetase gene expression system (the GS system) is a common approach for enhancing expression under certain conditions. The GS system is discussed in whole or part in connection with European Patent Nos. 0 216 846, 0 256 055, and 0 323 997 and European Patent Application No. 89303964.4.

[0089] It is likely that the IL-11 muteins expressed by different cell lines or in transgenic animals will have different glycosylation from each other. However, all IL-11 muteins encoded by the nucleic acid molecules provided herein, or comprising the amino acid

sequences provided herein are part of the invention, regardless of the glycosylation of the IL-11 muteins.

[0090] In a further aspect, the present invention provides a human or murine IL-11 muteins that have been further modified to enhance their pharmacokinetic properties and half life *in vivo*. Modifications include PEGylation with polyethylene glycol, (Clark *et al*, *J Biol Chem. 271(36)*:21969-77, 1996), fusions to large long lived proteins such as albumin (Yeh *et al*, *Proc Natl Acad Sci U S A. 89(5)*:1904-8, 1992) or the Fc portion of an Ig (Ashkenazi and Chamow, *Curr Opin Immunol. 9(2)*:195-200, 1997) and the introduction of glycosylation sites (Keyt *et al*, *Proc Natl Acad Sci USA. 91(9)*:3670-4, 1994).

[0091] An aspect of the invention provides IL-11 mutein antagonists that are PEGylated.

[0092] One aspect of the present invention provides IL-11 muteins having the amino acid sequences of SEQ ID NO:11, amino acids 10 to 178 of SEQ ID NO:11, amino acids 10 to 175 of SEQ ID NO:11, SEQ ID NO:12, amino acids 10 to 178 of SEQ ID NO:12 amino acids 10 to 175 of SEQ ID NO:12, SEQ ID NO:15, amino acids 10 to 178 of SEQ ID NO:15, amino acids 10 to 175 of SEQ ID NO:15, SEQ ID NO:19, amino acids 10 to 178 of SEQ ID NO:19, amino acids 10 to 175 of SEQ ID NO:19, SEQ ID NO:20, amino acids 10 to 178 of SEQ ID NO:20, amino acids 10 to 175 of SEQ ID NO:20, SEQ ID NO:21, amino acids 10 to 178 of SEQ ID NO:21 or amino acids 10 to 175 of SEQ ID NO:21 that are PEGylated.

[0093] The IL-11 muteins of the invention may be conveniently supplied in compositions suitable for pharmaceutical use. Such compositions are another aspect of the present invention.

[0094] Administration may be systemic or local. Systemic administration is particularly useful. Reference to "systemic administration" includes intra-articular, intravenous,

intraperitoneal, and subcutaneous injection, infusion, as well as administration *via* oral, rectal and nasal routes, or *via* inhalation.

[0095] Compositions suitable for systemic use include sterile aqueous solutions (where water soluble), sterile powders for the extemporaneous preparation of sterile injectable solutions, and sterile powders for inhalation. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be any pharmaceutically acceptable carriers and/or diluent, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof and vegetable oils. The proper fluidity can be maintained, for example, by the use of superfactants. Various anti-bacterial and anti-fungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thirmerosal and the like may be included. In many cases, it will be preferable to include agents to adjust tonicity, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

[0096] Sterile solutions are prepared by incorporating the active in the required amount in the appropriate solvent and optionally with other active ingredients and excipients as required, followed by filtered sterilization or other appropriate means of sterilization. In the case of sterile powders, suitable methods of preparation include vacuum drying and the freeze-drying technique which yield a powder of active ingredient plus any additionally desired ingredient which can be made at an appropriate particle size.

[0097] When the active is suitably protected, it may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets. For oral therapeutic administration, the active ingredient may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers and the like.

[0098] Dosage regimens may be adjusted to provide the optimum desired response (e.g. a therapeutic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage.

[0099] A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician or veterinarian could start doses of the antagonist, employed in the pharmaceutical composition, at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved. In general, a suitable dose of a composition of the invention may be that amount of the compound which is the lowest dose effective to produce a therapeutic effect.

[0100] For therapeutic applications, the IL-11 muteins of the present invention or compositions containing those muteins are administered to a mammal, preferably a human, in a pharmaceutically acceptable dosage form such as those discussed above, including those that may be administered to a human intravenously as a bolus or by continuous infusion over a period of time.

[0101] In one aspect, the present invention contemplates a method for the treatment of a condition mediated by IL-11, the method comprising administering to said subject an effective amount of an IL-11 mutein of the present invention.

[0102] The IL-11 muteins of the present invention that are agonists, and compositions comprising such muteins, may be used in a method for the treatment of IL-11-mediated conditions where IL-11 exerts a positive effect.

[0103] The IL-11 muteins of the present invention that are antagonists and compositions

comprising such muteins may be used in a method for the treatment of IL-11-mediated conditions where IL-11 exerts a negative effect.

[0104] The IL-11 muteins of the present invention and compositions comprising such muteins may be used in a method of manufacture of a medicament for the treatment of IL-11-mediated conditions.

[0105] Particular IL-11 mutein antagonists of the present invention are IL-11 muteins having the amino acid sequences of SEQ ID NO:11, amino acids 10 to 178 of SEQ ID NO:11, amino acids 10 to 175 of SEQ ID NO:11, SEQ ID NO:12, amino acids 10 to 178 of SEQ ID NO:12 amino acids 10 to 175 of SEQ ID NO:12, SEQ ID NO:15, amino acids 10 to 178 of SEQ ID NO:15, amino acids 10 to 175 of SEQ ID NO:15, SEQ ID NO:19, amino acids 10 to 178 of SEQ ID NO:19, amino acids 10 to 175 of SEQ ID NO:19, SEQ ID NO:20, amino acids 10 to 178 of SEQ ID NO:20, amino acids 10 to 175 of SEQ ID NO:20, SEQ ID NO:21, amino acids 10 to 178 of SEQ ID NO:21 or amino acids 10 to 175 of SEQ ID NO:21 and which may be PEGylated. Particularly PEGylation is *via* attachment to the cysteine residue corresponding to position 147 of SEQ ID NO's 11, 12, 15, 19, 20 or 21.

[0106] The invention further contemplates the use of an IL-11 mutein in the manufacture of a medicament for the treatment of an IL-11-mediated condition.

[0107] The present invention is further described by the following non-limiting Examples.

EXAMPLE 1

IL-11 mutant proteins

A. Recombinant production of soluble IL-11 mutant proteins

[0108] The IL-11 mutants 1.21 (SEQ ID NO:10), 1B.382 (SEQ ID NO:17) and mIL-11-W147A were cloned into a modified version of the pET15b vector (Novagen Cat # 69661-3). The pET15b vector was modified by replacing the thrombin cleavage site and multiple cloning sites with *AscI* and *EcoRI* restriction sites, and by inserting an M13 origin of replication so the vector could be used as a phagemid.

[0109] The corresponding N-terminal hexahistidine-tagged proteins were expressed in the *E.coli* strain BL21-CodonPlus [Registered trade mark] (DE3)-RIL *E.coli* (Strategene cat # 230245). Typically, 400 mL shake-flask cultures in superbroth containing 2% v/v glucose and 100 μg/mL ampicillin were grown to an optical density (600 nm) of 0.5. Protein expression was then induced by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 200 uM, and the cultures were incubated with shaking at 37°C for a further 4 hours. The recombinant proteins were purified from the bacterial cells (lysed in 7 M guanidinium hydrochloride) using immobilized nickel ion affinity chromatography, and refolded by dialysis into PBS. The refolded samples were further dialyzed against 0.15% aqueous trifluoroacetic acid. In some cases samples were also purified by reverse phase HPLC using acetonitrile gradients in 0.15% v/v trifluoroacetic acid before lyophilization. Samples were reconstituted in a small volume of water prior to dilution with buffer.

[0110] The affinity of the selected IL-11 mutants for IL-11Rα was determined in a competition ELISA experiment. 96 well plates coated with mIL-11Rα-Fc were incubated with a constant sub-saturating amount of the phage displayed IL-11 variants in the presence of different concentrations of soluble IL-11 proteins. After incubation for 2 hours at room temperature, the plates were washed and bound phage were then labeled with an anti-M13 polyclonal antibody conjugated to horseradish peroxidase. After the removal of excess antibody by washing with PBS containing 0.05% Tween 20, TMB substrate was added to each well and incubated for 10 minutes before the reaction was quenched by the

addition of 2M phosphoric acid. Absorbance at 450 nm was then determined for each well by analysis on a microtitre plate reader.

[0111] The W147A mutation has no effect on IL-11Rα binding but does prevent the recruitment of gp130 to the IL-11 receptor complex (Underhill-Day *et al*, 2003 *supra*) thereby preventing IL-11 signaling. W147A IL-11 is an antagonistic variant of IL-11.

[0112] Clear differences were observed between the affinities of the mIL-11-W147A and mutant proteins for binding to IL-11Rα-Fc. Relative to W147A IL-11, clone 1.21 (SEQ ID NO:10) bound to IL-11Rα with a 20-fold higher affinity, while clone 1B.382 (SEQ ID NO:17) also bound IL-11Rα with a 20-fold higher affinity.

B. *In vitro* activity of antagonist

[0113] An IL-11 responsive Ba/F3 cell line was generated to test the ability of the mutant IL-11 proteins to block IL-11 bioactivity. Ba/F3 cells, a murine pro B-lymphocyte cell line which does not normally express IL-11Rα or gp130 nor proliferate in response to IL-11 were stably transfected with constructs encoding wild-type murine IL-11Rα and the co-receptor murine gp130 and selected by growth in media containing IL-11. Clonal cell lines were derived by limit dilution cloning. A number of stably transfected clones were analyzed for their dose-responsive proliferation (using a MTT assay) when cultured in the presence of IL-11 and one was selected for further work.

[0114] IL-11 responsive Ba/F3 cells stably transfected with murine IL-11Rα/gp130 were seeded at $3x10^4$ cells/well in 50uL of Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum and increasing concentrations of mutant IL-11 proteins in the presence of a fixed, submaximal concentration of murine IL-11 (50 pM) in a total volume of 100 uL/well. After incubation for 48 hours, proliferation was measured colorimetrically at 570 nm using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich). Assays were always performed in duplicate and mean values for each assay point were then plotted.

[0115] W147A IL-11 has been previously characterized as an antagonist of IL-11 bioactivity (Underhill-Day *et al*, 2003 *supra*). When IL-11Rα/gp130 Ba/F3 cells were stimulated with a sub-maximal dose of IL-11, W147A IL-11 was able to inhibit cell proliferation in a dose-dependent manner. Several of the mutant IL-11 proteins were assayed for their ability to inhibit IL-11-induced proliferation and compared to W147A IL-11 (Table 4). The mutant IL-11 proteins were significantly more potent at blocking the proliferation induced IL-11 as measured in a standard MTT assay. Clones 1.21 (SEQ ID NO:10) and 1B.382 (SEQ ID NO:17) were both 20 to 30-fold more potent antagonists of IL-11 than W147A IL-11.

EXAMPLE 2

PEGylated IL-11 muteins

Production of PEGylated IL-11 muteins

[0116] The mature protein sequence of murine IL-11 contains an amino acid sequence that can be cleaved by thrombin resulting in the removal of the first nine amino acids. A comparison of the IL-11 mutein 1.21 (SEQ ID NO:10) with and without the first nine amino acids showed identical activity and indicated that the first nine residues of murine IL-11 are not required for IL-11Rα binding. The internal thrombin site was optimized by site directed mutagenesis to allow for efficient cleavage by mutation of residues 6 and 7 to Leu (L) and Val (V), respectively. For large scale production of the PEGylated IL-11 mutein of amino acids 10-178 of SEQ ID NO:12 the amino-terminal His-tag and the first nine residues of the modified mIL-11 sequence were removed by thrombin digestion.

[0117] For cleavage of the N-terminal hexahistidine tag, lyophilized samples of the relevant IL-11 mutein protein were re-suspended in thrombin cleavage buffer (150 mM NaCl, 2.5 mM $CaCl_2$, 20 mM Tris.HCl pH 8.4) at a concentration of 0.5 mg/mL and treated with 5 units of thrombin/mg protein for 4 hours at room temperature. Under these conditions, thrombin efficiently cleaves murine derived IL-11 mutein at the optimized internal site between residues Arg^9-Val^{10} and the thrombin digested samples have an N-terminal sequence of Val^{10}-Ser^{11}-Ser^{12}. Following treatment with thrombin the cleaved samples were purified by reverse phase HPLC as previously described.

Site-specific PEGylation

[0118] A limitation to the *in vivo* use of small proteins is their rapid clearance from circulation. One of the main routes of clearance is *via* filtration through the kidney, the efficiency of which is inversely proportional to the molecular weight. One strategy for reducing the *in vivo* clearance rate of small proteins is through chemical modification with polyethylene glycol (Tsutsumi *et al*, *Thjromb. Haemost. 77.1*:168-73, 1997), however, this can reduce or even eliminate the activity of a protein if attached at an inappropriate site.

[0119] To improve the potential pharmacokinetic properties of a mutant IL-11 protein for *in vivo* use, a strategy was designed for site-specific modification of the mutant IL-11 proteins with a 40 kDa polyethylene glycol moiety. The absence of cysteine with the sequence of IL-11 was exploited to introduce a single unique Cys residue at position 147 by site directed mutagenesis. This provided a chemically reactive sidechain which could be site-specifically modified with a maleimide-derivatized PEG reagent. Moreover, the site of PEG attachment corresponds to site III on the surface of IL-11, and should not interfere with binding of the mutant IL-11 proteins to IL-11Rα, or to the gp130 molecule which binds to the site II surface.

[0120] Modified forms of the mutant IL-11 proteins were generated containing a W147C mutation and the optimized internal thrombin site described above. The proteins were expressed in *E.coli* and purified and refolded as described in Example 1. The N-terminal His-tag and the first 9 N-terminal amino acids were then cleaved with thrombin as described above. The thrombin-treated samples were purified as described in Example 1 except that the samples was adjusted to pH 8.0 and reduced with 5 mM DTT prior to refolding in PBS containing 2mM EDTA and 2mM DTT.

[0121] Mutant IL-11 proteins containing an engineered Cys residue at the position corresponding to position 147 of SEQ ID NO:12 were then modified with 40 kDa maleimide-derivatized polyethylene glycol. Briefly, lyophilized thrombin-treated mutant IL-11 proteins were resuspended at a concentration of 5 mg/mL in 1 mM aqueous acetic acid containing 5 mM tris(2-carboxyethyl)phosphine, and mixed with 4 volumes of 12.5 mg/mL mPEG2-maleimide (Nektar Therapeutics cat #2D3YOTO1) in PBS. Reactions were incubated for 16 hours at room temperature and protein-PEG conjugates were then separated from unconjugated components by cation exchange chromatography on an SP Sepharose column, using a NaCl gradient in 20 mM sodium acetate, pH 5.5 buffer. Fractions containing the PEGylated products were pooled, dialyzed against 5 mM ammonium acetate buffer, pH 5.5, and then lyophilized.

[0122] A modified form of clone 1.21, containing a W147C mutation and an optimized internal thrombin site, was expressed in *E.coli*, and purified and refolded as described. The N-terminal His-tag and 9 amino acid residue fragment were cleaved with thrombin and then site-specifically PEGylated at Cys147 (where Cys147 is the position corresponding to position 147 of SEQ ID NO:12). Excess PEG reagent was removed by ion exchange chromatography. Analysis of the PEGylated and truncated modified form of clone 1.21 (referred to herein as Δ1.21) by SDS-PAGE showed a shift in apparent molecular weight consistent with attachment of a single 40 kDa PEG moiety.

[0123] The activity of Δ1.21 was tested in the IL-11Rα binding ELISA and the Ba/F3 cell assay, and compared to the activity of non-PEGylated 1.21 (containing Ala at position 147) and with non-PEGylated W147A IL-11. In both assays, the activity of Δ1.21 was reduced relative to non-PEGylated 1.21. IL-11Rα binding affinity was reduced approximately 5-fold, whilst the ability of Δ1.21 to antagonize IL-11-induced Ba/F3 cell proliferation was reduced approximately 10-fold. Moderate decreases in potency are commonly observed for PEGylated proteins and often result from a decrease in the rate of association between the protein and its target receptor. Despite the decrease in potency, Δ1.21was nevertheless more potent than non-PEGylated W147A IL-11 in both assays.

EXAMPLE 3

In vivo Half Life

[0124] Female C57BL/6J mice (around 8 weeks old and 20 +/- 2g) were used to determine the *in vivo* half life of PEGylated mutein (Δ1.21) and non-PEGylated thrombin cleaved mutein 1.21 (i.e. amino acids 10-178 of SEQ ID NO:10). Each mouse received 1 IP injection. Mice were injected with the thrombin cleaved mutein 1.21 at a dose of 1mg/kg (20 ug per animal) or with an equivalent molar dose of Δ1.21 at a dose of 3.2 mg/kg (64 ug per animal). At an appropriate time following the IP injection, the mice were killed by CO_2 inhalation, followed by cervical dislocation, and blood collected by cardiac puncture. Sera was separated from the blood by incubation at 37°C for 1 hour and then overnight at 4°C before centrifugation to pellet the red blood cells. Blood was collected at 5 minute, 10 minutes, 30 minutes, 1 hour, 2 hours and 5 hours from mice injected with thrombin cleaved mutein 1.21 and at 10 minutes, 1 hour, 2 hours, 6 hours, 24 hours, 48 hours and 72 hours from mice injected with Δ1.21. A total of 4 mice were used for each time point.

[0125] The concentration of the thrombin cleaved mutein 1.21 was quantitated using a capture ELISA. Briefly, ELISA plates were coated with 2 μg/ml mIL-11Rα-Fc (50μl/well) [R&D systems] overnight at 4°C in PBS and the plates were then blocked with PBS containing 5% w/v skim milk (200μl/well) for 2 hours at room temperature. After washing the blocked plates with PBS containing 0.05% v/v Tween 20, serum samples were serially diluted in Tris buffered saline containing 1% w/v BSA and 0.05% v/v Tween 20 (TBS+BT) and added to the plates (100μl/well). The plates were incubated overnight at 4°C. Plates were washed with PBS containing 0.05% v/v Tween 20 after the overnight binding of the mutein in the serum to the mIL-11Rα-Fc coated on the plate, and then incubated with polyclonal biotinylated anti-mIL-11 (50μl/well) [R&D systems, cat#BAF418] at 0.3μg/ml in TBS+BT for 2 hours at room temperature. After washing in PBS containing 0.05% v/v Tween 20 the plate was incubated with streptavidin-HRP (Sigma) [50μl/well] diluted 1000-fold in TBS+BT and incubated for 1 hour at room temperature. After washing in PBS containing 0.05% v/v Tween 20 TMB substrate was added to each well (100μl/well) and after 10 minutes incubation the reaction was stopped

with 2M phosphoric acids (50μl/well) and the plate read at a wavelength of 450 nm using a microtitre plate reader. A standard curve was generated for both thrombin cleaved mutein 1.21 and Δ1.21 using known concentrations of proteins. These standard curves were then used to convert the ELISA data into nM values for thrombin cleaved mutein 1.21 and Δ1.21 contained in the serum.

[0126] PEGylation of the mutein clearly improved the half-life. The highest concentration of unPEGylated thrombin cleaved mutein 1.21 in the serum was seen at the earliest time point of 5 minutes after administration and was in continual decline. Only minimal amounts of unPEGylated thrombin cleaved mutein 1.21 remained in the serum 5 hours after administration and was estimated to have a half-life of less than 1 hour. By comparison the highest concentration of Δ1.21 in the serum was seen at 6 hours post administration and was still present in measurable concentrations 72 hours post injection. The half-life of the Δ1.21 was estimated to be approximately 24 hours.

EXAMPLE 4

PEGylated human IL-11 mutein

[0127] A PEGylated human IL-11 mutein was prepared based on SEQ ID NO:11. This was expressed purified and refolded as described and then site-specifically PEGylated at Cys147 (numbering based on SEQ ID NO:11) using the general approach described above for the murine IL-11 mutein. Human IL-11 does not contain an internal thrombin site so the first 9 amino acids of the human IL-11 sequence as well as a tag sequence from the vector were retained to provide the mutein of SEQ ID NO:22.

[0128] The PEGylated human IL-11 mutein of SEQ ID NO:22 had equivalent activity to the pegylated mouse IL-11 mutein Δ1.21 described above in both ELISA and Ba/F3 assays.

[0129] Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to

be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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TABLE 3

Cell assay data

Clone ID	Sequence	IC_{50} (nM)	IC_{50} (mutant)/ IC_{50}(wild-type*)
wild-type*		14	1.0
1.21	^{58}P-A-I-D-Y^{62}	0.54	26
1B.382	^{58}F-M-Q-I-Q^{62}	0.49	29

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CLAIMS:

1. An IL-11 mutein wherein the amino acid sequence AMSAG at position 58 to 62 of wild-type mammalian IL-11 is replaced with the amino acid sequence PAIDY or FMQIQ.

2. The IL-11 mutein of Claim 1 wherein the amino acid sequence AMSAG is replaced by PAIDY.

3. The IL-11 mutein of Claim 1 wherein the amino acid sequence AMSAG is replaced by FMQIQ.

4. The IL-11 mutein of Claim 1 or 2 or 3 comprising a further mutation which inhibits or reduces its binding to gp130.

5. The IL-11 mutein of Claim 4 wherein the further mutation is an amino acid substitution of the tryptophan (W) at amino acid position 147 of the wild-type IL-11.

6. The IL-11 mutein of Claim 5 wherein the tryptophan (W) is substituted by an alanine (A) or cysteine (C).

7. The IL-11 mutein of Claim 1 comprising an amino acid sequence selected from SEQ ID NO:4, amino acids 10 to 178 of SEQ ID NO:4, amino acids 10 to 175 of SEQ ID NO:4, SEQ ID NO:5, amino acids 10 to 178 of SEQ ID NO:5, amino acids 10 to 175 of SEQ ID NO:5, SEQ ID NO:6, amino acids 10 to 178 of SEQ ID NO:6, amino acids 10 to 175 of SEQ ID NO:6, SEQ ID NO:7, amino acids 10 to 178 of SEQ ID NO:7, amino acids 10 to 175 of SEQ ID NO:7, SEQ ID NO:8, amino acids 10 to 178 of SEQ ID NO:8, amino acids 10 to 175 of SEQ ID NO:8, SEQ ID NO:13, amino acids 10 to 178 of SEQ ID NO:13 and amino acids 10 to 175 of SEQ ID NO:13.

8. The IL-11 mutein of Claim 1 comprising an amino acid sequence selected from SEQ ID NO:9, amino acids 10 to 178 of SEQ ID NO:9, amino acids 10 to 175 of SEQ ID

NO:9, SEQ ID NO:10, amino acids 10 to 178 of SEQ ID NO:10, amino acids 10 to 175 of SEQ ID NO:10, SEQ ID NO:11, amino acids 10 to 178 of SEQ ID NO:11, amino acids 10 to 175 of SEQ ID NO:11, SEQ ID NO:12, amino acids 10 to 178 of SEQ ID NO:12, amino acids 10 to 175 of SEQ ID NO:12, SEQ ID NO:14, amino acids 10 to 178 of SEQ ID NO:14, amino acids 10 to 175 of SEQ ID NO:14, SEQ ID NO:15, amino acids 10 to 178 of SEQ ID NO:15, amino acids 10 to 175 of SEQ ID NO:15, SEQ ID NO:16, amino acids 10 to 178 of SEQ ID NO:16, amino acids 10 to 175 of SEQ ID NO:16, SEQ ID NO:17, amino acids 10 to 178 of SEQ ID NO:17, amino acids 10 to 175 of SEQ ID NO:17, SEQ ID NO:18, amino acids 10 to 178 of SEQ ID NO:18, amino acids 10 to 175 of SEQ ID NO:18, SEQ ID NO:19, amino acids 10 to 178 of SEQ ID NO:19, amino acids 10 to 175 of SEQ ID NO:19, SEQ ID NO:20, amino acids 10 to 178 of SEQ ID NO:20, amino acids 10 to 175 of SEQ ID NO:20, SEQ ID NO:21, amino acids 10 to 178 of SEQ ID NO:21 and amino acids 10 to 175 of SEQ ID NO:21.

9. The IL-11 mutein of Claim 1 or 7 or 8 wherein said IL-11 mutein is PEGylated.

10. A pharmaceutical composition comprising the IL-11 mutein of any one of Claims 1 to 9 and one or more pharmaceutical acceptable carriers, diluents and/or excipients.

11. A method for the treatment of an IL-11-mediated condition, said method comprising administering to said subject an effective amount of an IL-11 mutein as claimed in any one of Claims 1 to 9.

12. Use of an IL-11 mutein of any one of Claims 1 to 9 in the manufacture of a medicament in the treatment of an IL-11-mediated condition.

Human: Mature IL-11 Sequence

(SEQ ID NO:1)

PGPPPGPPRV.SPDPRAELDS.TVLLTRSLLA.DTRQLAAQLR.DKFPADGDHN.LDSLPTLAMS
AGALGALQLP GVLTRLRADL LSYLRHVQWL RRAGGSSLKT LEPELGTLQA RLDRLLRRLQ
LLMSRLALPQ PPPDPPAPPL APPSSAWGGI RAAHAILGGL HLTLDWAVRG LLLLKTRL

Murine: Mature IL-11 Sequence

(SEQ ID NO:2)

PGPPAGSPRV SSDPRADLDS AVLLTRSLLA DTRQLAAQMR DKFPADGDHS LDSLPTLAMS
AGTLGSLQLP GVLTRLRVDL MSYLRHVQWL RRAGGPSLKT LEPELGALQA RLERLLRRLQ
LLMSRLALPQ AAPDQPVIPL GPPASAWGSI RAAHAILGGL HLTLDWAVRG LLLLKTRL

Macaque: Mature IL-11 Sequence

(SEQ ID NO:3)

PGPPPGSPRA SPDPRAELDS TVLLTRSLLE DTRQLTIQLK DKFPADGDHN LDSLPTLAMS
AGALGALQLP SVLTRLRADL LSYLRHVQWL RRAMGSSLKT LEPELGTLQT RLDRLLRRLQ
LLMSRLALPQ LPPDPPAPPL APPSSTWGGI RAAHAILGGL HLTLDWAVRG LLLLKTRL

Figure 1a

Macaque: PAIDY IL-11 Sequence

(SEQ ID NO:4)

PGPPPGSPRA SPDPRAELDS TVLLTRSLLE DTRQLTIQLK DKFPADGDHN LDSLPTLPAI
DYALGALQLP SVLTRLRADL LSYLRHVQWL RRAMGSSLKT LEPELGTLQT RLDRLLRRLQ
LLMSRLALPQ LPPDPPAPPL APPSSTWGGI RAAHAILGGL HLTLDWAVRG LLLLKTRL

Human: PAIDY IL-11 Mutein

(SEQ ID NO:5)

PGPPPGPPRV.SPDPRAELDS.TVLLTRSLLA.DTRQLAAQLR.DKFPADGDHN.LDSLPTLPAI
DYALGALQLP GVLTRLRADL LSYLRHVQWL RRAGGSSLKT LEPELGTLQA RLDRLLRRLQ
LLMSRLALPQ PPPDPPAPPL APPSSAWGGI RAAHAILGGL HLTLDWAVRG LLLLKTRL

Murine: PAIDY IL-11 Mutein

(SEQ ID NO:6)

PGPPAGSPRV SSDPRADLDS AVLLTRSLLA DTRQLAAQMR DKFPADGDHS LDSLPTLPAI
DYTLGSLQLP GVLTRLRVDL MSYLRHVQWL RRAGGPSLKT LEPELGALQA RLERLLRRLQ
LLMSRLALPQ AAPDQPVIPL GPPASAWGSI RAAHAILGGL HLTLDWAVRG LLLLKTRL

Figure 1b

Human: FMQIQ IL-11 Mutein

(SEQ ID NO:7)

PGPPPGPPRV.SPDPRAELDS.TVLLTRSLLA.DTRQLAAQLR.DKFPADGDHN.LDSLPTLFMQ
IQALGALQLP GVLTRLRADL LSYLRHVQWL RRAGGSSLKT LEPELGTLQA RLDRLLRRLQ
LLMSRLALPQ PPPDPPAPPL APPSSAWGGI RAAHAILGGL HLTLDWAVRG LLLLKTRL

Murine: FMQIQ IL-11 Mutein

(SEQ ID NO:8)

PGPPAGSPRV SSDPRADLDS AVLLTRSLLA DTRQLAAQMR DKFPADGDHS LDSLPTLFMQ
IQTLGSLQLP GVLTRLRVDL MSYLRHVQWL RRAGGPSLKT LEPELGALQA RLERLLRRLQ
LLMSRLALPQ AAPDQPVIPL GPPASAWGSI RAAHAILGGL HLTLDWAVRG LLLLKTRL

Macaque: FMQIQ IL-11 Sequence

(SEQ ID NO:13)

PGPPPGSPRA SPDPRAELDS TVLLTRSLLE DTRQLTIQLK DKFPADGDHN LDSLPTLFMQ
IQALGALQLP SVLTRLRADL LSYLRHVQWL RRAMGSSLKT LEPELGTLQT RLDRLLRRLQ
LLMSRLALPQ LPPDPPAPPL APPSSTWGGI RAAHAILGGL HLTLDWAVRG LLLLKTRL

Figure 1c

Human: PAIDY & W147A IL-11 Mutein

(SEQ ID NO:9)

PGPPPGPPRV.SPDPRAELDS.TVLLTRSLLA.DTRQLAAQLR.DKFPADGDHN.LDSLPTLPAI
DYALGALQLP GVLTRLRADL LSYLRHVQWL RRAGGSSLKT LEPELGTLQA RLDRLLRRLQ
LLMSRLALPQ PPPDPPAPPL APPSSAAGGI RAAHAILGGL HLTLDWAVRG LLLLKTRL

Murine: PAIDY & W147A IL-11 Mutein

(SEQ ID NO:10)

PGPPAGSPRV SSDPRADLDS AVLLTRSLLA DTRQLAAQMR DKFPADGDHS LDSLPTLPAI
DYTLGSLQLP GVLTRLRVDL MSYLRHVQWL RRAGGPSLKT LEPELGALQA RLERLLRRLQ
LLMSRLALPQ AAPDQPVIPL GPPASAAGSI RAAHAILGGL HLTLDWAVRG LLLLKTRL

Macaque: PAIDY & W147A IL-11 Sequence

(SEQ ID NO:14)

PGPPPGSPRA SPDPRAELDS TVLLTRSLLE DTRQLTIQLK DKFPADGDHN LDSLPTLPAI
DYALGALQLP SVLTRLRADL LSYLRHVQWL RRAMGSSLKT LEPELGTLQT RLDRLLRRLQ
LLMSRLALPQ LPPDPPAPPL APPSSTAGGI RAAHAILGGL HLTLDWAVRG LLLLKTRL

Figure 1d

Human: PAIDY & W147C IL-11 Mutein

(SEQ ID NO:11)

PGPPPGPPRV.SPDPRAELDS.TVLLTRSLLA.DTRQLAAQLR.DKFPADGDHN.LDSLPTLPAI
DYALGALQLP GVLTRLRADL LSYLRHVQWL RRAGGSSLKT LEPELGTLQA RLDRLLRRLQ
LLMSRLALPQ PPPDPPAPPL APPSSACGGI RAAHAILGGL HLTLDWAVRG LLLLKTRL

Murine: PAIDY & W147C IL-11 Mutein

(SEQ ID NO:12)

PGPPAGSPRV SSDPRADLDS AVLLTRSLLA DTRQLAAQMR DKFPADGDHS LDSLPTLPAI
DYTLGSLQLP GVLTRLRVDL MSYLRHVQWL RRAGGPSLKT LEPELGALQA RLERLLRRLQ
LLMSRLALPQ AAPDQPVIPL GPPASACGSI RAAHAILGGL HLTLDWAVRG LLLLKTRL

Macaque: PAIDY & W147C IL-11 Sequence

(SEQ ID NO:15)

PGPPPGSPRA SPDPRAELDS TVLLTRSLLE DTRQLTIQLK DKFPADGDHN LDSLPTLPAI
DYALGALQLP SVLTRLRADL LSYLRHVQWL RRAMGSSLKT LEPELGTLQT RLDRLLRRLQ
LLMSRLALPQ LPPDPPAPPL APPSSTCGGI RAAHAILGGL HLTLDWAVRG LLLLKTRL

Figure 1e

Human: FMQIQ & W147A IL-11 Mutein

(SEQ ID NO:16)

PGPPPGPPRV.SPDPRAELDS.TVLLTRSLLA.DTRQLAAQLR.DKFPADGDHN.LDSLPTLFMQ
IQALGALQLP GVLTRLRADL LSYLRHVQWL RRAGGSSLKT LEPELGTLQA RLDRLLRRLQ
LLMSRLALPQ PPPDPPAPPL APPSSAAGGI RAAHAILGGL HLTLDWAVRG LLLLKTRL

Murine: FMQIQ & W147A IL-11 Mutein

(SEQ ID NO:17)

PGPPAGSPRV SSDPRADLDS AVLLTRSLLA DTRQLAAQMR DKFPADGDHS LDSLPTLFMQ
IQTLGSLQLP GVLTRLRVDL MSYLRHVQWL RRAGGPSLKT LEPELGALQA RLERLLRRLQ
LLMSRLALPQ AAPDQPVIPL GPPASAAGSI RAAHAILGGL HLTLDWAVRG LLLLKTRL

Macaque: FMQIQ & W147A IL-11 Sequence

(SEQ ID NO:18)

PGPPPGSPRA SPDPRAELDS TVLLTRSLLE DTRQLTIQLK DKFPADGDHN LDSLPTLFMQ
IQALGALQLP SVLTRLRADL LSYLRHVQWL RRAMGSSLKT LEPELGTLQT RLDRLLRRLQ
LLMSRLALPQ LPPDPPAPPL APPSSTAGGI RAAHAILGGL HLTLDWAVRG LLLLKTRL

Figure 1f

Human: FMQIQ & W147C IL-11 Mutein

(SEQ ID NO:19)

PGPPPGPPRV.SPDPRAELDS.TVLLTRSLLA.DTRQLAAQLR.DKFPADGDHN.LDSLPTLFMQ
IQALGALQLP GVLTRLRADL LSYLRHVQWL RRAGGSSLKT LEPELGTLQA RLDRLLRRLQ
LLMSRLALPQ PPPDPPAPPL APPSSACGGI RAAHAILGGL HLTLDWAVRG LLLLKTRL

Murine: FMQIQ & W147C IL-11 Mutein

(SEQ ID NO:20)

PGPPAGSPRV SSDPRADLDS AVLLTRSLLA DTRQLAAQMR DKFPADGDHS LDSLPTLFMQ
IQTLGSLQLP GVLTRLRVDL MSYLRHVQWL RRAGGPSLKT LEPELGALQA RLERLLRRLQ
LLMSRLALPQ AAPDQPVIPL GPPASACGSI RAAHAILGGL HLTLDWAVRG LLLLKTRL

Macaque: FMQIQ & W147C IL-11 Sequence

(SEQ ID NO:21)

PGPPPGSPRA SPDPRAELDS TVLLTRSLLE DTRQLTIQLK DKFPADGDHN LDSLPTLFMQ
IQALGALQLP SVLTRLRADL LSYLRHVQWL RRAMGSSLKT LEPELGTLQT RLDRLLRRLQ
LLMSRLALPQ LPPDPPAPPL APPSSTCGGI RAAHAILGGL HLTLDWAVRG LLLLKTRL

Human: N-terminally tagged PAIDY & W147C IL-11 Sequence

(SEQ ID NO:22)

MGSHHHHHHG ARQPGPPPGP PRVSPDPRAE LDSTVLLTRS LLADTRQLAA QLRDKFPADG
DHNLDSLPTL PAIDYALGAL QLPGVLTRLR ADLLSYLRHV QWLRRAGGSS LKTLEPELGT
LQARLDRLLR RLQLLMSRLA LPQPPPDPPA PPLAPPSSAC GGIRAAHAIL GGLHLTLDWA
VRGLLLLKTR L

Figure 1g

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2008/001587

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

C07K 14/54 (2006.01) ***A61P 15/16*** (2006.01) ***A61P 19/08*** (2006.01)
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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
GenomeQuest: Sequence search based on SEQ ID NO: 4-8 and 13
CA, Medline & WPIDS & keywords: Interleukin-11, antagonist, binding and like terms

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CZUPRYN, M.J., et al. Structure-Function Relationships in Human Interleukin-11. Journal of Biological Chemistry. 1995. vol 270, pages 978-985. See whole document, especially page 979- page 980 section 'Modification of Methionine Residues'.	
A	UNDERHILL-DAY, N., et al. Functional Characterisation of W147A: A High-Affinity Interleukin-11 Antagonist. Endocrinology. 2003. vol. 144, pages 3406-3414. See whole document especially figure 5, page 3413, column 1, lines 27-34.	

☐ Further documents are listed in the continuation of Box C ☐ See patent family annex

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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"P" document published prior to the international filing date but later than the priority date claimed
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"&" document member of the same patent family

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27 November 2008	7 - JAN 2009
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(54) Title: IL-11R BINDING PROTEINS AND USES THEREOF

(57) Abstract: The present disclosure provides proteins comprising antigen binding sites of antibodies that bind to interleukin-11 (IL-11) receptor alpha (IL-11Rα) and uses thereof, e.g., in therapy.

"IL-11R BINDING PROTEINS AND USES THEREOF"

RELATED APPLICATION DATA

The present application claims priority from Australian Patent Application No. 2013900389 entitled "IL-11R binding proteins and uses thereof" filed on 7 February 2013 and from US Patent Application No. 61/764,756 entitled "IL-11R binding proteins and uses thereof" filed on 14 February 2013. The entire contents of those applications are hereby incorporated by reference.

SEQUENCE LISTING

The present application is accompanied by a Sequence Listing filed in electronic format. The entire contents of that Sequence Listing are hereby incorporated by reference.

FIELD

The present disclosure relates to proteins comprising antigen binding sites of antibodies that bind to interleukin-11 (IL-11) receptor alpha (IL-11Rα) and uses thereof, e.g., in therapy.

BACKGROUND

IL-11 is a member of the IL-6 cytokine family which also comprises IL-27, IL-31, leukemia inhibitory factor (LIF), oncostatin M (OSM) and ciliary neurotrophic factor (CNTF) amongst others. IL-6 family cytokines induce signal transduction via a common signal-transducing receptor β-subunit, gp130 and a specific receptor α-subunit. In the case of IL-11, binding of this cytokine to its specific receptor α-subunit, IL-11Rα, induces gp130 homodimerization. Dimerization of gp130 activates the JAK/STAT signaling pathway and leads to the activation of signal transducer and activator of transcription (STAT) 3 (STAT3) and to a lesser extent, STAT1.

IL-11 signaling is known to play a role in hematopoiesis, immune response, inflammation, adipogenesis, osteoclastogenesis, neurogenesis, megakaryocyte maturation and platelet production. IL-11 is used clinically or is in development for treating a variety of conditions, e.g., chemotherapy-induced thrombocytopenia, and various inflammatory disorders including arthritis, inflammatory bowel disease, radiation-induced lung damage, sepsis and psoriasis. However, clinical use of IL-11 has been restricted due to reports of serious adverse events including edema. Moreover, IL-11 has been shown to have deleterious effects in various conditions.

For example, IL-11 has been found to act as an inhibitor of bone formation, and is critical for osteoclast formation and activity and bone resorption. Thus, blocking the activity of IL-11 has been proposed as a treatment for osteoporosis and for preventing bone resorption/promoting bone formation in other conditions such as metastatic bone cancer, myeloma, Paget's disease of bone, and bone fracture and healing.

IL-11 signaling has been implicated as having a pathogenic role during the early phase of tuberculosis. Blocking IL-11 with an anti-IL-11 antibody was shown to diminish histopathology and neutrophilic infiltration of the lung tissue in mice infected with *Mycobacterium tuberculosis*.

Antagonism of IL-11 has also been proposed as a method of treating Th2-mediated disorders including asthma, chronic obstructive pulmonary disease (COPD), rhinitis, allergies and atopic dermatitis. In this regard, blocking IL-11 signaling using a mutant form of IL-11 that does not induce signal transduction was shown to be of therapeutic benefit in a mouse model of asthma.

IL-11 and/or IL-11Rα is overexpressed in liver cancer, pancreatic cancer, gastric cancer, osteosarcoma, endometrial cancer and ovarian cancer. Moreover, as discussed above, IL-11 induced gp130 dimerization leads to activation of STAT3, which induces expression of genes associated with angiogenesis (e.g. VEGF), cell cycle progression (e.g. cyclin D1) and cell survival (e.g. Bcl-XL, survival). Persistent STAT3 activity appears to be associated with hematologic malignancies and tumors of epithelial origin. Excessive STAT3 activation promotes the growth and survival of gastric cells, is associated with increased gastric angiogenesis and leads to gastric tumorigenesis in mice. However, gastric inflammation, hyperplasia and tumor formation are suppressed in IL-11 unresponsive mice or in mice treated with a non-signaling mutant of IL-11.

IL-11 is also involved in other biological processes, such as, inhibition of adipogenesis, induction of cachexia (e.g., cancer cachexia), induction of a febrile response, modulation of extracellular matrix metabolism, stimulation of acute-phase reactants and embryo implantation.

It will be apparent to the skilled artisan from the foregoing that reagents that neutralize IL-11 signaling are desirable for their potential to provide a therapeutic benefit in any of a number of diverse conditions. Reagents that bind to the IL-11Rα are also desirable since they have the advantage of being capable of specifically targeting cells *in vivo* as opposed to needing to bind to and neutralize soluble IL-11 throughout a subject.

Despite this desirability, many reagents (e.g., antibodies) that bind to IL-11Rα do not neutralize IL-11 signaling. For example, Blanc *et al* (*Journal of Immunological*

Methods 241: 43-59, 2000) described a panel of 14 mouse monoclonal antibodies raised against human IL-11Rα but none of them were capable of inhibiting IL-11-induced proliferation of BaF3/gp130/IL-11R cells, indicating that the antibodies do not neutralize IL-11 signaling. Commercially available anti-IL-11Rα antibodies, e.g., 4D12 available from Santa Cruz Biotechnology, Inc., also do not neutralize IL-11 signaling.

SUMMARY

In producing the present invention, the inventors sought to produce reagents (e.g., antibodies and proteins comprising antigen binding domains thereof) that bind to IL-11Rα and neutralize IL-11 signaling. The inventors produced a series of antibodies having such activity, some of which potently neutralize IL-11 signaling, e.g., prevent proliferation of IL-11-dependent BaF3 cell proliferation. These antibodies were shown to be cross-reactive with human IL-11Rα (hIL-11Rα) and cynomolgus monkey IL-11Rα (cynoIL-11Rα), meaning that they may be used in primate models of human disease. The antibodies were also found to bind to overlapping epitopes. The inventors then affinity matured one of these antibodies and produced a series of additional antibodies having additional desirable properties, e.g., neutralization of IL-11 signaling and/or improved affinity and/or sequences similar to human germline (e.g., having a reduced likelihood of inducing an immune response when administered to a human).

Based on the foregoing, it will be apparent to the skilled artisan that the inventors have produced a protein comprising an antigen binding domain of an antibody, the antigen binding domain capable of binding to or specifically binding to IL-11Rα and neutralizing IL-11 signaling.

In one example, the present disclosure provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling, wherein the antigen binding domain is capable of binding to hIL-11Rα and cynoIL-11Rα.

In one example, the IL-11Rα-binding protein neutralizes human IL-11 (hIL-11) and/or cynomolgus monkey IL-11 (cynoIL-11) signaling.

The present disclosure additionally or alternatively provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and the protein inhibits IL-11 (e.g., hIL-11 or cynoIL-11)-mediated proliferation of BaF3 cells expressing IL-11Rα and gp130 with an IC_{50} of 10μg/ml or less. In one example, the IC_{50} is 5μg/ml or less. For example, the IC_{50} is 4μg/ml or

less or 3.5μg/ml or less. In one example, the IC_{50} is 3μg/ml or less or 2μg/ml or less. For example, the IC_{50} is 1μg/ml or less. For example, the IC_{50} is 0.9μg/ml or less or 0.8μg/ml or less or 0.7μg/ml. In one example, the IC_{50} is 0.7μg/ml or less. In one example, relating to each of the foregoing examples, the IC_{50} can be 10pg/ml or more or 10ng/ml or more.

In one example, the IC_{50} is 10nM or less. For example, the IC_{50} is 8nM or less or 7nM or less. In one example, the IC_{50} is 6nM or less or 6.5nM or less. For example, the IC_{50} is 5nM or less. For example, the IC_{50} is 4.5nM or less. For example, the IC_{50} is 4nM or less. In one example, relating to each of the foregoing examples, the IC_{50} can be 10pM or more.

In one example, the IL-11Rα-binding protein inhibits IL-11 (e.g., hIL-11 or cynoIL-11)-mediated proliferation of BaF3 cells expressing IL-11Rα and gp130 with an IC_{50} at least about 1.5 fold greater than antibody 8E2 (comprising a heavy chain comprising a sequence set forth in SEQ ID NO: 83 and a light chain comprising a sequence set forth in SEQ ID NO: 84). In one example, the IC_{50} is at least about 2 fold greater or at least about 2.5 fold greater or at least about 3 fold greater than antibody 8E2.

In one example, the IL-11Rα-binding protein inhibits IL-11 (e.g., hIL-11)-mediated proliferation of BaF3 cells expressing IL-11Rα and gp130 with an IC_{50} at least about 1.5 fold greater (i.e., the IC50 value is about 1.5 fold less than) or 2 fold greater or 3 fold greater than an antagonistic hIL-11 mutein (e.g., comprising a sequence set forth in SEQ ID NO: 110), wherein the IC_{50} is measured in nM. In one example, the IC_{50} is at least about 3.5 fold greater or at least about 4 fold greater than the mutein.

In one example, the IC_{50} is determined by culturing BaF3 cells expressing IL-11Rα and gp130 (e.g., genetically modified to express IL-11Rα and/or gp130) (e.g., about $1x10^4$ cells) in the presence of from about 0.3ng/mL hIL-11 to about 5ng/mL hIL-11 (e.g., in the presence of about 0.3ng/mL hIL-11 or about 0.5ng/mL hIL-11 or about 5ng/mL hIL-11) for about 48-50 hours, or about 48 hours or about 50 hours. In one example, the cells are cultured in the presence of a protein of the disclosure (e.g., for about 30 minutes) prior to addition of the IL-11. In one example, proliferation is determined by measuring incorporation of 3H-thymidine into DNA during the last 6 hours of culture. In assays performed to determine neutralization of cynoIL-11, the cells can be cultured in the presence of from about 0.5ng/mL cynoIL-11 to about 5ng/mL cynoIL-11 (e.g., in the presence of about 0.5ng/mL cynoIL-11 or about 5ng/mL cynoIL-11).

The present disclosure additionally or alternatively provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and the level of binding of the IL-11Rα-binding protein to a polypeptide of SEQ ID NO: 86 is lower than the level of binding of the IL-11Rα-binding to a polypeptide of SEQ ID NO: 3 and/or 85.

The present disclosure additionally or alternatively provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and the level of binding of the IL-11Rα-binding protein to a polypeptide of SEQ ID NO: 89 is lower than the level of binding of the IL-11Rα-binding to a polypeptide of SEQ ID NO: 3 and/or 85.

In one example, the level of binding is determined by Western Blotting and/or by fluorescence-activated cell sorting (FACS) of cells expressing the polypeptide.

In one example, the level of binding of the IL-11Rα-binding protein to the polypeptide of SEQ ID NO: 86 or 89 is reduced by at least about 10 fold or 20 fold or 50 fold or 100 fold or 150 fold or 200 fold compared to the binding of the IL-11Rα-binding protein to the polypeptide of SEQ ID NO: 3 and/or 85.

In one example, the IL-11Rα-binding protein does not detectably bind to the polypeptide of SEQ ID NO: 86 or 89.

In one example, the IL-11Rα-binding protein binds to a polypeptide of SEQ ID NO: 87 or 88. For example, the level of binding of the IL-11Rα-binding protein binds to a polypeptide of SEQ ID NO: 87 or 88 is similar to or about the same as (e.g., within about 20% or 15% or 10% or 5%) of the level of binding to the polypeptide of SEQ ID NO: 3 and/or 85.

The present disclosure additionally or alternatively provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and the antigen binding domain binds to an epitope comprising residues within the first fibronectin III domain of IL-11Rα.

In one example, the epitope comprises residues within the immunoglobulin-like domain and the first fibronectin III domain of IL-11Rα

In one example, the epitope comprises residues between amino acids 111-215 of SEQ ID NO: 1.

In one example, the epitope comprises residues between amino acids 1-215 of SEQ ID NO: 1.

The present disclosure additionally or alternatively provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and wherein the protein competitively inhibits binding of antibody 8E2 (comprising a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 5) to hIL-11Rα and/or to a polypeptide of SEQ ID NO: 3 and/or 85.

The present disclosure additionally or alternatively provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and wherein the protein competitively inhibits binding of antibody 8E4 (comprising a V_H comprising a sequence set forth in SEQ ID NO: 74 and a V_L comprising a sequence set forth in SEQ ID NO: 73) to hIL-11Rα and/or to a polypeptide of SEQ ID NO: 3 and/or 85.

The present disclosure additionally or alternatively provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and wherein the protein competitively inhibits binding of antibody 8D10 (comprising a V_H comprising a sequence set forth in SEQ ID NO: 76 and a V_L comprising a sequence set forth in SEQ ID NO: 75) to hIL-11Rα and/or to a polypeptide of SEQ ID NO: 3 and/or 85.

The present disclosure additionally or alternatively provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and wherein the protein competitively inhibits binding of antibody 8E2 (comprising a heavy chain comprising a V_H comprising a sequence set forth in SEQ ID NO: 37 and a human IgG4 constant region and a light chain comprising a V_L comprising a sequence set forth in SEQ ID NO: 5 and a human light chain constant region) to hIL-11Rα and/or to a polypeptide of SEQ ID NO: 3 and/or 85.

The present disclosure additionally or alternatively provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and wherein the protein competitively inhibits binding of antibody 8E4 (comprising a heavy chain comprising a V_H comprising a sequence set forth in SEQ ID NO: 74 and a human IgG4 constant region and a light chain comprising a V_L

comprising a sequence set forth in SEQ ID NO: 73 and a human light chain constant region) to hIL-11Rα and/or to a polypeptide of SEQ ID NO: 3 and/or 85.

The present disclosure additionally or alternatively provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and wherein the protein competitively inhibits binding of antibody 8D10 (comprising a heavy chain comprising a V_H comprising a sequence set forth in SEQ ID NO: 76 and a human IgG4 constant region and a light chain comprising a V_L comprising a sequence set forth in SEQ ID NO: 75 and a human light chain constant region) to hIL-11Rα and/or to a polypeptide of SEQ ID NO: 3 and/or 85.

The present disclosure additionally or alternatively provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and wherein the protein competitively inhibits binding of antibody 8E2 (comprising a heavy chain comprising a sequence set forth in SEQ ID NO: 83 and a light chain comprising a sequence set forth in SEQ ID NO: 84) to hIL-11Rα and/or to a polypeptide of SEQ ID NO: 3 and/or 85.

The present disclosure additionally or alternatively provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and wherein the protein competitively inhibits binding of antibody 8E4 (comprising a heavy chain comprising a sequence set forth in SEQ ID NO: 92 and a light chain comprising a sequence set forth in SEQ ID NO: 91) to hIL-11Rα and/or to a polypeptide of SEQ ID NO: 3 and/or 85.

The present disclosure additionally or alternatively provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and wherein the protein competitively inhibits binding of antibody 8D10 ((comprising a heavy chain comprising a sequence set forth in SEQ ID NO: 94 and a light chain comprising a sequence set forth in SEQ ID NO: 93) to hIL-11Rα and/or to a polypeptide of SEQ ID NO: 3 and/or 85.

The present disclosure additionally or alternatively provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and wherein the level of binding of the IL-11Rα-binding protein to a

polypeptide of SEQ ID NO: 95 is lower than the level of binding of the IL-11Rα-binding protein to a polypeptide of SEQ ID NO: 85.

The present disclosure additionally or alternatively provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and wherein the level of binding of the IL-11Rα-binding protein to a polypeptide of SEQ ID NO: 96 is lower than the level of binding of the IL-11Rα-binding protein to a polypeptide of SEQ ID NO: 85.

In one example, the level of binding of the IL-11Rα-binding protein to the polypeptide comprising the substitution is reduced by at least about 1.5 fold or 2 fold.

In one example, the level of binding of the IL-11Rα-binding protein to the polypeptide comprising the substitution is reduced by at least about 3 fold or 4 fold or 5 fold or 10 fold.

In one example, the IL-11Rα-binding protein does not detectably bind to the polypeptide comprising the substitution.

In one example, the level of binding is assessed using a biosensor, e.g., by surface plasmon resonance. For example, the IL-11Rα-binding protein is immobilized and the level of binding to a polypeptide of SEQ ID NO: 85, 95 or 96 is determined. As exemplified herein, by assessing the level of binding at several concentrations an affinity can be determined.

In another example, the level of binding is assessed using FACS. For example, binding of the IL-11Rα-binding protein to a cell expressing a polypeptide of SEQ ID NO: 85, 95 or 96 or to a form of the IL-11Rα comprising a substitution described herein.

In one example, the IL-11Rα-binding protein preferentially binds to a polypeptide of SEQ ID NO: 85 relative to its ability to bind to a polypeptide of SEQ ID NO: 95 or 96.

In one example, the IL-11Rα-binding protein preferentially binds to a polypeptide of SEQ ID NO: 85 relative to its ability to bind to a polypeptide of SEQ ID NO: 95.

In one example, the IL-11Rα-binding protein competitively inhibits binding of antibody 8E2 (comprising a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 5) and/or 8E4 (comprising a V_H comprising a sequence set forth in SEQ ID NO: 74 and a V_L comprising a sequence set forth in SEQ ID NO: 73) and/or 8D10 (comprising a V_H comprising a sequence set

forth in SEQ ID NO: 76 and a V_L comprising a sequence set forth in SEQ ID NO: 75) to a polypeptide of SEQ ID NO: 3 and/or 85.

In one example, the antigen binding domain cross-reacts with:

(i) a polypeptide of SEQ ID NO: 97; and/or

(ii) a polypeptide of SEQ ID NO: 98; and/or

(iii) a polypeptide of SEQ ID NO: 99.

In one example, the IL-11Rα-binding protein does not detectably bind to mouse IL-11Rα (SEQ ID NO: 82 or 102) and/or to a polypeptide of SEQ ID NO: 90.

In one example, the IL-11Rα-binding protein cross-reacts with mouse IL-11Rα (SEQ ID NO: 82 or 102) and/or to a polypeptide of SEQ ID NO: 90.

In one example, the IL-11Rα-binding protein has an affinity constant (K_D) for human IL-11Rα and/or the polypeptide of SEQ ID NO: 3 and/or 85 of about $9x10^{-9}M$ or less. For example, the K_D is about $8x10^{-9}M$ or less or about $7x10^{-9}M$ or less or about $6x10^{-9}M$ or less or about $5x10^{-9}M$ or less. In one example, the K_D is about $4.8x10^{-9}M$ or less.

In another example, the IL-11Rα-binding protein has an affinity constant (K_D) for human IL-11Rα and/or the polypeptide of SEQ ID NO: 3 and/or 85 of about $2x10^{-9}M$ or less. For example, the K_D is about $1x10^{-9}M$ or less or about $9x10^{-10}M$ or less or about $8x10^{-10}M$ or less or about $7x10^{-10}M$ or less. In one example, the K_D is about $5x10^{-10}M$ or less. In one example, the K_D is about $4x10^{-10}M$ or less. In one example, the K_D is about $3x10^{-10}M$ or less. In one example, the K_D is about $2x10^{-10}M$ or less. In one example, the K_D is about $1.5x10^{-10}M$ or less.

In one example, relating to each of the foregoing examples, the K_D can be $0.1x10^{-12}M$ or more or $1x10-^{12}M$ or more.

In one example, the K_D is assessed using a biosensor, e.g., by surface plasmon resonance. For example, the IL-11Rα-binding protein is immobilized and the level of binding to a polypeptide of SEQ ID NO: 85 is determined.

In one example, the IL-11Rα-binding protein has a thermal transition midpoint (Tm) of about 60°C or greater. For example, the Tm is about 61°C or greater, for example, about 62°C or greater, such as, about 63°C or greater. For example, the Tm is about 65°C or greater. For example, the Tm is about 69°C or greater. For example, the Tm is about 70°C or greater. Proteins having higher Tms are considered to be more stable, thereby providing for better storage and/or reduced effects upon administration, e.g., due to aggregation.

The present disclosure additionally or alternatively provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen

binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and wherein the antigen binding domain comprises at least one of:

(i) a V_H comprising a complementarity determining region (CDR) 1 comprising a sequence at least about 40% identical (or 50% identical or 60% identical or 70% identical or 80% identical or 90% identical or 95% identical or 98% identical or 99% identical or 100% identical) to a sequence set forth between amino acids 31-35 of SEQ ID NO: 37, a CDR2 comprising a sequence at least about 76% identical (or 80% identical or 90% identical or 95% identical or 98% identical or 99% identical or 100% identical) to a sequence set forth between amino acids 50-66 of SEQ ID NO: 37 and a CDR3 comprising a sequence at least about 55% identical (or 60% identical or 70% identical or 80% identical or 90% identical or 95% identical or 98% identical or 99% identical or 100% identical) to a sequence set forth between amino acids 99-107 of SEQ ID NO: 37;

(ii) a V_H comprising a sequence at least about 95% or 96% or 97% or 98% or 99% identical to a sequence set forth in SEQ ID NO: 37;

(iii) a V_L comprising a CDR1 comprising a sequence at least about 45% identical (or 50% identical or 55% identical or 60% identical or 70% identical or 80% identical or 90% identical or 95% identical or 98% identical or 99% identical or 100% identical) to a sequence set forth between amino acids 24-34 of SEQ ID NO: 5, a CDR2 comprising a sequence set forth between amino acids 50-56 of SEQ ID NO: 5 and a CDR3 comprising a sequence at least about 44% identical (or 56% identical or 60% identical or 70% identical or 80% identical or 90% identical or 95% identical or 98% identical or 99% identical or 100% identical) to a sequence set forth between amino acids 89-97 of SEQ ID NO: 5;

(iv) a V_L comprising a sequence at least about 94% identical (or 95% identical or 96% identical or 97% identical or 98% identical or 99% identical) to a sequence set forth in SEQ ID NO: 5;

(v) a V_H comprising a CDR1 comprising a sequence set forth between amino acids 31-35 of SEQ ID NO: 74, a CDR2 comprising a sequence set forth between amino acids 50-66 of SEQ ID NO: 74 and a CDR3 comprising a sequence set forth between amino acids 99-115 of SEQ ID NO: 74;

(vi) a V_H comprising a sequence set forth in SEQ ID NO: 74;

(vii) a V_L comprising a CDR1 comprising a sequence set forth between amino acids 23-36 of SEQ ID NO: 73, a CDR2 comprising a sequence set forth between amino acids 52-58 of SEQ ID NO: 73 and a CDR3 comprising a sequence set forth between amino acids 91-101 of SEQ ID NO: 73;

(viii) a V_L comprising a sequence set forth in SEQ ID NO: 73;

(ix) a V_H comprising a CDR1 comprising a sequence set forth between amino acids 31-35 of SEQ ID NO: 76, a CDR2 comprising a sequence set forth between amino acids 50-66 of SEQ ID NO: 76 and a CDR3 comprising a sequence set forth between amino acids 99-107 of SEQ ID NO: 76;

(x) a V_H comprising a sequence set forth in SEQ ID NO: 76;

(xi) a V_L comprising a CDR1 comprising a sequence set forth between amino acids 24-34 of SEQ ID NO: 75, a CDR2 comprising a sequence set forth between amino acids 50-57 of SEQ ID NO: 75 and a CDR3 comprising a sequence set forth between amino acids 89-97 of SEQ ID NO: 75;

(xii) a V_L comprising a sequence set forth in SEQ ID NO: 75;

(xiii) a V_H as set forth in (i) and a V_L as set forth in (iii);

(xiv) a V_H as set forth in (i) and a V_L as set forth in (iv);

(xv) a V_H as set forth in (ii) and a V_L as set forth in (iii);

(xvi) a V_H as set forth in (ii) and a V_L as set forth in (iv);

(xvii) a V_H as set forth in (v) and a V_L as set forth in (vii);

(xviii) a V_H as set forth in (v) and a V_L as set forth in (viii);

(xix) a V_H as set forth in (vi) and a V_L as set forth in (vii);

(xx) a V_H as set forth in (vi) and a V_L as set forth in (viii);

(xxi) a V_H as set forth in (ix) and a V_L as set forth in (xi);

(xxii) a V_H as set forth in (ix) and a V_L as set forth in (xii);

(xxiii) a V_H as set forth in (x) and a V_L as set forth in (xi); or

(xxiv) a V_H as set forth in (x) and a V_L as set forth in (xii).

In one example, the antigen binding domain comprises:

(i) a V_H comprising a CDR1 comprising a sequence set forth between amino acids 31-35 of SEQ ID NO: 37, a CDR2 comprising a sequence at least about 80% identical (or 90% identical or 95% identical or 98% identical or 99% identical or 100% identical) to a sequence set forth between amino acids 50-66 of SEQ ID NO: 37 and a CDR3 comprising a sequence at least about 55% identical (or 60% identical or 70% identical or 80% identical or 90% identical or 95% identical or 98% identical or 99% identical or 100% identical) to a sequence set forth between amino acids 99-107 of SEQ ID NO: 37 and a V_L comprising a CDR1 comprising a sequence set forth between amino acids 24-34 of SEQ ID NO: 5, a CDR2 comprising a sequence set forth between amino acids 50-56 of SEQ ID NO: 5 and a CDR3 comprising a sequence set forth between amino acids 89-97 of SEQ ID NO: 5 or a V_L comprising a sequence set forth in SEQ ID NO: 5;

(ii) a V_H comprising a CDR1 comprising a sequence set forth between amino acids 31-35 of SEQ ID NO: 37, a CDR2 comprising a sequence at least about 80% identical (or 90% identical or 95% identical or 98% identical or 99% identical or 100% identical) to a sequence set forth between amino acids 50-66 of SEQ ID NO: 37 and a CDR3 comprising a sequence set forth between amino acids 99-107 of SEQ ID NO: 37 and a V_L comprising a CDR1 comprising a sequence set forth between amino acids 24-34 of SEQ ID NO: 5, a CDR2 comprising a sequence set forth between amino acids 50-56 of SEQ ID NO: 5 and a CDR3 comprising a sequence set forth between amino acids 89-97 of SEQ ID NO: 5 or a V_L comprising a sequence set forth in SEQ ID NO: 5; or

(iii) a V_H comprising a CDR1 comprising a sequence set forth between amino acids 31-35 of SEQ ID NO: 37, a CDR2 comprising a sequence set forth between amino acids 50-66 of SEQ ID NO: 37 and a CDR3 comprising a sequence at least about 55% identical (or 60% identical or 70% identical or 80% identical or 90% identical or 95% identical or 98% identical or 99% identical or 100% identical) to a sequence set forth between amino acids 99-107 of SEQ ID NO: 37 and a V_L comprising a CDR1 comprising a sequence set forth between amino acids 24-34 of SEQ ID NO: 5, a CDR2 comprising a sequence set forth between amino acids 50-56 of SEQ ID NO: 5 and a CDR3 comprising a sequence set forth between amino acids 89-97 of SEQ ID NO: 5 or a V_L comprising a sequence set forth in SEQ ID NO: 5.

In one example, the antigen binding domain comprises:

(i) a V_H comprising a CDR1 comprising a sequence set forth between amino acids 31-35 of SEQ ID NO: 37, a CDR2 comprising a sequence set forth between amino acids 50-66 of SEQ ID NO: 37 and a CDR3 comprising a sequence set forth between amino acids 99-107 of SEQ ID NO: 37 or a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a CDR1 comprising a sequence at least about 54% identical (or 60% identical or 70% identical or 80% identical or 90% identical or 95% identical or 98% identical or 99% identical or 100% identical) to a sequence set forth between amino acids 24-34 of SEQ ID NO: 5, a CDR2 comprising a sequence set forth between amino acids 50-56 of SEQ ID NO: 5 and a CDR3 comprising a sequence at least about 66% identical (or 70% identical or 80% identical or 90% identical or 95% identical or 98% identical or 99% identical or 100% identical) to a sequence set forth between amino acids 89-97 of SEQ ID NO: 5;

(ii) a V_H comprising a CDR1 comprising a sequence set forth between amino acids 31-35 of SEQ ID NO: 37, a CDR2 comprising a sequence set forth between amino acids 50-66 of SEQ ID NO: 37 and a CDR3 comprising a sequence set forth between amino acids 99-107 of SEQ ID NO: 37 or a V_H comprising a sequence set forth in SEQ

ID NO: 37 and a V_L comprising a CDR1 comprising a sequence at least about 54% identical (or 60% identical or 70% identical or 80% identical or 90% identical or 95% identical or 98% identical or 99% identical or 100% identical) to a sequence set forth between amino acids 24-34 of SEQ ID NO: 5, a CDR2 comprising a sequence set forth between amino acids 50-56 of SEQ ID NO: 5 and a CDR3 comprising a sequence set forth between amino acids 89-97 of SEQ ID NO: 5; or

(iii) a V_H comprising a CDR1 comprising a sequence set forth between amino acids 31-35 of SEQ ID NO: 37, a CDR2 comprising a sequence set forth between amino acids 50-66 of SEQ ID NO: 37 and a CDR3 comprising a sequence set forth between amino acids 99-107 of SEQ ID NO: 37 or a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a CDR1 comprising a sequence set forth between amino acids 24-34 of SEQ ID NO: 5, a CDR2 comprising a sequence set forth between amino acids 50-56 of SEQ ID NO: 5 and a CDR3 comprising a sequence at least about 66% identical (or 70% identical or 80% identical or 90% identical or 95% identical or 98% identical or 99% identical or 100% identical) to a sequence set forth between amino acids 89-97 of SEQ ID NO: 5.

In one example, the protein comprises a V_L comprising a CDR1, CDR2 and CDR3 of a V_L of antibody 8E2 and V_H comprising a CDR1 and a CDR2 of a V_H of antibody 8E2.

In one example, the protein comprises a V_L comprising a CDR1, CDR2 and CDR3 of a V_L of antibody 8E2 and a CDR1 and a V_H comprising a CDR1 and a CDR3 of a V_H of antibody 8E2.

In one example, the protein comprises a V_L comprising a CDR1, CDR2 and CDR3 of a V_L of antibody 8E2 and a V_H comprising a CDR2 and a CDR3 of a V_H of antibody 8E2.

In one example, the protein comprises a V_L comprising a CDR1 and a CDR3 of a V_L of antibody 8E2 and a V_H comprising a CDR1, a CDR2 and a CDR3 of a V_H of antibody 8E2.

In one example, the antigen binding domain comprises:

(i) a V_H comprising a sequence set forth in SEQ ID NO: 71 and a V_L comprising a sequence set forth in SEQ ID NO: 35; or

(ii) a V_H comprising a sequence set forth in SEQ ID NO: 72 and a V_L comprising a sequence set forth in SEQ ID NO: 36.

Such an IL-11Rα-binding protein can exhibit any one or more of the functional activities described herein, e.g., preferential binding to a polypeptide of SEQ ID NO: 3

relative to the level of binding of a polypeptide of SEQ ID NO: 3 comprising a substitution as described above.

In one example, differences between the recited sequence and the IL-11Rα-binding protein are substitutions.

The skilled artisan will be capable of determining sites for mutations to an IL-11Rα-binding protein of the disclosure, e.g., within a framework region of a variable region containing protein. Moreover, the inventors have identified numerous sites in a V_H CDR1, CDR2 and/or CDR3 and a V_L CDR1 and/or CDR3 that can be mutated as well as numerous mutations that maintain or improve activity of an IL-11Rα-binding protein of the disclosure. For example a mutation, e.g., a substitution is within HCDR1 and/or one or more (e.g., 2 or 3 or 4) of HCDR2 and/or one or more (e.g., 2 or 3 or 4 or 5 or 6) of the six N-terminal or the six C-terminal residues of HCDR3 of antibody 8E2. For example a mutation, e.g., a substitution is within at least one (e.g., 2 or 3 or 4 or 5 or 6) of the six C-terminal amino acids of LCDR1 and/or one or more (e.g., 2 or 3 or 4 or 5) of the five N-terminal of LCDR3 of antibody 8E2.

In one example, an IL-11Rα-binding protein of the disclosure comprises a light chain CDR1 comprising the sequence:

$QASQDX_1X_2X_3X_4X_5X_6$ (SEQ ID NO: 77)

wherein X_1=I or V; X_2=N or D or G or S or A or H; X_3=N or Y or I or K or M or Q or G or H; X_4=Y or W; X_5=L or V or I or M and X_6=N or E.

In one example, an IL-11Rα-binding protein of the disclosure comprises a light chain CDR3 comprising the sequence:

$X_1QX_2X_3X_4X_5X_6PX_7$ (SEQ ID NO: 78)

wherein X_1=Q or E or S or T; X_2=Y or H or F or N or S or W; X_3=D or E; X_4=N, D, F, S, E o T; X_5=L or Q; X_6=S or A or W or T or M or Q and X_7=T or E or F or A or L or F or N or Q.

In one example, X_1 is Q and X_2 is Y.

In one example, X_6 is S and X_7 is T.

In one example, an IL-11Rα-binding protein of the disclosure comprises a heavy chain CDR1 comprising the sequence:

$X_1X_2SX_3X_4$

wherein X_1=W or R; X_2=Y or W or F; X_4=M or I or V or T or L and X_5=T or A

In one example, an IL-11Rα-binding protein of the disclosure comprises a heavy chain CDR2 comprising the sequence:

$SIVPX_1X_2X_3X_4TQYADSVKG$

wherein X_1=S or W or Y or H; X_2=G or A; X_3=G or D or T and X_4=H or Y or L or I or F

In one example, an IL-11Rα-binding protein of the disclosure comprises a heavy chain CDR3 comprising the sequence:

$X_1X_2X_3WGX_4FX_5X_6$

wherein X_1=G or P; X_2=P or E or V or L or N or H; X_3=G or D; X_4=S or M or R or L; X_5=D or A or W and X_6=L or V or F or Q or E or Y or T

In one example, X_1 is G, X_2 is P and X_3 is G.

In one example, X_5 is D and X_6 is L.

In one example, the IL-11Rα-binding protein comprises one of the foregoing consensus sequences as a CDR and the remaining CDRs are from antibody 8E2.

The present disclosure also provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and wherein the antigen binding domain comprises a V_H comprising a sequence set forth in any one of SEQ ID NOs: 37 to 70, 74 or 76 and/or a V_L comprising a sequence set forth in any one of SEQ ID NOs: 5 to 34, 73 or 75.

The present disclosure also provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and wherein the antigen binding domain comprises:

(i) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(ii) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(iii) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 38 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(iv) a V_H comprising a sequence set forth in SEQ ID NO: 38 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(v) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 39 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(vi) a V_H comprising a sequence set forth in SEQ ID NO: 39 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(vii) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 40 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(viii) a V_H comprising a sequence set forth in SEQ ID NO: 40 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(ix) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 41 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(x) a V_H comprising a sequence set forth in SEQ ID NO: 41 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xi) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 42 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(xii) a V_H comprising a sequence set forth in SEQ ID NO: 42 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xiii) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 43 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(xiv) a V_H comprising a sequence set forth in SEQ ID NO: 43 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xv) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 44 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(xvi) a V_H comprising a sequence set forth in SEQ ID NO: 44 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xvii) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 45 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(xviii) a V_H comprising a sequence set forth in SEQ ID NO: 45 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xix) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 46 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(xx) a V_H comprising a sequence set forth in SEQ ID NO: 46 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xxi) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 47 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(xxii) a V_H comprising a sequence set forth in SEQ ID NO: 47 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xxiii) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 48 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(xxiv) a V_H comprising a sequence set forth in SEQ ID NO: 48 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xxv) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 49 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(xxvi) a V_H comprising a sequence set forth in SEQ ID NO: 49 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xxvii)a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 50 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(xxviii) a V_H comprising a sequence set forth in SEQ ID NO: 50 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xxix) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 51 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(xxx) a V_H comprising a sequence set forth in SEQ ID NO: 51 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xxxi) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 52 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(xxxii)a V_H comprising a sequence set forth in SEQ ID NO: 52 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xxxiii) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 53 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(xxxiv) a V_H comprising a sequence set forth in SEQ ID NO: 53 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xxxv) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 54 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(xxxvi) a V_H comprising a sequence set forth in SEQ ID NO: 54 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xxxvii) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 55 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(xxxviii) a V_H comprising a sequence set forth in SEQ ID NO: 55 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xxxix) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 56 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(xl) a V_H comprising a sequence set forth in SEQ ID NO: 56 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xli) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 57 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(xlii) a V_H comprising a sequence set forth in SEQ ID NO: 57 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xliii) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 58 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(xliv) a V_H comprising a sequence set forth in SEQ ID NO: 58 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xlv) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 59 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(xlvi) a V_H comprising a sequence set forth in SEQ ID NO: 59 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xlvii) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 60 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(xlviii) a V_H comprising a sequence set forth in SEQ ID NO: 60 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xlix) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 61 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(l) a V_H comprising a sequence set forth in SEQ ID NO: 61 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(li) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 62 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(lii) a V_H comprising a sequence set forth in SEQ ID NO: 62 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(liii) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 63 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(liv) a V_H comprising a sequence set forth in SEQ ID NO: 63 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(lv) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 64 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(lvi) a V_H comprising a sequence set forth in SEQ ID NO: 64 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(lvii) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 65 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(lviii) a V_H comprising a sequence set forth in SEQ ID NO: 65 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(lix) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 66 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(lx) a V_H comprising a sequence set forth in SEQ ID NO: 66 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(lxi) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 67 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(lxii) a V_H comprising a sequence set forth in SEQ ID NO: 67 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(lxiii) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 68 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(lxiv) a V_H comprising a sequence set forth in SEQ ID NO: 68 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(lxv) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 69 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(lxvi) a V_H comprising a sequence set forth in SEQ ID NO: 69 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(lxvii) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 70 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(lxviii)a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(lxix) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 70 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(lxx) a V_H comprising a sequence set forth in SEQ ID NO: 70 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(lxxi) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 6;

(lxxii) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 6;

(lxxiii)a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 7;

(lxxiv)a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 7;

(lxxv) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 8;

(lxxvi)a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 8;

(lxxvii) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 9;

(lxxviii) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 9;

(lxxix)a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 10;

(lxxx) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 10;

(lxxxi)a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 11;

(lxxxii) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 11;

(lxxxiii) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 12;

(lxxxiv) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 12;

(lxxxv) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 13;

(lxxxvi) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 13;

(lxxxvii) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 14;

(lxxxviii)a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 14;

(lxxxix) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 15;

(xc) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 15;

(xci) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 16;

(xcii) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 16;

(xciii) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 17;

(xciv) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 17;

(xcv) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 18;

(xcvi) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 18;

(xcvii) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 19;

(xcviii) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 19;

(xcix) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 20;

(c) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 20;

(ci) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 21;

(cii) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 21;

(ciii) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 22;

(civ) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 22;

(cv) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 23;

(cvi) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 23;

(cvii) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 24;

(cviii) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 24;

(cix) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 25;

(cx) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 25;

(cxi) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 26;

(cxii) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 26;

(cxiii) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 27;

(cxiv) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 27;

(cxv) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 28;

(cxvi) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 28;

(cxvii) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 29;

(cxviii) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 29;

(cxix) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 30;

(cxx) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 30;

(cxxi) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 31;

(cxxii) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 31;

(cxxiii) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 32;

(cxxiv) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 32;

(cxxv) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 33;

(cxxvi) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 33;

(cxxvii) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 34; or

(cxxviii) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 34.

The present disclosure also provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and wherein the antigen binding domain comprises:

(i) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 49 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(ii) a V_H comprising a sequence set forth in SEQ ID NO: 49 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(iii) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 53 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(iv) a V_H comprising a sequence set forth in SEQ ID NO: 53 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(v) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 57 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(vi) a V_H comprising a sequence set forth in SEQ ID NO: 57 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(vii) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 58 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(viii) a V_H comprising a sequence set forth in SEQ ID NO: 58 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(ix) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 8;

(x) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 8;

(xi) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 15;

(xii) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 15;

(xiii) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 16;

(xiv) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 16;

(xv) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 18;

(xvi) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 18;

(xvii) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 29; or

(xviii) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 29.

In one example, the CDRs are as follows:

(i) Heavy chain CDR1: amino acids 31-35 of the recited sequence;

(ii) Heavy chain CDR2: amino acids 50-66 of the recited sequence (optionally, wherein any one or more of the five C-terminal amino acids are substituted with another naturally-occurring amino acid);

(iii) Heavy chain CDR3: amino acids 99-107 of the recited sequence;

(iv) Light chain CDR1: amino acids 24-34 of the recited sequence;

(v) Light chain CDR2: amino acids 50-56 of the recited sequence; and

(vi) Light chain CDR3: amino acids 89-97 of the recited sequence.

The present disclosure also provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and wherein the antigen binding domain comprises a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 49 and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5.

The present disclosure also provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and wherein the antigen binding domain comprises a V_H comprising a sequence set forth in SEQ ID NO: 49 and a V_L comprising a sequence set forth in SEQ ID NO: 5.

The present disclosure also provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and wherein the antigen binding domain comprises a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 53 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5.

The present disclosure also provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and wherein the antigen binding domain comprises a V_H comprising a sequence set forth in SEQ ID NO: 53 and a V_L comprising a sequence set forth in SEQ ID NO: 5.

The present disclosure also provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and wherein the antigen binding domain comprises a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 57 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5.

The present disclosure also provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and wherein the antigen binding domain comprises a V_H comprising a sequence set forth in SEQ ID NO: 57 and a V_L comprising a sequence set forth in SEQ ID NO: 5.

The present disclosure also provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and wherein the antigen binding domain comprises a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 58 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5.

The present disclosure also provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and wherein the antigen binding domain comprises a V_H comprising a sequence set forth in SEQ ID NO: 58 and a V_L comprising a sequence set forth in SEQ ID NO: 5.

The present disclosure also provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and wherein the antigen binding domain comprises a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 8.

The present disclosure also provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and wherein the antigen binding domain comprises a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 8.

The present disclosure also provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and wherein the antigen

binding domain comprises a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 15.

The present disclosure also provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and wherein the antigen binding domain comprises a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 15.

The present disclosure also provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and wherein the antigen binding domain comprises a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 16.

The present disclosure also provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and wherein the antigen binding domain comprises a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 16.

The present disclosure also provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and wherein the antigen binding domain comprises a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 18.

The present disclosure also provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and wherein the antigen binding domain comprises a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 18.

The present disclosure also provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and wherein the antigen binding domain comprises a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 29.

The present disclosure also provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and wherein the antigen binding domain comprises a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 29.

The present disclosure also provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and wherein the antigen binding domain comprises V_H comprising a sequence set forth in SEQ ID NO: 37. In one example, the V_L comprises CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 18.

The present disclosure also provides an IL-11Rα-binding protein comprising an antigen binding domain of an antibody, wherein the antigen binding domain binds to or specifically binds to IL-11Rα and neutralizes IL-11 signaling and wherein the antigen binding domain comprises a V_L comprising a sequence set forth in SEQ ID NO: 18. In one example, the VH comprises CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37.

In one example, the CDRs are as follows:

(i) Heavy chain CDR1: amino acids 31-35 of the recited sequence;

(ii) Heavy chain CDR2: amino acids 50-66 of the recited sequence (optionally, wherein any one or more of the five C-terminal amino acids are substituted with another naturally-occurring amino acid);

(iii) Heavy chain CDR3: amino acids 99-107 of the recited sequence;

(iv) Light chain CDR1: amino acids 24-34 of the recited sequence;

(v) Light chain CDR2: amino acids 50-56 of the recited sequence; and

(vi) Light chain CDR3: amino acids 89-97 of the recited sequence.

In one example, an IL-11Rα-binding protein described herein comprises at least a V_H and a V_L, wherein the V_H and V_L bind to form a Fv comprising an antigen binding domain. The skilled artisan will understand that the antigen binding domain comprises the binding site of the antibody.

In one example, the V_H and the V_L are in a single polypeptide chain. For example, the protein is:

(i) a single chain Fv fragment (scFv);

(ii) a dimeric scFv (di-scFv);

(iii) one of (i) or (ii) linked to a constant region of an antibody, Fc or a heavy chain constant domain (C_H) 2 and/or C_H3; or

(iv) one of (i) or (ii) linked to a protein that binds to an immune effector cell.

In one example, the V_L and V_H are in separate polypeptide chains.

For example, the protein is:

(i) a diabody;

(ii) a triabody;

(iii) a tetrabody;

(iv) a Fab;

(v) a $F(ab')_2$;

(vi) a Fv;

(vii) one of (i) to (vi) linked to a constant region of an antibody, Fc or a heavy chain constant domain (C_H) 2 and/or C_H3;

(viii) one of (i) to (vi) linked to a protein that binds to an immune effector cell.

The foregoing proteins (described in the previous two lists) can also be referred to as antigen binding domains of antibodies.

In one example, the protein is an antibody, for example, a monoclonal antibody. In one example, the antibody is a naked antibody.

In one example, a protein (or antibody) is chimeric, de-immunized, humanized, human or primatized.

In one example, the protein or antibody is human.

The present disclosure additionally or alternatively provides an antibody that binds to IL-11Rα and neutralizes IL-11 signaling, the antibody comprising:

(i) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 49 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(ii) a V_H comprising a sequence set forth in SEQ ID NO: 49 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(iii) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 53 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(iv) a V_H comprising a sequence set forth in SEQ ID NO: 53 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(v) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 57 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(vi) a V_H comprising a sequence set forth in SEQ ID NO: 57 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(vii) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 58 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5;

(viii) a V_H comprising a sequence set forth in SEQ ID NO: 58 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(ix) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 8;

(x) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 8;

(xi) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 15;

(xii) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 15;

(xiii) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 16;

(xiv) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 16;

(xv) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 18;

(xvi) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 18;

(xvii) a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 29; or

(xviii) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 29.

The present disclosure also provides an antibody that binds to IL-11Rα and neutralizes IL-11 signaling, the antibody comprising V_H comprising a sequence set forth in SEQ ID NO: 37. In one example, the V_L comprises CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 18.

(xix) The present disclosure also provides an antibody that binds to IL-11Rα and neutralizes IL-11 signaling, the antibody comprising a V_L comprising a sequence set

forth in SEQ ID NO: 18. In one example, the VH comprises CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37.

In one example, the CDRs are as follows:

(i) Heavy chain CDR1: amino acids 31-35 of the recited sequence;

(ii) Heavy chain CDR2: amino acids 50-66 of the recited sequence (optionally, wherein any one or more of the five C-terminal amino acids are substituted with another naturally-occurring amino acid);

(iii) Heavy chain CDR3: amino acids 99-107 of the recited sequence;

(iv) Light chain CDR1: amino acids 24-34 of the recited sequence;

(v) Light chain CDR2: amino acids 50-56 of the recited sequence; and

(vi) Light chain CDR3: amino acids 89-97 of the recited sequence.

Sequences of exemplary V_H and V_L are described in Table 1.

The present disclosure also provides an antibody that binds to IL-11Rα and neutralizes IL-11 signaling, the antibody comprising a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 49 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5.

The present disclosure also provides an antibody that binds to IL-11Rα and neutralizes IL-11 signaling, the antibody comprising a V_H comprising a sequence set forth in SEQ ID NO: 49 and a V_L comprising a sequence set forth in SEQ ID NO: 5.

The present disclosure also provides an antibody that binds to IL-11Rα and neutralizes IL-11 signaling, the antibody comprising a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 53 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5.

The present disclosure also provides an antibody that binds to IL-11Rα and neutralizes IL-11 signaling, the antibody comprising a V_H comprising a sequence set forth in SEQ ID NO: 53 and a V_L comprising a sequence set forth in SEQ ID NO: 5.

The present disclosure also provides an antibody that binds to IL-11Rα and neutralizes IL-11 signaling, the antibody comprising a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 57 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5.

The present disclosure also provides an antibody that binds to IL-11Rα and neutralizes IL-11 signaling, the antibody comprising a V_H comprising a sequence set forth in SEQ ID NO: 57 and a V_L comprising a sequence set forth in SEQ ID NO: 5.

The present disclosure also provides an antibody that binds to IL-11Rα and neutralizes IL-11 signaling, the antibody comprising a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 58 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 5.

The present disclosure also provides an antibody that binds to IL-11Rα and neutralizes IL-11 signaling, the antibody comprising a V_H comprising a sequence set forth in SEQ ID NO: 58 and a V_L comprising a sequence set forth in SEQ ID NO: 5.

The present disclosure also provides an antibody that binds to IL-11Rα and neutralizes IL-11 signaling, the antibody comprising a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 8.

The present disclosure also provides an antibody that binds to IL-11Rα and neutralizes IL-11 signaling, the antibody comprising a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 8.

The present disclosure also provides an antibody that binds to IL-11Rα and neutralizes IL-11 signaling, the antibody comprising a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 15.

The present disclosure also provides an antibody that binds to IL-11Rα and neutralizes IL-11 signaling, the antibody comprising a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 15.

The present disclosure also provides an antibody that binds to IL-11Rα and neutralizes IL-11 signaling, the antibody comprising a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 16.

The present disclosure also provides an antibody that binds to IL-11Rα and neutralizes IL-11 signaling, the antibody comprising a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 16.

The present disclosure also provides an antibody that binds to IL-11Rα and neutralizes IL-11 signaling, the antibody comprising a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid

N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 18.

The present disclosure also provides an antibody that binds to IL-11Rα and neutralizes IL-11 signaling, the antibody comprising a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 18.

The present disclosure also provides an antibody that binds to IL-11Rα and neutralizes IL-11 signaling, the antibody comprising a V_H comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 37 (optionally, also comprising the amino acid N-terminal to CDR1) and a V_L comprising CDRs 1, 2 and 3 of a sequence set forth in SEQ ID NO: 29.

The present disclosure also provides an antibody that binds to IL-11Rα and neutralizes IL-11 signaling, the antibody comprising a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 29.

In one example, the CDRs are as follows:

(i) Heavy chain CDR1: amino acids 31-35 of the recited sequence;

(ii) Heavy chain CDR2: amino acids 50-66 of the recited sequence (optionally, wherein any one or more of the five C-terminal amino acids are substituted with another naturally-occurring amino acid);

(iii) Heavy chain CDR3: amino acids 99-107 of the recited sequence;

(iv) Light chain CDR1: amino acids 24-34 of the recited sequence;

(v) Light chain CDR2: amino acids 50-56 of the recited sequence; and

(vi) Light chain CDR3: amino acids 89-97 of the recited sequence.

Reference herein to a protein or antibody that "binds to" IL-11Rα provides literal support for a protein or antibody that "binds specifically to" or "specifically binds to" IL-11R.

The present disclosure also provides antigen binding domains or antigen binding fragments of the foregoing antibodies.

In one example, a protein or antibody as described herein comprises a human constant region, e.g., an IgG constant region, such as an IgG1, IgG2, IgG3 or IgG4 constant region or mixtures thereof. In the case of an antibody or protein comprising a V_H and a V_L, the V_H can be linked to a heavy chain constant region and the V_L can be linked to a light chain constant region.

The C-terminal lysine of the heavy chain constant region of a whole antibody (or am IL-11Rα-binding protein comprising a constant region or a C_H3) of the disclosure may be removed, for example, during production or purification of the antibody or protein, or by recombinantly engineering the nucleic acid encoding a heavy

chain of the antibody. Accordingly, whole antibodies (or IL-11R-binding proteins) may comprise populations with all C-terminal lysine residues removed, populations with no C-terminal lysine residues removed, and/or populations having a mixture of protein with and without the C-terminal lysine residue. In some examples, the populations may additionally comprise protein in which the C-terminal lysine residue is removed in one of the heavy chain constant regions. Similarly, a composition of whole antibodies may comprise the same or a similar mix of antibody populations with or without the C-terminal lysine residue.

In one example, a protein or antibody as described herein comprises a constant region of an IgG4 antibody or a stabilized constant region of an IgG4 antibody. In one example, the protein or antibody comprises an IgG4 constant region with a proline at position 241 (according to the numbering system of Kabat (Kabat *et al.*, Sequences of Proteins of Immunological Interest Washington DC United States Department of Health and Human Services, 1987 and/or 1991)).

In one example, the heavy chain constant region comprises a sequence from position 119 to position 445 of SEQ ID NO: 83. In one example a protein or antibody as described herein or a composition of a protein or antibody as described herein, comprises a heavy chain constant region, including a stabilized heavy chain constant region, comprising a mixture of sequences fully or partially with or without the C-terminal lysine residue.

In one example, an antibody of the disclosure comprises a V_H disclosed herein linked or fused to an IgG4 constant region or stabilized IgG4 constant region (e.g., as discussed above) and the V_L is linked to or fused to a kappa light chain constant region.

The functional characteristics of an IL-11Rα-binding protein of the disclosure will be taken to apply *mutatis mutandis* to an antibody of the disclosure.

In one example, an IL-11Rα-binding protein or antibody as described herein is isolated and/or recombinant.

In one example, an IL-11Rα-binding protein or antibody of the disclosure is conjugated to another compound, for example, a detectable label or a compound that extends the half-life of the protein or antibody, such as polyethylene glycol or an albumin binding protein. Other suitable compounds are described herein.

The present disclosure also provides a nucleic acid encoding the IL-11Rα-binding protein or antibody of the present disclosure or a polypeptide thereof.

In one example, such a nucleic acid is included in an expression construct in which the nucleic acid is operably linked to a promoter. Such an expression construct can be in a vector, e.g., a plasmid.

In examples of the disclosure directed to single polypeptide chain IL-11Rα-binding proteins, the expression construct may comprise a promoter linked to a nucleic acid encoding that polypeptide chain.

In examples directed to multiple polypeptide chains that form an IL-11Rα-binding protein, an expression construct comprises a nucleic acid encoding a polypeptide comprising, e.g., a V_H operably linked to a promoter and a nucleic acid encoding a polypeptide comprising, e.g., a V_L operably linked to a promoter.

In another example, the expression construct is a bicistronic expression construct, e.g., comprising the following operably linked components in 5' to 3' order:

(i) a promoter

(ii) a nucleic acid encoding a first polypeptide;

(iii) an internal ribosome entry site; and

(iv) a nucleic acid encoding a second polypeptide,

wherein the first polypeptide comprises a V_H and the second polypeptide comprises a V_L, or *vice versa*.

The present disclosure also contemplates separate expression constructs one of which encodes a first polypeptide comprising a V_H and another of which encodes a second polypeptide comprising a V_L. For example, the present disclosure also provides a composition comprising:

(i) a first expression construct comprising a nucleic acid encoding a polypeptide comprising a V_H operably linked to a promoter; and

(ii) a second expression construct comprising a nucleic acid encoding a polypeptide comprising a V_L operably linked to a promoter.

The present disclosure also provides an isolated or recombinant cell expressing an IL-11Rα-binding protein of the disclosure.

In one example, the cell comprises the expression construct of the disclosure or:

(i) a first expression construct comprising a nucleic acid encoding a polypeptide comprising a V_H operably linked to a promoter; and

(ii) a second expression construct comprising a nucleic acid encoding a polypeptide comprising a V_L operably linked to a promoter,

wherein the first and second polypeptides associate to form an IL-11Rα-binding protein of the present disclosure.

Examples of cells of the present disclosure include bacterial cells, yeast cells, insect cells or mammalian cells.

The present disclosure additionally provides methods for producing an IL-11Rα-binding protein or antibody of the disclosure. For example, such a method involves

maintaining the expression construct(s) of the disclosure under conditions sufficient for the IL-11Rα-binding protein or antibody to be produced.

In one example, a method for producing an IL-11Rα-binding protein or antibody of the disclosure comprises culturing the cell of the disclosure under conditions sufficient for the IL-11Rα-binding protein or antibody to be produced and, optionally, secreted.

In one example, the method for producing an IL-11Rα-binding protein or antibody of the disclosure additionally comprises isolating the protein or antibody and, optionally, formulating the IL-11Rα-binding protein or antibody into a pharmaceutical composition.

The present disclosure additionally provides a composition comprising an IL-11Rα-binding protein or antibody of the disclosure and a pharmaceutically acceptable carrier.

In some examples, the composition comprises:

(i) an antibody of the disclosure comprising a C-terminal lysine residue from the heavy chain;

(ii) an antibody of the disclosure lacking a C-terminal lysine residue from the heavy chain; and/or

(iii) an antibody of the disclosure comprising a C-terminal lysine residue on one heavy chain and lacking a C-terminal lysine residue on another (or the other) heavy chain,

and, optionally, a pharmaceutically acceptable carrier.

The present disclosure also provides a method for treating or preventing an IL-11-mediated condition in a subject, the method comprising administering the IL-11Rα-binding protein, antibody or composition of the disclosure. In this regard, an IL-11Rα-binding protein, antibody or composition can be used to prevent a relapse of a condition, and this is considered preventing the condition.

In one example, the IL-11-mediated condition is an autoimmune condition, an inflammatory condition, a wasting condition, bone conditions or cancer.

Exemplary autoimmune conditions include arthritis, inflammatory bowel disease and psoriasis.

Exemplary inflammatory conditions include infection-induced inflammation (e.g., inflammation induced by *M. tuberculosis*), gastric inflammation (e.g., associated with gastric cancer), inflammatory airway conditions (e.g., asthma, chronic obstructive pulmonary disease (COPD), rhinitis or allergy) or inflammatory dermatitis (e.g., atopic dermatitis).

Exemplary wasting conditions include cachexia (e.g., cancer cachexia or cachexia caused by renal failure) or sarcopenia.

Exemplary bone conditions include osteoporosis (including post-menopausal osteoporosis), bone fracture, bone resorption/damage caused by cancer (e.g., metastatic bone cancer, myeloma or Paget's disease of bone) and bone resorption/damage caused by treatment of cancer (e.g., chemotherapy, hormone ablation or hormone inhibition).

Exemplary cancers include hematologic cancers, cancers of epithelial origin, liver cancer, pancreatic cancer, gastric cancer, osteosarcoma, endometrial cancer and ovarian cancer.

In one example, the cancer or the bone condition is metastasis of a cancer to bone.

The present disclosure also provides a method for inhibiting or neutralizing IL-11 in a subject, the method comprising administering the IL-11Rα-binding protein, antibody or composition of the disclosure. In one example, the subject suffers from an IL-11-mediated condition.

The present disclosure also provides a method for preventing pregnancy in a subject, the method comprising administering the IL-11Rα-binding protein, antibody or composition of the disclosure.

In one example, a method described herein comprises administering between about 0.05mg/kg and 30mg/kg of the IL-11Rα-binding protein or antibody. For example, the method comprising administering between 0.1mg/kg and 10mg/kg or between 0.2mg/kg and 5mg/kg of the IL-11Rα-binding protein or antibody. In one example, the method comprises administering about 0.5-2.0mg/kg of the IL-11Rα-binding protein or antibody.

The present disclosure also provides for use of an IL-11Rα-binding protein or antibody as described herein in any example in medicine.

The present disclosure also provides for use of an IL-11Rα-binding protein or antibody as described herein according to any example in the manufacture of a medicament to treat an IL-11-mediated condition. Exemplary conditions are described herein.

The present disclosure also provides a method for localizing and/or detecting and/or diagnosing and/or prognosing an IL-11-mediated condition associated with a cell expressing IL-11Rα, the method comprising detecting *in vivo* an IL-11Rα-binding protein or antibody as described herein bound to the IL-11Rα expressing cell, if present, wherein the IL-11Rα-binding protein or antibody is conjugated to a detectable tag.

In one example, the method additionally comprises administering the IL-11Rα-binding protein to the subject.

The present disclosure also provides a method for detecting IL-11Rα or a cell expressing same in a sample, the method comprising contacting the sample with a protein or antibody as described herein according to any example such that a complex forms and detecting the complex, wherein detection of the complex is indicative of IL-11Rα or a cell expressing same in the sample. In one example, the method is performed *ex vivo* or *in vitro*.

The present disclosure also provides a method for diagnosing or prognosing a IL-11-mediated condition, the method comprising performing a method as described herein according to any example to detect IL-11Rα or a cell expressing same, wherein detection of the IL-11Rα or cell expressing same is diagnostic or prognostic of the condition. In one example, the method is performed *ex vivo* or *in vitro*. Exemplary IL-11Rα-mediated conditions are described herein.

The present disclosure also provides a kit (e.g., a package or article of manufacture) comprising an IL-11Rα-binding protein or antibody as described herein according to any example, optionally, packaged with instructions for use in a method as described herein.

KEY TO SEQUENCE LISTING

SEQ ID NO 1: amino acid sequence of *Homo sapiens* pre-IL-11Rα

SEQ ID NO 2: amino acid sequence of *Macaca fascicularis* pre-IL-11 Rα

SEQ ID NO 3: amino acid sequence of polypeptide comprising amino acids 23 to 363 of SEQ ID NO: 1, a 8xHIS tag and a serine at a position corresponding to position 248 of SEQ ID NO: 1 (also referred to as "WT F/L")

SEQ ID NO 4: amino acid sequence of *Homo sapiens* gp130

SEQ ID NO 5: amino acid sequence of V_L chain of antibody 8E2

SEQ ID NO 6: amino acid sequence of V_L chain of antibody TS-303

SEQ ID NO 7: amino acid sequence of V_L chain of antibody TS-305

SEQ ID NO 8: amino acid sequence of V_L chain of antibody TS-306

SEQ ID NO 9: amino acid sequence of V_L chain of antibody TS-307

SEQ ID NO 10: amino acid sequence of V_L chain of antibody TS-310

SEQ ID NO 11: amino acid sequence of V_L chain of antibody TS-311

SEQ ID NO 12: amino acid sequence of V_L chain of antibody TS-312

SEQ ID NO 13: amino acid sequence of V_L chain of antibody TS-313

SEQ ID NO 14: amino acid sequence of V_L chain of antibody TS-322

SEQ ID NO 15: amino acid sequence of V_L chain of antibody TS-2
SEQ ID NO 16: amino acid sequence of V_L chain of antibody TS-4
SEQ ID NO 17: amino acid sequence of V_L chain of antibody TS-6
SEQ ID NO 18: amino acid sequence of V_L chain of antibody TS-7
SEQ ID NO 19: amino acid sequence of V_L chain of antibody TS-9
SEQ ID NO 20: amino acid sequence of V_L chain of antibody TS-13
SEQ ID NO 21: amino acid sequence of V_L chain of antibody TS-14
SEQ ID NO 22: amino acid sequence of V_L chain of antibody TS-17
SEQ ID NO 23: amino acid sequence of V_L chain of antibody TS-20
SEQ ID NO 24: amino acid sequence of V_L chain of antibody TS-21
SEQ ID NO 25: amino acid sequence of V_L chain of antibody TS-22
SEQ ID NO 26: amino acid sequence of V_L chain of antibody TS-29
SEQ ID NO 27: amino acid sequence of V_L chain of antibody TS-32
SEQ ID NO 28: amino acid sequence of V_L chain of antibody TS-49
SEQ ID NO 29: amino acid sequence of V_L chain of antibody TS-51
SEQ ID NO 30: amino acid sequence of V_L chain of antibody TS-55
SEQ ID NO 31: amino acid sequence of V_L chain of antibody TS-57
SEQ ID NO 32: amino acid sequence of V_L chain of antibody TS-58
SEQ ID NO 33: amino acid sequence of V_L chain of antibody TS-63
SEQ ID NO 34: amino acid sequence of V_L chain of antibody TS-64
SEQ ID NO 35: amino acid sequence of consensus of V_L chain of 8E2 antibody and derivatives
SEQ ID NO 36: amino acid sequence of consensus of V_L chain of 8E2 antibody and select derivatives
SEQ ID NO 37: amino acid sequence of V_H chain of antibody 8E2
SEQ ID NO 38: amino acid sequence of V_H chain of antibody TS-66
SEQ ID NO 39: amino acid sequence of V_H chain of antibody TS-69
SEQ ID NO 40: amino acid sequence of V_H chain of antibody TS-71
SEQ ID NO 41: amino acid sequence of V_H chain of antibody TS-76
SEQ ID NO 42: amino acid sequence of V_H chain of antibody TS-79
SEQ ID NO 43: amino acid sequence of V_H chain of antibody TS-82
SEQ ID NO 44: amino acid sequence of V_H chain of antibody TS-88
SEQ ID NO 45: amino acid sequence of V_H chain of antibody TS-89
SEQ ID NO 46: amino acid sequence of V_H chain of antibody TS-91
SEQ ID NO 47: amino acid sequence of V_H chain of antibody TS-92
SEQ ID NO 48: amino acid sequence of V_H chain of antibody TS-97

SEQ ID NO 49: amino acid sequence of V_H chain of antibody TS-101
SEQ ID NO 50: amino acid sequence of V_H chain of antibody TS-103
SEQ ID NO 51: amino acid sequence of V_H chain of antibody TS-104
SEQ ID NO 52: amino acid sequence of V_H chain of antibody TS-107
SEQ ID NO 53: amino acid sequence of V_H chain of antibody TS-108
SEQ ID NO 54: amino acid sequence of V_H chain of antibody TS-115
SEQ ID NO 55: amino acid sequence of V_H chain of antibody TS-129
SEQ ID NO 56: amino acid sequence of V_H chain of antibody TS-133
SEQ ID NO 57: amino acid sequence of V_H chain of antibody TS-134
SEQ ID NO 58: amino acid sequence of V_H chain of antibody TS-135
SEQ ID NO 59: amino acid sequence of V_H chain of antibody TS-136
SEQ ID NO 60: amino acid sequence of V_H chain of antibody TS-140
SEQ ID NO 61: amino acid sequence of V_H chain of antibody TS-143
SEQ ID NO 62: amino acid sequence of V_H chain of antibody TS-151
SEQ ID NO 63: amino acid sequence of V_H chain of antibody TS-156
SEQ ID NO 64: amino acid sequence of V_H chain of antibody TS-213
SEQ ID NO 65: amino acid sequence of V_H chain of antibody TS-214
SEQ ID NO 66: amino acid sequence of V_H chain of antibody TS-215
SEQ ID NO 67: amino acid sequence of V_H chain of antibody TS-218
SEQ ID NO 68: amino acid sequence of V_H chain of antibody TS-221
SEQ ID NO 69: amino acid sequence of V_H chain of antibody TS-222
SEQ ID NO 70: amino acid sequence of V_H chain of antibody TS-224
SEQ ID NO 71: amino acid sequence of consensus of V_H chain of 8E2 antibody and derivatives
SEQ ID NO 72: amino acid sequence of consensus of V_H chain of 8E2 antibody and select derivatives
SEQ ID NO 73: amino acid sequence of V_L chain of antibody 8E4
SEQ ID NO 74: amino acid sequence of V_H chain of antibody 8E4
SEQ ID NO 75: amino acid sequence of V_L chain of antibody 8D10
SEQ ID NO 76: amino acid sequence of V_H chain of antibody 8D10
SEQ ID NO 77: amino acid sequence of consensus of CDR1 of V_L chain of 8E2 antibody and derivatives
SEQ ID NO 78: amino acid sequence of consensus of CDR3 of V_L chain of 8E2 antibody and derivatives
SEQ ID NO 79: amino acid sequence of consensus of CDR1 of V_H chain of 8E2 antibody and derivatives

SEQ ID NO 80: amino acid sequence of consensus of CDR2 of V_H chain of 8E2 antibody and derivatives

SEQ ID NO 81: amino acid sequence of consensus of CDR3 of V_H chain of 8E2 antibody and derivatives

SEQ ID NO 82: amino acid sequence of *Mus musculus* pre-IL-11Rα

SEQ ID NO 83: amino acid sequence of heavy chain of antibody 8E2

SEQ ID NO 84: amino acid sequence of light chain of antibody 8E2

SEQ ID NO 85: amino acid sequence of a polypeptide comprising amino acids 23 to 318 of *Homo sapiens* IL-11Rα (SEQ ID NO: 1), a 8xHIS tag and a serine at a position corresponding to position 248 of SEQ ID NO:1 (also referred to as "WT D1/2")

SEQ ID NO: 86: amino acid sequence of a polypeptide comprising amino acids 23-110 of *Homo sapiens* IL-11Rα (SEQ ID NO: 1) and amino acids 111-367 of *Mus musculus* IL-11Rα (SEQ ID NO: 82) (in which there is a serine at a position corresponding to position 206 of SEQ ID NO: 82) and a 8xHIS tag

SEQ ID NO: 87: amino acid sequence of a polypeptide comprising amino acids 23-215 of *Homo sapiens* IL-11Rα (SEQ ID NO: 1) and amino acids 216-367 of *Mus musculus* IL-11Rα (SEQ ID NO: 82) and a 8xHIS tag

SEQ ID NO: 88: amino acid sequence of a polypeptide comprising amino acids 23-318 of *Homo sapiens* IL-11Rα (SEQ ID NO: 1) (in which there is a serine at a position corresponding to position 248 of SEQ ID NO: 1) and amino acids 319-367 of *Mus musculus* IL-11Rα (SEQ ID NO: 82) and a 8xHIS tag

SEQ ID NO: 89: amino acid sequence of a polypeptide comprising amino acids 24-215 of *Mus musculus* IL-11Rα (SEQ ID NO: 82) (in which there is a serine at a position corresponding to position 206 of SEQ ID NO: 82) and amino acids 216-363 of *Homo sapiens* IL-11Rα (SEQ ID NO: 1) and a 8xHIS tag

SEQ ID NO: 90: amino acid sequence of a polypeptide comprising amino acids 24 to 367 of *Mus musculus* sIL-11Rα (SEQ ID NO: 82), a 8xHIS tag and a serine at a position corresponding to position 206 of SEQ ID NO: 82.

SEQ ID NO 91: amino acid sequence of light chain of antibody 8E4

SEQ ID NO 92: amino acid sequence of heavy chain of antibody 8E4

SEQ ID NO 93: amino acid sequence of light chain of antibody 8D10

SEQ ID NO 94: amino acid sequence of heavy chain of antibody 8D10

SEQ ID NO: 95: amino acid sequence of SEQ ID NO: 85 comprising a glutamic acid at a position corresponding to position 117 of SEQ ID NO: 1.

SEQ ID NO: 96: amino acid sequence of SEQ ID NO: 85 comprising an arginine at a position corresponding to position 66 of SEQ ID NO: 1.

SEQ ID NO: 97: amino acid sequence of SEQ ID NO: 85 comprising a serine at a position corresponding to position 65 of SEQ ID NO: 1.

SEQ ID NO: 98: amino acid sequence of SEQ ID NO: 85 comprising a serine at a position corresponding to position 101 of SEQ ID NO: 1.

SEQ ID NO: 99: amino acid sequence of SEQ ID NO: 85 comprising an alanine at a position corresponding to position 178 of SEQ ID NO: 1.

SEQ ID NO: 100: amino acid sequence of mature *Homo sapiens* IL-11Rα

SEQ ID NO: 101: amino acid sequence of mature *Macaca fascicularis* IL-11 Rα

SEQ ID NO: 102: amino acid sequence of mature *Mus musculus* sIL-11Rα

SEQ ID NO: 103: amino acid sequence of consensus of 8E2L1 as depicted in Figure 1

SEQ ID NO: 104: amino acid sequence of consensus of 8E2L3.1 as depicted in Figure 1

SEQ ID NO: 105: amino acid sequence of consensus of 8E2L3.2 as depicted in Figure 1

SEQ ID NO: 106: amino acid sequence of consensus of 8E2H1 as depicted in Figure 2

SEQ ID NO: 107: amino acid sequence of consensus of 8E2H2 as depicted in Figure 2

SEQ ID NO: 108: amino acid sequence of consensus of 8E2H3.1 as depicted in Figure 2

SEQ ID NO: 109: amino acid sequence of consensus of 8E2H3.2 as depicted in Figure 2

SEQ ID NO: 110: amino acid sequence of antagonistic IL-11 mutein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagrammatic representation showing an alignment of CDRs of the V_L of antibody 8E2 and affinity matured forms thereof. The consensus sequences listed (SEQ ID NOs: 103-105) represents the most commonly occurring amino acid at each position and was determined using the MegAlign software. Other than the consensus sequences, the sequences are derived from the antibodies set out in Table 1.

Figure 2 is a diagrammatic representation showing an alignment of CDRs of the V_H of antibody 8E2 and affinity matured forms thereof. The consensus sequences listed (SEQ ID NOs: 106-109) represents the most commonly occurring amino acid at each position and was determined using the MegAlign software. Other than the consensus sequences, the sequences are derived from the antibodies set out in Table 1.

Figures 3A, B, C and D are diagrammatic representations showing sequences of variable regions of the 8E2 antibody and derivatives. Figure 3A shows sequences of V_L regions of 8E2 and its antibody derivatives and the consensus sequence of V_L

regions of 8E2 and its antibody derivatives. Figure 3B shows sequences of V_L regions of 8E2 and select antibody derivatives and the consensus sequence of V_L regions of 8E2 and select antibody derivatives. Figure 3C shows sequences of V_H regions of 8E2 and its antibody derivatives and the consensus sequence of V_H regions of 8E2 and its antibody derivatives. Figure 3D shows sequences of V_H regions of 8E2 and select antibody derivatives and the consensus sequence of V_H regions of 8E2 and select antibody derivatives. Boxed regions contain CDRs (as indicated) as defined by the Kabat numbering system.

Figure 4 includes a series of graphical representations showing that IL-11 stimulates the phosphorylation of STAT-3 in the human colorectal adenocarcinoma cell line DLD-1 and stomach adenocarcinoma cell line MKN-28, and that an antibody of the disclosure can inhibit this IL-11 mediated phosphorylation. Panels A and B show the level of STAT-3 phosphorylation in DLD-1 and MKN-28 cells, respectively, following stimulation with increasing concentrations of hIL-11 for 15 minutes. Panels C and D show inhibition of IL-11-mediated STAT-3 phosphorylation in DLD-1 and MKN-28 cells, respectively, by increasing concentrations of 8E2 anti-IL-11R antibody (O). In contrast, the BM4 isotype control antibody (Δ) had no effect on IL-11-mediated STAT-3 phosphorylation. (Antibodies were added to cells prior to stimulation with hIL-11 (50 ng/ml) . Mean and standard deviations are shown.)

DETAILED DESCRIPTION

General

Throughout this specification, unless specifically stated otherwise or the context requires otherwise, reference to a single step, composition of matter, group of steps or group of compositions of matter shall be taken to encompass one and a plurality (i.e. one or more) of those steps, compositions of matter, groups of steps or groups of compositions of matter.

Those skilled in the art will appreciate that the present disclosure is susceptible to variations and modifications other than those specifically described. It is to be understood that the disclosure includes all such variations and modifications. The disclosure also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations or any two or more of said steps or features.

The present disclosure is not to be limited in scope by the specific examples described herein, which are intended for the purpose of exemplification only.

Functionally-equivalent products, compositions and methods are clearly within the scope of the present disclosure.

Any example of the present disclosure herein shall be taken to apply *mutatis mutandis* to any other example of the disclosure unless specifically stated otherwise.

Unless specifically defined otherwise, all technical and scientific terms used herein shall be taken to have the same meaning as commonly understood by one of ordinary skill in the art (for example, in cell culture, molecular genetics, immunology, immunohistochemistry, protein chemistry, and biochemistry).

Unless otherwise indicated, the recombinant protein, cell culture, and immunological techniques utilized in the present disclosure are standard procedures, well known to those skilled in the art. Such techniques are described and explained throughout the literature in sources such as, J. Perbal, A Practical Guide to Molecular Cloning, John Wiley and Sons (1984), J. Sambrook *et al.* Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory Press (1989), T.A. Brown (editor), Essential Molecular Biology: A Practical Approach, Volumes 1 and 2, IRL Press (1991), D.M. Glover and B.D. Hames (editors), DNA Cloning: A Practical Approach, Volumes 1-4, IRL Press (1995 and 1996), and F.M. Ausubel *et al.* (editors), Current Protocols in Molecular Biology, Greene Pub. Associates and Wiley-Interscience (1988, including all updates until present), Ed Harlow and David Lane (editors) Antibodies: A Laboratory Manual, Cold Spring Harbour Laboratory, (1988), and J.E. Coligan *et al.* (editors) Current Protocols in Immunology, John Wiley & Sons (including all updates until present).

The description and definitions of variable regions and parts thereof, immunoglobulins, antibodies and fragments thereof herein may be further clarified by the discussion in Kabat *Sequences of Proteins of Immunological Interest*, National Institutes of Health, Bethesda, Md., 1987 and 1991, Bork *et al., J Mol. Biol. 242*, 309-320, 1994, Chothia and Lesk *J. Mol Biol. 196*:901 -917, 1987, Chothia *et al. Nature 342*, 877-883, 1989 and/or or Al-Lazikani *et al., J Mol Biol 273*, 927-948, 1997.

The term "and/or", e.g., "X and/or Y" shall be understood to mean either "X and Y" or "X or Y" and shall be taken to provide explicit support for both meanings or for either meaning.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

As used herein the term "derived from" shall be taken to indicate that a specified integer may be obtained from a particular source albeit not necessarily directly from that source.

Reference herein to a range of, e.g., residues, will be understood to be inclusive. For example, reference to "a region comprising amino acids 56 to 65" will be understood in an inclusive manner, i.e., the region comprises a sequence of amino acids as numbered 56, 57, 58, 59, 60, 61, 62, 63, 64 and 65 in a specified sequence.

Selected Definitions

For the purposes of nomenclature only and not limitation an exemplary sequence of a human precursor IL-11Rα (pre-IL-11Rα) is set out in NCBI Reference Sequence: NP_001136256.1 (and set out in SEQ ID NO: 1). A sequence of a mature human IL-11Rα is set forth in SEQ ID NO: 100. In the case of the sequence set forth in NP_001136256.1, a mature protein lacks amino acids 1 to 22. Positions of amino acids are often referred to herein by reference to pre-IL-11Rα. The positions in mature IL-11Rα is readily determined by accounting for the signal sequence (amino acids 1-22 in the case of SEQ ID NO: 1). An exemplary sequence of a cynomolgus monkey pre-IL-11Rα is set out in SEQ ID NO: 2 and a mature IL-11Rα in SEQ ID NO: 101. An exemplary sequence of a mouse pre-IL-11Rα is set out in SEQ ID NO: 82 and a mature IL-11Rα in SEQ ID NO: 102. The sequence of IL-11Rα from other species can be determined using sequences provided herein and/or in publicly available databases and/or determined using standard techniques (e.g., as described in Ausubel *et al.,* (editors), Current Protocols in Molecular Biology, Greene Pub. Associates and Wiley-Interscience (1988, including all updates until present) or Sambrook *et al.,* Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press (1989)) Reference to human IL-11Rα may be abbreviated to hIL-11Rα, reference to cynomolgus monkey IL-11Rα may be abbreviated to cynoIL-11Rα, and reference to mouse IL-11Rα may be abbreviated to mIL-11Rα. Reference to soluble IL-11Rα refers to polypeptides comprising the extracellular region of IL-11Rα, e.g., amino acids 23-363 or 23-318 of SEQ ID NO: 1. In the present studies soluble forms of the receptor comprising amino acids 23-363 or 23-318 of SEQ ID NO: 1 with a serine substitution at position 248 were used (e.g. SEQ ID NO: 3 or 85) and the corresponding segment of mIL-11Rα with the serine substitution at a position corresponding to position 206 of SEQ ID NO: 82 (e.g., SEQ ID NO 90) for studies on the mouse receptor. These serine mutations were introduced into the soluble polypeptides to improve expression and prevent aggregation of the polypeptides. Various point

mutations of the soluble receptor of SEQ ID NO: 3 and SEQ ID NO: 85 have also been utilized (e.g., see SEQ ID NOs: 95-99). These soluble polypeptides are representative of hIL-11Rα or mIL-11Rα as demonstrated by the ability of the IL-11Rα-binding proteins of the disclosure to bind to the relevant receptor when expressed on the surface of a cell. Accordingly, studies using the mutant polypeptides are a model of studies using hIL-11Rα and/or mIL-11Rα.

Reference herein to IL-11 includes native forms of IL-11 and mutant forms thereof retaining an ability to bind to IL-11Rα (e.g., hIL-11Rα) and induce signaling.

Reference herein to a particular domain of hIL-11Rα will be understood to mean the following:

- Immunoglobulin-like (IG-like) domain: amino acids 23-110 of SEQ ID NO: 1;
- First fibronectin III domain: amino acids 111-215 of SEQ ID NO: 1;
- Second fibronectin III domain: amino acids 216-370 of SEQ ID NO: 1;
- Transmembrane domain: amino acids 371-391 of SEQ ID NO: 1; and
- Cytoplasmic domain: amino acids 392-422 of SEQ ID NO: 1.

As used herein, the term "hIL-11 mutein" includes a mutant form of hIL-11 in which wildtype residues at positions 58-62 (AMSAG) are replaced with PAIDY and the tryptophan at residue 147 is replaced with an alanine (W147A). For example, the mutein comprises or consists of a sequence set forth in SEQ ID NO: 110. Other examples of hIL-11 muteins can be found in WO2009/052588. Optionally, the mutein comprises an additional sequence, e.g., a hexa-HIS tag.

The term "isolated protein" or "isolated polypeptide" is a protein or polypeptide that by virtue of its origin or source of derivation is not associated with naturally-associated components that accompany it in its native state; is substantially free of other proteins from the same source. A protein may be rendered substantially free of naturally associated components or substantially purified by isolation, using protein purification techniques known in the art. By "substantially purified" is meant the protein is substantially free of contaminating agents, e.g., at least about 70% or 75% or 80% or 85% or 90% or 95% or 96% or 97% or 98% or 99% free of contaminating agents.

The term "recombinant" shall be understood to mean the product of artificial genetic recombination. Accordingly, in the context of a recombinant protein comprising an antibody antigen binding domain, this term does not encompass an antibody naturally-occurring within a subject's body that is the product of natural recombination that occurs during B cell maturation. However, if such an antibody is isolated, it is to be considered an isolated protein comprising an antibody antigen

binding domain. Similarly, if nucleic acid encoding the protein is isolated and expressed using recombinant means, the resulting protein is a recombinant protein comprising an antibody antigen binding domain. A recombinant protein also encompasses a protein expressed by artificial recombinant means when it is within a cell, tissue or subject, e.g., in which it is expressed.

The term "protein" shall be taken to include a single polypeptide chain, i.e., a series of contiguous amino acids linked by peptide bonds or a series of polypeptide chains covalently or non-covalently linked to one another (i.e., a polypeptide complex). For example, the series of polypeptide chains can be covalently linked using a suitable chemical or a disulphide bond. Examples of non-covalent bonds include hydrogen bonds, ionic bonds, Van der Waals forces, and hydrophobic interactions.

The term "polypeptide" or "polypeptide chain" will be understood from the foregoing paragraph to mean a series of contiguous amino acids linked by peptide bonds.

As used herein, the term "antigen binding domain" shall be taken to mean a region of an antibody that is capable of specifically binding to an antigen, i.e., a V_H or a V_L or an Fv comprising both a V_H and a V_L. The antigen binding domain need not be in the context of an entire antibody, e.g., it can be in isolation (e.g., a domain antibody) or in another form, e.g., as described herein, such as a scFv.

For the purposes for the present disclosure, the term "antibody" includes a protein capable of specifically binding to one or a few closely related antigens (e.g., IL-11Rα) by virtue of an antigen binding domain contained within a Fv. This term includes four chain antibodies (e.g., two light chains and two heavy chains), recombinant or modified antibodies (e.g., chimeric antibodies, humanized antibodies, human antibodies, CDR-grafted antibodies, primatized antibodies, de-immunized antibodies, synhumanized antibodies, half-antibodies, bispecific antibodies). An antibody generally comprises constant domains, which can be arranged into a constant region or constant fragment or fragment crystallizable (Fc). Exemplary forms of antibodies comprise a four-chain structure as their basic unit. Full-length antibodies comprise two heavy chains (~50 to 70 kD) covalently linked and two light chains (~23 kDa each). A light chain generally comprises a variable region (if present) and a constant domain and in mammals is either a κ light chain or a λ light chain. A heavy chain generally comprises a variable region and one or two constant domain(s) linked by a hinge region to additional constant domain(s). Heavy chains of mammals are of one of the following types α, δ, ε, γ, or μ. Each light chain is also covalently linked to one of the heavy chains. For example, the two heavy chains and the heavy and light

chains are held together by inter-chain disulfide bonds and by non-covalent interactions. The number of inter-chain disulfide bonds can vary among different types of antibodies. Each chain has an N-terminal variable region (V_H or V_L wherein each are ~110 amino acids in length) and one or more constant domains at the C- terminus. The constant domain of the light chain (C_L which is ~110 amino acids in length) is aligned with and disulfide bonded to the first constant domain of the heavy chain (C_H1 which is 330 to 440 amino acids in length). The light chain variable region is aligned with the variable region of the heavy chain. The antibody heavy chain can comprise 2 or more additional C_H domains (such as, C_H2, C_H3 and the like) and can comprise a hinge region between the C_H1 and C_H2 constant domains. Antibodies can be of any type (e.g., IgG, IgE, IgM, IgD, IgA, and IgY), class (e.g., IgG_1, IgG_2, IgG_3, IgG_4, IgA_1 and IgA_2) or subclass. In one example, the antibody is a murine (mouse or rat) antibody or a primate (such as, human) antibody. In one example the antibody heavy chain is missing a C-terminal lysine residue. In one example, the antibody is humanized, synhumanized, chimeric, CDR-grafted or deimmunized.

The terms "full-length antibody," "intact antibody" or "whole antibody" are used interchangeably to refer to an antibody in its substantially intact form, as opposed to an antigen binding fragment of an antibody. Specifically, whole antibodies include those with heavy and light chains including an Fc region. The constant domains may be wild-type sequence constant domains (e.g., human wild-type sequence constant domains) or amino acid sequence variants thereof.

As used herein, "variable region" refers to the portions of the light and/or heavy chains of an antibody as defined herein that is capable of specifically binding to an antigen and, includes amino acid sequences of complementarity determining regions (CDRs); i.e., CDRl, CDR2, and CDR3, and framework regions (FRs). For example, the variable region comprises three or four FRs (e.g., FR1, FR2, FR3 and optionally FR4) together with three CDRs. V_H refers to the variable region of the heavy chain. V_L refers to the variable region of the light chain.

As used herein, the term "complementarity determining regions" (*syn.* CDRs; i.e., CDRl, CDR2, and CDR3) refers to the amino acid residues of an antibody variable region the presence of which are major contributors to specific antigen binding. Each variable region domain (V_H or V_L) typically has three CDRs identified as CDRl, CDR2 and CDR3. In one example, the amino acid positions assigned to CDRs and FRs are defined according to Kabat *Sequences of Proteins of Immunological Interest*, National Institutes of Health, Bethesda, Md., 1987 and 1991 (also referred to herein as "the Kabat numbering system"). In another example, the amino acid positions assigned to CDRs and FRs are defined according to the Enhanced Chothia Numbering Scheme

(http://www.bioinfo.org.uk/mdex.html). According to the numbering system of Kabat, V_H FRs and CDRs are positioned as follows: residues 1 to 30 (FR1), 31 to 35 (CDR1), 36 to 49 (FR2), 50 to 65 (CDR2), 66 to 94 (FR3), 95 to 102 (CDR3) and 103 to 113 (FR4). According to the numbering system of Kabat, V_L FRs and CDRs are positioned as follows: residues 1 to 23 (FR1), 24 to 34 (CDR1), 35 to 49 (FR2), 50 to 56 (CDR2), 57 to 88 (FR3), 89 to 97 (CDR3) and 98 to 107 (FR4). The present disclosure is not limited to FRs and CDRs as defined by the Kabat numbering system, but includes all numbering systems, including the canonical numbering system or of Chothia and Lesk *J. Mol. Biol. 196*: 901-917, 1987; Chothia *et al., Nature 342*: 877-883, 1989; and/or Al-Lazikani *et al., J. Mol. Biol. 273*: 927-948, 1997; the numbering system of Honnegher and Plükthun *J. Mol. Biol. 309*: 657-670, 2001; or the IMGT system discussed in Giudicelli *et al., Nucleic Acids Res. 25*: 206-211 1997. In one example, the CDRs are defined according to the Kabat numbering system. Optionally, heavy chain CDR2 according to the Kabat numbering system does not comprise the five C-terminal amino acids listed herein or any one or more of those amino acids are substituted with another naturally-occurring amino acid. In this regard, Padlan *et al., FASEB J., 9*: 133-139, 1995 established that the five C-terminal amino acids of heavy chain CDR2 are not generally involved in antigen binding.

"Framework regions" (FRs) are those variable region residues other than the CDR residues.

As used herein, the term "Fv" shall be taken to mean any protein, whether comprised of multiple polypeptides or a single polypeptide, in which a V_L and a V_H associate and form a complex having an antigen binding domain, i.e., capable of specifically binding to an antigen. The V_H and the V_L which form the antigen binding domain can be in a single polypeptide chain or in different polypeptide chains. Furthermore, an Fv of the disclosure (as well as any protein of the disclosure) may have multiple antigen binding domains which may or may not bind the same antigen. This term shall be understood to encompass fragments directly derived from an antibody as well as proteins corresponding to such a fragment produced using recombinant means. In some examples, the V_H is not linked to a heavy chain constant domain (C_H) 1 and/or the V_L is not linked to a light chain constant domain (C_L). Exemplary Fv containing polypeptides or proteins include a Fab fragment, a Fab' fragment, a F(ab') fragment, a scFv, a diabody, a triabody, a tetrabody or higher order complex, or any of the foregoing linked to a constant region or domain thereof, e.g., C_H2 or C_H3 domain, e.g., a minibody. A "Fab fragment" consists of a monovalent antigen-binding fragment of an immunoglobulin, and can be produced by digestion of a whole antibody with the enzyme papain, to yield a fragment consisting of an intact light chain and a portion of a

heavy chain or can be produced using recombinant means. A "Fab' fragment" of an antibody can be obtained by treating a whole antibody with pepsin, followed by reduction, to yield a molecule consisting of an intact light chain and a portion of a heavy chain comprising a V_H and a single constant domain. Two Fab' fragments are obtained per antibody treated in this manner. A Fab' fragment can also be produced by recombinant means. A "F(ab')2 fragment" of an antibody consists of a dimer of two Fab' fragments held together by two disulfide bonds, and is obtained by treating a whole antibody molecule with the enzyme pepsin, without subsequent reduction. A "Fab_2" fragment is a recombinant fragment comprising two Fab fragments linked using, for example a leucine zipper or a C_H3 domain. A "single chain Fv" or "scFv" is a recombinant molecule containing the variable region fragment (Fv) of an antibody in which the variable region of the light chain and the variable region of the heavy chain are covalently linked by a suitable, flexible polypeptide linker.

As used herein, the term "binds" in reference to the interaction of an IL-11Rα-binding protein or an antigen binding domain thereof with an antigen means that the interaction is dependent upon the presence of a particular structure (e.g., an antigenic determinant or epitope) on the antigen. For example, an antibody recognizes and binds to a specific protein structure rather than to proteins generally. If an antibody binds to epitope "A", the presence of a molecule containing epitope "A" (or free, unlabeled "A"), in a reaction containing labeled "A" and the protein, will reduce the amount of labeled "A" bound to the antibody.

As used herein, the term "specifically binds" or "binds specifically" shall be taken to mean that an IL-11Rα-binding protein of the disclosure reacts or associates more frequently, more rapidly, with greater duration and/or with greater affinity with a particular antigen or cell expressing same than it does with alternative antigens or cells. For example, an IL-11Rα-binding protein binds to IL-11Rα (e.g., hIL-11Rα or a polypeptide comprising a region thereof, e.g., a polypeptide of SEQ ID NO: 3 or 85) with materially greater affinity (e.g., 1.5 fold or 2 fold or 5 fold or 10 fold or 20 fold or 40 fold or 60 fold or 80 fold to 100 fold or 150 fold or 200 fold) than it does to other interleukin receptors or to antigens commonly recognized by polyreactive natural antibodies (i.e., by naturally occurring antibodies known to bind a variety of antigens naturally found in humans). In an example of the present disclosure, an IL-11Rα-binding protein that "specifically binds" to one form of hIL-11Rα or a polypeptide comprising a region thereof (e.g., the extracellular region of hIL-11Rα) or a polypeptide a sequence set forth in SEQ ID NO: 3 or 85 with an affinity at least 1.5 fold or 2 fold or greater (e.g., 5 fold or 10 fold or 20 fold r 50 fold or 100 fold or 200

fold) than it does to a mutant form of SEQ ID NO: 3 comprising a sequence set forth in SEQ ID NO: 95. Generally, but not necessarily, reference to binding means specific binding, and each term shall be understood to provide explicit support for the other term.

As used herein, the term "does not detectably bind" shall be understood to mean that an IL-11Rα-binding protein, e.g., an antibody, binds to a candidate antigen at a level less than 10%, or 8% or 6% or 5% above background. The background can be the level of binding signal detected in the absence of the protein and/or in the presence of a negative control protein (e.g., an isotype control antibody) and/or the level of binding detected in the presence of a negative control antigen. The level of binding is detected using biosensor analysis (e.g. Biacore) in which the IL-11Rα-binding protein is immobilized and contacted with an antigen.

As used herein, the term "does not significantly bind" shall be understood to mean that the level of binding of an IL-11Rα-binding protein of the disclosure to a polypeptide is not statistically significantly higher than background, e.g., the level of binding signal detected in the absence of the IL-11Rα-binding protein and/or in the presence of a negative control protein (e.g., an isotype control antibody) and/or the level of binding detected in the presence of a negative control polypeptide. The level of binding is detected using biosensor analysis (e.g. Biacore) in which the IL-11Rα-binding protein is immobilized and contacted with an antigen.

As used herein, phrases referring to "reduced binding" or "binding being at a lower level" in relation to an antigen will be understood to mean that an IL-11Rα-binding protein, e.g., antibody, binds to an antigen (e.g., a mutant of SEQ ID NO: 3 as described herein, such as a mutant comprising the sequence set forth in SEQ ID NO: 95) with an affinity at least about 1.5 fold or 2 fold or 5 fold or 10 fold or 20 fold or 50 fold or 100 fold or 200 fold less than a control epitope or antigen (e.g. SEQ ID NO: 3).

An IL-11Rα-binding protein or antibody may be considered to "preferentially bind" to a polypeptide if it binds that polypeptide with a dissociation constant (K_D) that is less than the protein's or antibody's K_D for another polypeptide. In one example, an IL-11Rα-binding protein or antibody is considered to preferentially bind to a polypeptide if it binds the polypeptide with an affinity (i.e., K_D) that is at least about 1.5 fold or 2 fold or 5 fold or 10 fold or 20 fold or 50 fold or 100 fold or 200 fold more than the protein's or antibody's K_D for another polypeptide.

As used herein, the term "capable of binding to hIL-11Rα and cynoIL-11Rα" will be understood to mean that an IL-11Rα-binding protein cross-reacts with hIL-11Rα and cynoIL-11Rα, i.e., binding to either protein.

For the purposes of clarification and as will be apparent to the skilled artisan based on the exemplified subject matter herein, reference to "affinity" in this specification is a reference to K_D of a protein or antibody.

For the purposes of clarification and as will be apparent to the skilled artisan based on the description herein, reference to an "affinity of at least about" will be understood to mean that the affinity (or K_D) is equal to the recited value or higher (i.e., the value recited as the affinity is lower), i.e., an affinity of 2nM is greater than an affinity of 3nM. Stated another way, this term could be "an affinity of X or less", wherein X is a value recited herein.

An "IC_{50} of at least about" will be understood to mean that the IC_{50} is equal to the recited value or lower (i.e., the value recited as the IC_{50} is lower), i.e., an IC_{50} of 2μg/ml is greater than an IC_{50} of 1μg/ml. Stated another way, this term could be "an IC_{50} of X or less", wherein X is a value recited herein.

As used herein, the term "epitope" (*syn.* "antigenic determinant") shall be understood to mean a region of IL-11Rα to which an IL-11Rα-binding protein comprising an antigen binding domain of an antibody binds. This term is not necessarily limited to the specific residues or structure to which the IL-11Rα-binding protein makes contact. For example, this term includes the region spanning amino acids contacted by the IL-11Rα-binding protein and 5-10 (or more) or 2-5 or 1-3 amino acids outside of this region. In some examples, the epitope comprises a series of discontinuous amino acids that are positioned close to one another when IL-11Rα-binding protein is folded, i.e., a "conformational epitope". The skilled artisan will also be aware that the term "epitope" is not limited to peptides or polypeptides. For example, the term "epitope" includes chemically active surface groupings of molecules such as sugar side chains, phosphoryl side chains, or sulfonyl side chains, and, in certain examples, may have specific three dimensional structural characteristics, and/or specific charge characteristics.

The term "competitively inhibits" shall be understood to mean that an IL-11Rα-binding protein of the disclosure (or an antigen binding domain thereof) reduces or prevents binding of a recited antibody or IL-11Rα-binding protein to IL-11Rα, e.g., to hIL-11Rα. This may be due to the IL-11Rα-binding protein (or antigen binding domain) and antibody binding to the same or an overlapping epitope. It will be apparent from the foregoing that the IL-11Rα-binding protein need not completely inhibit binding of the antibody, rather it need only reduce binding by a statistically significant amount, for example, by at least about 10% or 20% or 30% or 40% or 50% or 60% or 70% or 80% or 90% or 95%. Preferably, the IL-11Rα-binding protein

reduces binding of the antibody by at least about 30%, more preferably by at least about 50%, more preferably, by at least about 70%, still more preferably by at least about 75%, even more preferably, by at least about 80% or 85% and even more preferably, by at least about 90%. Methods for determining competitive inhibition of binding are known in the art and/or described herein. For example, the antibody is exposed to IL-11Rα either in the presence or absence of the IL-11Rα-binding protein. If less antibody binds in the presence of the IL-11Rα-binding protein than in the absence of the IL-11Rα-binding protein, the protein is considered to competitively inhibit binding of the antibody. In one example, the competitive inhibition is not due to steric hindrance.

"Overlapping" in the context of two epitopes shall be taken to mean that two epitopes share a sufficient number of amino acid residues to permit an IL-11Rα-binding protein (or antigen binding domain thereof) that binds to one epitope to competitively inhibit the binding of an IL-11Rα-binding protein (or antigen binding domain) that binds to the other epitope. For example, the "overlapping" epitopes share at least 1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 or 9 or 20 amino acids.

As used herein, the term "neutralize" shall be taken to mean that a protein is capable of blocking, reducing or preventing IL-11-mediated signaling in a cell through the IL-11Rα. Methods for determining neutralization are known in the art and/or described herein.

As used herein, the term "condition" refers to a disruption of or interference with normal function, and is not to be limited to any specific condition, and will include diseases or disorders.

As used herein, an "IL-11-associated condition" refers to any condition that is caused by or associated with an excess of IL-11 or cells expressing IL-11 or with administration of IL-11. The skilled artisan will be readily able to determine such conditions. Exemplary conditions are described herein.

As used herein, the terms "preventing", "prevent" or "prevention" include administering an IL-11Rα-binding protein of the disclosure to thereby stop or hinder the development of at least one symptom of a condition. This term also encompasses treatment of a subject in remission to prevent or hinder relapse.

As used herein, the terms "treating", "treat" or "treatment" include administering an IL-11Rα-binding protein described herein to thereby reduce or eliminate at least one symptom of a specified disease or condition.

As used herein, the term "subject" shall be taken to mean any animal including humans, for example a mammal. Exemplary subjects include but are not limited to humans and non-human primates. For example, the subject is a human.

Antibodies

In one example, an IL-11Rα-binding protein as described herein according to any example is an antibody.

Methods for generating antibodies are known in the art and/or described in Harlow and Lane (editors) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, (1988). Generally, in such methods IL-11Rα (e.g., hIL-11Rα) or a region thereof (e.g., an extracellular region) or immunogenic fragment or epitope thereof or a cell expressing and displaying same (i.e., an immunogen), optionally formulated with any suitable or desired carrier, adjuvant, or pharmaceutically acceptable excipient, is administered to a non-human animal, for example, a mouse, chicken, rat, rabbit, guinea pig, dog, horse, cow, goat or pig. The immunogen may be administered intranasally, intramuscularly, sub-cutaneously, intravenously, intradermally, intraperitoneally, or by other known route.

The production of polyclonal antibodies may be monitored by sampling blood of the immunized animal at various points following immunization. One or more further immunizations may be given, if required to achieve a desired antibody titer. The process of boosting and titering is repeated until a suitable titer is achieved. When a desired level of immunogenicity is obtained, the immunized animal is bled and the serum isolated and stored, and/or the animal is used to generate monoclonal antibodies (mAbs).

Monoclonal antibodies are one exemplary form of antibody contemplated by the present disclosure. The term "monoclonal antibody" or "mAb" refers to a homogeneous antibody population capable of binding to the same antigen(s), for example, to the same epitope within the antigen. This term is not intended to be limited with regard to the source of the antibody or the manner in which it is made.

For the production of mAbs any one of a number of known techniques may be used, such as, for example, the procedure exemplified in US4196265 or Harlow and Lane (1988), *supra*.

For example, a suitable animal is immunized with an immunogen under conditions sufficient to stimulate antibody producing cells. Rodents such as rabbits, mice and rats are exemplary animals. Mice genetically-engineered to express human

antibodies and, for example, do not express murine antibodies, can also be used to generate an antibody of the present disclosure (e.g., as described in WO2002/066630).

Following immunization, somatic cells with the potential for producing antibodies, specifically B lymphocytes (B cells), are selected for use in the mAb generating protocol. These cells may be obtained from biopsies of spleens, tonsils or lymph nodes, or from a peripheral blood sample. The B cells from the immunized animal are then fused with cells of an immortal myeloma cell, generally derived from the same species as the animal that was immunized with the immunogen.

Hybrids are amplified by culture in a selective medium comprising an agent that blocks the *de novo* synthesis of nucleotides in the tissue culture media. Exemplary agents are aminopterin, methotrexate and azaserine.

The amplified hybridomas are subjected to a functional selection for antibody specificity and/or titer, such as, for example, by flow cytometry and/or immunohistochemstry and/or immunoassay (e.g. radioimmunoassay, enzyme immunoassay, cytotoxicity assay, plaque assay, dot immunoassay, and the like).

Alternatively, ABL-MYC technology (NeoClone, Madison WI 53713, USA) is used to produce cell lines secreting MAbs (e.g., as described in Largaespada *et al, J. Immunol. Methods. 197*: 85-95, 1996).

Antibodies can also be produced or isolated by screening a display library, e.g., a phage display library, e.g., as described in US6300064 and/or US5885793. For example, the present inventors have isolated fully human antibodies from a phage display library.

As described herein, some IL-11Rα-binding proteins of the present disclosure that bind hIL-11Rα cross-react with cynoIL-11Rα and/or bind to some mutant forms of hIL-11Rα or polypeptides comprising regions of hIL-11Rα that have been mutated and/or not others. These characteristics can be used in the generation of an antibody or an IL-11Rα-binding protein.

For example, a phage display library is screened with a polypeptide comprising SEQ ID NO: 3 to identify proteins that bind thereto. Mutant forms of the polypeptide (e.g., wherein valine at a position corresponding to position 117 of SEQ ID NO: 1 is substituted with glutamic acid (e.g., comprising a sequence of SEQ ID NO: 95)) to which the IL-11Rα-binding protein is not to detectably bind are then used to remove cross-reactive proteins and/or mutant forms of the polypeptide (e.g., comprising a sequence of SEQ ID NO: 97, 98 or 99) to which the IL-11Rα-binding protein is to bind are used to isolate proteins that are correctly cross-reactive. A screening process for immunization of a non-human mammal can also be devised based on the foregoing.

In another example, a phage display library is screened or an animal is immunized with a polypeptide comprising the extracellular domain (or a region corresponding to amino acids 23-215 or 110-215 of SEQ ID NO: 1) of cynoIL-11Rα and identified IL-11Rα-binding protein and/or antibodies are screened to identify those that are cross-reactive with hIL-11Rα and/or a polypeptide of SEQ ID NO: 3 and/or 85.

In a further example, an IL-11Rα or an extracellular region thereof (optionally a mutant form to which 8E2 or 8D10 or 8E4 binds) is contacted with one of the foregoing antibodies. A phage display library is then brought into contact with the IL-11Rα or region and phage expressing proteins that can compete with the antibody for binding selected.

In a still further example, a chimeric protein comprising, e.g., a mouse IL-11Rα in which an epitope of interest from a hIL-11Rα is substituted for the corresponding mouse sequence. This chimeric protein is then used to immunize mice (which are less likely to induce an immune response against the mouse protein) and/or to screen a phage display library. The resulting antibodies/proteins are then screened to identify those that bind to hIL-11Rα (particularly at the epitope of interest) and not mouse IL-11Rα.

The antibody of the present disclosure may be a synthetic antibody. For example, the antibody is a chimeric antibody, a humanized antibody, a human antibody synhumanized antibody, primatized antibody or a de-immunized antibody.

Deimmunized, Chimeric, CDR Grafted, Humanized, Synhumanized, Primatized, Human and Composite IL-11Rα-Binding Proteins

The IL-11Rα-binding proteins of the present disclosure may be CDR grafted proteins which include CDRs from an antibody from a non-human species (e.g., mouse or rat or non-human primate) grafted onto or inserted into FRs from a human antibody or which include CDRs from an antibody from one type of antibody (e.g., one type of human antibody) grafted onto or inserted into FRs from another type of antibody (e.g., another type of human antibody). This term also encompasses a composite IL-11Rα-binding protein comprising, for example, one or more CDR grafted variable regions and one or more, e.g., human variable regions, chimeric variable regions, synhumanized variable regions or primatized variable regions.

The IL-11Rα-binding proteins of the present disclosure may be a humanized protein.

The term "humanized protein" shall be understood to refer to a protein comprising a human-like variable region, which includes CDRs from an antibody from a non-human species (e.g., mouse or rat or non-human primate) grafted onto or inserted

into FRs from a human antibody (this type of antibody is falls within the class of "CDR-grafted antibody"). Humanized IL-11Rα-binding proteins also include proteins in which one or more residues of the human protein are modified by one or more amino acid substitutions and/or one or more FR residues of the human protein are replaced by corresponding non-human residues. Humanized proteins may also comprise residues which are found in neither the human antibody or in the non-human antibody. Any additional regions of the protein (e.g., Fc region) are generally human. Humanization can be performed using a method known in the art, e.g., US5225539, US6054297, US7566771 or US5585089. The term "humanized protein" also encompasses a super-humanized protein, e.g., as described in US7732578. This term also encompasses a composite protein comprising, for example, one or more humanized variable regions and one or more, e.g., human variable regions, chimeric variable regions, synhumanized variable regions or primatized variable regions.

The IL-11Rα-binding proteins of the present disclosure may be human IL-11Rα-binding proteins. The term "human protein" as used herein refers to proteins having variable and, optionally, constant antibody regions found in humans, e.g. in the human germline or somatic cells or from libraries produced using such regions. The "human" proteins can include amino acid residues not encoded by human sequences, e.g. mutations introduced by random or site directed mutations *in vitro* (in particular mutations which involve conservative substitutions or mutations in a small number of residues of the protein, e.g. in 1, 2, 3, 4 or 5 of the residues of the protein). These "human proteins" do not necessarily need to be generated as a result of an immune response of a human, rather, they can be generated using recombinant means (e.g., screening a phage display library) and/or by a transgenic animal (e.g., a mouse) comprising nucleic acid encoding human antibody constant and/or variable regions and/or using guided selection (e.g., as described in US5565332). This term also encompasses affinity matured forms of such antibodies. For the purposes of the present disclosure, a human protein will also be considered to include a protein comprising FRs from a human antibody or FRs comprising sequences from a consensus sequence of human FRs and in which one or more of the CDRs are random or semi-random, e.g., as described in US6300064 and/or US6248516.

Exemplary human IL-11Rα-binding proteins are antibodies comprising the following pairs of variable regions:

(i) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(ii) a V_H comprising a sequence set forth in SEQ ID NO: 38 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(iii) a V_H comprising a sequence set forth in SEQ ID NO: 39 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(iv) a V_H comprising a sequence set forth in SEQ ID NO: 40 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(v) a V_H comprising a sequence set forth in SEQ ID NO: 41 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(vi) a V_H comprising a sequence set forth in SEQ ID NO: 42 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(vii) a V_H comprising a sequence set forth in SEQ ID NO: 43 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(viii) a V_H comprising a sequence set forth in SEQ ID NO: 44 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(ix) a V_H comprising a sequence set forth in SEQ ID NO: 45 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(x) a V_H comprising a sequence set forth in SEQ ID NO: 46 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xi) a V_H comprising a sequence set forth in SEQ ID NO: 47 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xii) a V_H comprising a sequence set forth in SEQ ID NO: 48 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xiii) a V_H comprising a sequence set forth in SEQ ID NO: 49 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xiv) a V_H comprising a sequence set forth in SEQ ID NO: 50 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xv) a V_H comprising a sequence set forth in SEQ ID NO: 51 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xvi) a V_H comprising a sequence set forth in SEQ ID NO: 52 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xvii) a V_H comprising a sequence set forth in SEQ ID NO: 53 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xviii) a V_H comprising a sequence set forth in SEQ ID NO: 54 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xix) a V_H comprising a sequence set forth in SEQ ID NO: 55 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xx) a V_H comprising a sequence set forth in SEQ ID NO: 56 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xxi) a V_H comprising a sequence set forth in SEQ ID NO: 57 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xxii) a V_H comprising a sequence set forth in SEQ ID NO: 58 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xxiii) a V_H comprising a sequence set forth in SEQ ID NO: 59 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xxiv) a V_H comprising a sequence set forth in SEQ ID NO: 60 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xxv) a V_H comprising a sequence set forth in SEQ ID NO: 61 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xxvi) a V_H comprising a sequence set forth in SEQ ID NO: 62 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xxvii) a V_H comprising a sequence set forth in SEQ ID NO: 63 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xxviii) a V_H comprising a sequence set forth in SEQ ID NO: 64 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xxix) a V_H comprising a sequence set forth in SEQ ID NO: 65 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xxx) a V_H comprising a sequence set forth in SEQ ID NO: 66 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xxxi) a V_H comprising a sequence set forth in SEQ ID NO: 67 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xxxii) a V_H comprising a sequence set forth in SEQ ID NO: 68 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xxxiii) a V_H comprising a sequence set forth in SEQ ID NO: 69 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xxxiv) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xxxv) a V_H comprising a sequence set forth in SEQ ID NO: 70 and a V_L comprising a sequence set forth in SEQ ID NO: 5;

(xxxvi) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 6;

(xxxvii) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 7;

(xxxviii) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 8;

(xxxix) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 9;

(xl) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 10;

(xli) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 11;

(xlii) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 12;

(xliii) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 13;

(xliv) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 14;

(xlv) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 15;

(xlvi) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 16;

(xlvii) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 17;

(xlviii)a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 18;

(xlix) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 19;

(l) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 20;

(li) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 21;

(lii) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 22;

(liii) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 23;

(liv) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 24;

(lv) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 25;

(lvi) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 26;

(lvii) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 27;

(lviii) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 28;

(lix) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 29;

(lx) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 30;

(lxi) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 31;

(lxii) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 32;

(lxiii) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 33; or

(lxiv) a V_H comprising a sequence set forth in SEQ ID NO: 37 and a V_L comprising a sequence set forth in SEQ ID NO: 34.

Optionally, the V_H is linked to a heavy chain constant region, e.g., an IgG4 heavy chain constant region. In one example, the heavy chain constant region lacks the C-terminal lysine residue.

Optionally, the V_L is linked to a light chain constant region.

The IL-11Rα-binding proteins of the present disclosure may be synhumanized proteins. The term "synhumanized protein" refers to a protein prepared by a method described in WO2007/019620. A synhumanized IL-11Rα-binding protein includes a variable region of an antibody, wherein the variable region comprises FRs from a New World primate antibody variable region and CDRs from a non-New World primate antibody variable region. For example, a synhumanized IL-11Rα-binding protein includes a variable region of an antibody, wherein the variable region comprises FRs from a New World primate antibody variable region and CDRs from a mouse or rat antibody. In one example, the synhumanized IL-11Rα-binding protein is a IL-11Rα-binding antibody in which one or both of the variable regions are synhumanized. This term also encompasses a composite protein comprising, for example, one or more synhumanized variable regions and one or more, e.g., human variable regions or humanized variable regions or chimeric variable regions.

The IL-11Rα-binding proteins of the present disclosure may be primatized proteins. A "primatized protein" comprises variable region(s) from an antibody generated following immunization of a non-human primate (e.g., a cynomolgus macaque). Optionally, the variable regions of the non-human primate antibody are

linked to human constant regions to produce a primatized antibody. Exemplary methods for producing primatized antibodies are described in US6113898. This term also encompasses a composite protein comprising, for example, one or more primatized variable regions and one or more, e.g., human variable regions or humanized variable regions or chimeric variable regions.

In one example an IL-11Rα-binding protein of the disclosure is a chimeric protein. The term "chimeric proteins" refers to proteins in which an antigen binding domain is from a particular species (e.g., murine, such as mouse or rat) or belonging to a particular antibody class or subclass, while the remainder of the protein is from a protein derived from another species (such as, for example, human or non-human primate) or belonging to another antibody class or subclass. In one example, a chimeric protein is a chimeric antibody comprising a V_H and/or a V_L from a non-human antibody (e.g., a murine antibody) and the remaining regions of the antibody are from a human antibody. The production of such chimeric proteins is known in the art, and may be achieved by standard means (as described, e.g., in US6331415; US5807715; US4816567 and US4816397). This term also encompasses a composite protein comprising, for example, one or more chimeric variable regions and one or more, e.g., human variable regions or humanized variable regions or chimeric variable regions.

The present disclosure also contemplates a deimmunized IL-11Rα-binding protein, e.g., as described in WO2000/34317 and WO2004/108158. De-immunized antibodies and proteins have one or more epitopes, e.g., B cell epitopes or T cell epitopes removed (i.e., mutated) to thereby reduce the likelihood that a subject will raise an immune response against the antibody or protein. For example, an IL-11Rα-binding protein of the disclosure is analyzed to identify one or more B or T cell epitopes and one or more amino acid residues within the epitope is mutated to thereby reduce the immunogenicity of the IL-11Rα-binding protein.

It will be apparent to the skilled artisan from the foregoing disclosure that a "composite" protein comprises one form of V_H (e.g., human) and another form of V_L (e.g., humanized). The present disclosure explicitly encompasses all combinations of forms of V_H and V_L.

Antibody Binding Domain Containing Proteins

Single-Domain Antibodies

In some examples, a protein of the disclosure is or comprises a single-domain antibody (which is used interchangeably with the term "domain antibody" or "dAb"). A single-domain antibody is a single polypeptide chain comprising all or a portion of the heavy chain variable region of an antibody. In certain examples, a single-domain

antibody is a human single-domain antibody (Domantis, Inc., Waltham, MA; see, e.g., US6248516).

Diabodies, Triabodies, Tetrabodies

In some examples, a protein of the disclosure is or comprises a diabody, triabody, tetrabody or higher order protein complex such as those described in WO98/044001 and/or WO94/007921.

For example, a diabody is a protein comprising two associated polypeptide chains, each polypeptide chain comprising the structure V_L-X-V_H or V_H-X-V_L, wherein V_L is an antibody light chain variable region, V_H is an antibody heavy chain variable region, X is a linker comprising insufficient residues to permit the V_H and V_L in a single polypeptide chain to associate (or form an Fv) or is absent, and wherein the V_H of one polypeptide chain binds to a V_L of the other polypeptide chain to form an antigen binding domain, i.e., to form a Fv molecule capable of specifically binding to one or more antigens. The V_L and V_H can be the same in each polypeptide chain or the V_L and V_H can be different in each polypeptide chain so as to form a bispecific diabody (i.e., comprising two Fvs having different specificity).

Single Chain Fv (scFv)

The skilled artisan will be aware that scFvs comprise V_H and V_L regions in a single polypeptide chain and a polypeptide linker between the V_H and V_L which enables the scFv to form the desired structure for antigen binding (i.e., for the V_H and V_L of the single polypeptide chain to associate with one another to form a Fv). For example, the linker comprises in excess of 12 amino acid residues with $(Gly_4Ser)_3$ being one of the more favored linkers for a scFv.

The present disclosure also contemplates a disulfide stabilized Fv (or diFv or dsFv), in which a single cysteine residue is introduced into a FR of V_H and a FR of V_L and the cysteine residues linked by a disulfide bond to yield a stable Fv.

Alternatively, or in addition, the present disclosure encompasses a dimeric scFv, i.e., a protein comprising two scFv molecules linked by a non-covalent or covalent linkage, e.g., by a leucine zipper domain (e.g., derived from Fos or Jun). Alternatively, two scFvs are linked by a peptide linker of sufficient length to permit both scFvs to form and to bind to an antigen, e.g., as described in US20060263367.

Heavy Chain Antibodies

Heavy chain antibodies differ structurally from many other forms of antibodies, in so far as they comprise a heavy chain, but do not comprise a light chain. Accordingly, these antibodies are also referred to as "heavy chain only antibodies". Heavy chain antibodies are found in, for example, camelids and cartilaginous fish (also called IgNAR).

The variable regions present in naturally occurring heavy chain antibodies are generally referred to as "V_{HH} domains" in camelid antibodies and V-NAR in IgNAR, in order to distinguish them from the heavy chain variable regions that are present in conventional 4-chain antibodies (which are referred to as "V_H domains") and from the light chain variable regions that are present in conventional 4-chain antibodies (which are referred to as "V_L domains").

A general description of heavy chain antibodies from camelids and the variable regions thereof and methods for their production and/or isolation and/or use is found *inter alia* in the following references WO94/04678, WO97/49805 and WO 97/49805.

A general description of heavy chain antibodies from cartilaginous fish and the variable regions thereof and methods for their production and/or isolation and/or use is found *inter alia* in WO2005/118629.

Other Antibodies and Proteins Comprising Antigen Binding Domains Thereof

The present disclosure also contemplates other antibodies and proteins comprising antigen-binding domains thereof, such as:

(i) "key and hole" bispecific proteins as described in US5731168;

(ii) heteroconjugate proteins, e.g., as described in US4676980;

(iii) heteroconjugate proteins produced using a chemical cross-linker, e.g., as described in US4676980; and

(iv) Fab_3 (e.g., as described in EP19930302894).

Mutations to Proteins

The present disclosure also provides a IL-11Rα-binding protein or a nucleic acid encoding same having at least 80% identity to a sequence disclosed herein. In one example, a IL-11Rα-binding protein or nucleic acid of the disclosure comprises sequence at least about 85% or 90% or 95% or 97% or 98% or 99% identical to a sequence disclosed herein, wherein the protein specifically binds to IL-11Rα as described herein according to any example.

Alternatively, or additionally, the IL-11Rα-binding protein comprises a CDR (e.g., three CDRs) at least about 80% or 85% or 90% or 95% or 97% or 98% or 99% identical to CDR(s) of a V_H or V_L as described herein according to any example,

wherein the protein is capable of specifically binding to IL-11Rα as described herein according to any example. In this regard, the inventors have produced numerous antibodies having diverse sequences within their CDRs. Methods for determining binding of a protein to IL-11Rα are described herein.

For example, the inventors have identified a group of IL-11Rα-binding proteins sharing at least 40% identity in their HCDR1 (and optionally, the amino acid N-terminal to the HCDR1) according to the Kabat numbering system and another subgroup of proteins sharing 80% identity in their HCDR1.

The inventors have also identified a class of IL-11Rα-binding protein sharing 77% identity in their HCDR2 according to the Kabat numbering system and a subclass of IL-11Rα-binding proteins sharing at least about 82% identity in their HCDR2 according to the Kabat numbering system.

As discussed herein, it is also known in the art that the five C-terminal residues of heavy chain CDR2 can be mutated to conservative or non-conservative amino acid substitutions (31% of residues) (Padlan *et al., FASEB J. 9*: 133-139, 1995). Thus, a protein can comprise a CDR2 having at least about 47% identity to a heavy chain CDR2 sequence disclosed herein.

For example, the inventors have identified a group of IL-11Rα-binding proteins sharing at least about 44% identity in their HCDR3 according to the Kabat numbering system.

For example, the inventors have identified several residues in a V_H comprising a sequence set forth in SEQ ID NO: 37 that can be substituted without loss of function or that result in improved function. In one example, the IL-11Rα-binding protein comprises between 1 and 11 amino acid substitutions compared to SEQ ID NO: 37. For example, the IL-11Rα-binding protein comprises 1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 or 9 or 10 or 11 amino acid substitutions compared to SEQ ID NO: 37. For example, the IL-11Rα-binding protein comprises 3 amino acid substitutions compared to SEQ ID NO: 37. For example, the IL-11Rα-binding protein comprises 4 amino acid substitutions compared to SEQ ID NO: 37.

In one example, the IL-11Rα-binding protein comprises between 1 and 4 amino acid substitutions in CDR3 compared to SEQ ID NO: 37. For example, the IL-11Rα-binding protein comprises 1 or 2 or 3 or 4 amino acid substitutions in the CDR3 compared to SEQ ID NO: 37.

In one example, the IL-11Rα-binding protein comprises between 1 and 3 amino acid substitutions in CDR2 compared to SEQ ID NO: 37. For example, the IL-11Rα-binding protein comprises 1 or 2 or 3 amino acid substitutions in the CDR3 compared to SEQ ID NO: 37.

In one example, a IL-11Rα-binding protein of the disclosure comprises a mutant of a sequence set forth in SEQ ID NO: 37, wherein the mutant sequence at least comprises a tryptophan at position 54 of SEQ ID NO: 37.

In one example, an IL-11Rα-binding protein of the disclosure comprises a mutant of a sequence set forth in SEQ ID NO: 37, wherein the mutant sequence at least comprises a threonine at position 56 of SEQ ID NO: 37.

In one example, an IL-11Rα-binding protein of the disclosure comprises a mutant of a sequence set forth in SEQ ID NO: 37, wherein the mutant sequence at least comprises an aspartic acid at position 57 of SEQ ID NO: 37.

In one example, an IL-11Rα-binding protein of the disclosure comprises a mutant of a sequence set forth in SEQ ID NO: 37, wherein the mutant sequence at least comprises a tryptophan at position 57 of SEQ ID NO: 37.

In one example, an IL-11Rα-binding protein of the disclosure comprises a mutant of a sequence set forth in SEQ ID NO: 37, wherein the mutant sequence at least comprises a leucine at position 57 of SEQ ID NO: 37.

In one example, an IL-11Rα-binding protein of the disclosure comprises a mutant of a sequence set forth in SEQ ID NO: 37, wherein the mutant sequence at least comprises a proline at position 99 of SEQ ID NO: 37.

In one example, an IL-11Rα-binding protein of the disclosure comprises a mutant of a sequence set forth in SEQ ID NO: 37, wherein the mutant sequence at least comprises a glutamic acid at position 100 of SEQ ID NO: 37.

In one example, an IL-11Rα-binding protein of the disclosure comprises a mutant of a sequence set forth in SEQ ID NO: 37, wherein the mutant sequence at least comprises a leucine at position 100 of SEQ ID NO: 37.

In one example, an IL-11Rα-binding protein of the disclosure comprises a mutant of a sequence set forth in SEQ ID NO: 37, wherein the mutant sequence at least comprises an aspartic acid at position 101 of SEQ ID NO: 37.

In one example, an IL-11Rα-binding protein of the disclosure comprises a mutant of a sequence set forth in SEQ ID NO: 37, wherein the mutant sequence at least comprises a leucine at position 104 of SEQ ID NO: 37.

In one example, an IL-11Rα-binding protein of the disclosure comprises a mutant of a sequence set forth in SEQ ID NO: 37, wherein the mutant sequence at least comprises an arginine at position 104 of SEQ ID NO: 37.

In one example, an IL-11Rα-binding protein of the disclosure comprises a mutant of a sequence set forth in SEQ ID NO: 37, wherein the mutant sequence at least comprises a tryptophan at position 54, an aspartic acid at position 56 and a leucine at position 57 each in relation to SEQ ID NO: 37.

In one example, an IL-11Rα-binding protein of the disclosure comprises a mutant of a sequence set forth in SEQ ID NO: 37, wherein the mutant sequence at least comprises a tryptophan at position 54, an aspartic acid at position 56 and a leucine at position 57 each in relation to SEQ ID NO: 37.

In one example, an IL-11Rα-binding protein of the disclosure comprises a mutant of a sequence set forth in SEQ ID NO: 37, wherein the mutant sequence at least comprises a tryptophan at position 54, a threonine at position 56 and a leucine at position 57 each in relation to SEQ ID NO: 37.

In one example, an IL-11Rα-binding protein of the disclosure comprises a mutant of a sequence set forth in SEQ ID NO: 37, wherein the mutant sequence at least comprises a proline at position 99, a glutamic acid at position 100, an aspartic acid at position 101 and a leucine at position 104 each in relation to SEQ ID NO: 37.

In one example, an IL-11Rα-binding protein of the disclosure comprises a mutant of a sequence set forth in SEQ ID NO: 37, wherein the mutant sequence at least comprises a proline at position 99, a leucine at position 100, an aspartic acid at position 101 and an arginine at position 104 each in relation to SEQ ID NO: 37.

For example, the inventors have identified a group of IL-11Rα-binding proteins sharing at least 45% identity in their LCDR1 according to the Kabat numbering system and another subgroup of proteins sharing about 54% identity in their LCDR1.

The inventors have also identified a class of IL-11Rα-binding protein sharing at least about 55% or 56% identity in their LCDR3 according to the Kabat numbering system.

For example, the inventors have identified several residues in a V_L comprising a sequence set forth in SEQ ID NO: 5 that can be substituted without loss of function or that result in improved function. In one example, the IL-11Rα-binding protein comprises between 1 and 11 amino acid substitutions compared to SEQ ID NO: 5. For example, the IL-11Rα-binding protein comprises 1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 or 9 or 10 or 11 amino acid substitutions compared to SEQ ID NO: 5. For example, the IL-11Rα-binding protein comprises 3 amino acid substitutions compared to SEQ ID NO: 5. For example, the IL-11Rα-binding protein comprises 4 amino acid substitutions compared to SEQ ID NO: 5. For example, the IL-11Rα-binding protein comprises 5 amino acid substitutions compared to SEQ ID NO: 5.

In one example, the IL-11Rα-binding protein comprises between 1 and 4 amino acid substitutions in CDR3 compared to SEQ ID NO: 5. For example, the IL-11Rα-binding protein comprises 1 or 2 or 3 or 4 amino acid substitutions in the CDR3 compared to SEQ ID NO: 5.

In one example, the IL-11Rα-binding protein comprises between 1 and 5 amino acid substitutions in CDR1 compared to SEQ ID NO: 5. For example, the IL-11Rα-

binding protein comprises 1 or 2 or 3 or 4 or 5 amino acid substitutions in the CDR3 compared to SEQ ID NO: 5.

In one example, a IL-11Rα-binding protein of the disclosure comprises a mutant of a sequence set forth in SEQ ID NO: 5, wherein the mutant sequence at least comprises a valine at position 29 of SEQ ID NO: 5.

In one example, a IL-11Rα-binding protein of the disclosure comprises a mutant of a sequence set forth in SEQ ID NO: 5, wherein the mutant sequence at least comprises an aspartic acid at position 30 of SEQ ID NO: 5.

In one example, a IL-11Rα-binding protein of the disclosure comprises a mutant of a sequence set forth in SEQ ID NO: 5, wherein the mutant sequence at least comprises a lysine at position 31 of SEQ ID NO: 5.

In one example, a IL-11Rα-binding protein of the disclosure comprises a mutant of a sequence set forth in SEQ ID NO: 5, wherein the mutant sequence at least comprises a valine at position 33 of SEQ ID NO: 5.

In one example, a IL-11Rα-binding protein of the disclosure comprises a mutant of a sequence set forth in SEQ ID NO: 5, wherein the mutant sequence at least comprises a glutamic acid at position 34 of SEQ ID NO: 5.

In one example, a IL-11Rα-binding protein of the disclosure comprises a mutant of a sequence set forth in SEQ ID NO: 5, wherein the mutant sequence at least comprises an alanine at position 91 of SEQ ID NO: 5.

In one example, a IL-11Rα-binding protein of the disclosure comprises a mutant of a sequence set forth in SEQ ID NO: 5, wherein the mutant sequence at least comprises a histidine at position 91 of SEQ ID NO: 5.

In one example, a IL-11Rα-binding protein of the disclosure comprises a mutant of a sequence set forth in SEQ ID NO: 5, wherein the mutant sequence at least comprises a glutamic acid at position 91 of SEQ ID NO: 5.

In one example, a IL-11Rα-binding protein of the disclosure comprises a mutant of a sequence set forth in SEQ ID NO: 5, wherein the mutant sequence at least comprises an aspartic acid at position 93 of SEQ ID NO: 5.

In one example, a IL-11Rα-binding protein of the disclosure comprises a mutant of a sequence set forth in SEQ ID NO: 5, wherein the mutant sequence at least comprises a phenylalanine at position 93 of SEQ ID NO: 5.

In one example, a IL-11Rα-binding protein of the disclosure comprises a mutant of a sequence set forth in SEQ ID NO: 5, wherein the mutant sequence at least comprises a serine at position 93 of SEQ ID NO: 5.

In one example, a IL-11Rα-binding protein of the disclosure comprises a mutant of a sequence set forth in SEQ ID NO: 5, wherein the mutant sequence at least comprises a glutamine at position 94 of SEQ ID NO: 5.

In one example, an IL-11Rα-binding protein of the disclosure comprises a mutant of a sequence set forth in SEQ ID NO: 5, wherein the mutant sequence at least comprises a valine at position 29, an aspartic acid at position 30 a lysine at position 31 a valine at position 33 and a glutamic acid at position 34 each in relation to SEQ ID NO: 5.

In one example, an IL-11Rα-binding protein of the disclosure comprises a mutant of a sequence set forth in SEQ ID NO: 5, wherein the mutant sequence at least comprises an alanine at position 91, a glutamic acid at position 92, an aspartic acid at position 93 and a glutamine at position 94 each in relation to SEQ ID NO: 5.

In one example, an IL-11Rα-binding protein of the disclosure comprises a mutant of a sequence set forth in SEQ ID NO: 5, wherein the mutant sequence at least comprises a histidine at position 91, a glutamic acid in position 92, a phenylalanine at position 93 and a glutamine at position 94 each in relation to SEQ ID NO: 5.

In one example, an IL-11Rα-binding protein of the disclosure comprises a mutant of a sequence set forth in SEQ ID NO: 5, wherein the mutant sequence at least comprises a histidine at position 91, a glutamic acid at position 92 and a glutamine at position 94 each in relation to SEQ ID NO: 5.

In one example, an IL-11Rα-binding protein of the disclosure comprises a mutant of a sequence set forth in SEQ ID NO: 5, wherein the mutant sequence at least comprises a histidine at position 91, a glutamic acid at position 92 and a glutamine at position 94 each in relation to SEQ ID NO: 5.

In one example, an IL-11Rα-binding protein of the disclosure comprises a mutant of a sequence set forth in SEQ ID NO: 5, wherein the mutant sequence at least comprises a glutamic acid at position 92, a serine at position 93 and a glutamine at position 94 each in relation to SEQ ID NO: 5.

In another example, a nucleic acid of the disclosure comprises a sequence at least about 80% or 85% or 90% or 95% or 97% or 98% or 99% identical to a sequence set forth herein and encoding an IL-11Rα-binding protein having a function as described herein according to any example. The present disclosure also encompasses nucleic acids encoding an IL-11Rα-binding protein of the disclosure, which differs from a sequence exemplified herein as a result of degeneracy of the genetic code.

The % identity of a nucleic acid or polypeptide is determined by GAP (Needleman and Wunsch. *Mol. Biol. 48*, 443-453, 1970) analysis (GCG program) with a gap creation penalty=5, and a gap extension penalty=0.3. The query sequence is at least 50 residues in length, and the GAP analysis aligns the two sequences over a region of at least 50 residues. For example, the query sequence is at least 100 residues in length and the GAP analysis aligns the two sequences over a region of at least 100 residues. For example, the two sequences are aligned over their entire length.

The present disclosure also contemplates a nucleic acid that hybridizes under stringent hybridization conditions to a nucleic acid encoding an IL-11Rα-binding protein described herein. A "moderate stringency" is defined herein as being a hybridization and/or washing carried out in 2 x SSC buffer, 0.1% (w/v) SDS at a temperature in the range 45°C to 65°C, or equivalent conditions. A "high stringency" is defined herein as being a hybridization and/or wash carried out in 0.1 x SSC buffer, 0.1% (w/v) SDS, or lower salt concentration, and at a temperature of at least 65°C, or equivalent conditions. Reference herein to a particular level of stringency encompasses equivalent conditions using wash/hybridization solutions other than SSC known to those skilled in the art. For example, methods for calculating the temperature at which the strands of a double stranded nucleic acid will dissociate (also known as melting temperature, or Tm) are known in the art. A temperature that is similar to (e.g., within 5°C or within 10°C) or equal to the Tm of a nucleic acid is considered to be high stringency. Medium stringency is to be considered to be within 10°C to 20°C or 10°C to 15°C of the calculated Tm of the nucleic acid.

The present disclosure also contemplates mutant forms of an IL-11Rα-binding protein of the disclosure comprising one or more conservative amino acid substitutions compared to a sequence set forth herein. In some examples, the IL-11Rα-binding protein comprises 10 or fewer, e.g., 9 or 8 or 7 or 6 or 5 or 4 or 3 or 2 or 1 conservative amino acid substitutions. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain and/or hydropathicity and/or hydrophilicity.

Families of amino acid residues having similar side chains have been defined in the art, including basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), β-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Hydropathic indices are described, for example in Kyte and Doolittle *J. Mol. Biol., 157*: 105-132, 1982 and hydrophylic indices are described in, e.g., US4554101.

The present disclosure also contemplates non-conservative amino acid changes. For example, of particular interest are substitutions of charged amino acids with another charged amino acid and with neutral or positively charged amino acids. In some examples, the IL-11Rα-binding protein comprises 10 or fewer, e.g., 9 or 8 or 7 or 6 or 5 or 4 or 3 or 2 or 1 non-conservative amino acid substitutions.

In one example, the mutation(s) occur within a FR of an antigen binding domain of an IL-11Rα-binding protein of the disclosure. In another example, the mutation(s) occur within a CDR of an IL-11Rα-binding protein of the disclosure.

Exemplary methods for producing mutant forms of an IL-11Rα-binding protein include:

- mutagenesis of DNA (Thie *et al.*, *Methods Mol. Biol. 525*: 309-322, 2009) or RNA (Kopsidas *et al.*, *Immunol. Lett. 107*:163-168, 2006; Kopsidas *et al. BMC Biotechnology*, *7*: 18, 2007; and WO1999/058661);
- introducing a nucleic acid encoding the polypeptide into a mutator cell, e.g., XL-1Red, XL-mutS and XL-mutS-Kanr bacterial cells (Stratagene);
- DNA shuffling, e.g., as disclosed in Stemmer, *Nature 370*: 389-91, 1994; and
- site directed mutagenesis, e.g., as described in Dieffenbach (ed) and Dveksler (ed) (*In*: PCR Primer: A Laboratory Manual, Cold Spring Harbor Laboratories, NY, 1995).

Exemplary methods for determining biological activity of the mutant IL-11Rα-binding proteins of the disclosure will be apparent to the skilled artisan and/or described herein, e.g., antigen binding. For example, methods for determining antigen binding, competitive inhibition of binding, affinity, association, dissociation and therapeutic efficacy are described herein.

Constant Regions

The present disclosure encompasses IL-11Rα-binding proteins and/or antibodies described herein comprising a constant region of an antibody. This includes antigen binding fragments of an antibody fused to a Fc.

Sequences of constant regions useful for producing the proteins of the present disclosure may be obtained from a number of different sources. In some examples, the constant region or portion thereof of the protein is derived from a human antibody. The constant region or portion thereof may be derived from any antibody class, including IgM, IgG, IgD, IgA and IgE, and any antibody isotype, including IgG1, IgG2, IgG3 and IgG4. In one example, the constant region is human isotype IgG4 or a stabilized IgG4 constant region.

In one example, the Fc region of the constant region has a reduced ability to induce effector function, e.g., compared to a native or wild-type human IgG1 or IgG3 Fc region. In one example, the effector function is antibody-dependent cell-mediated cytotoxicity (ADCC) and/or antibody-dependent cell-mediated phagocytosis (ADCP) and/or complement-dependent cytotoxicity (CDC). Methods for assessing the level of