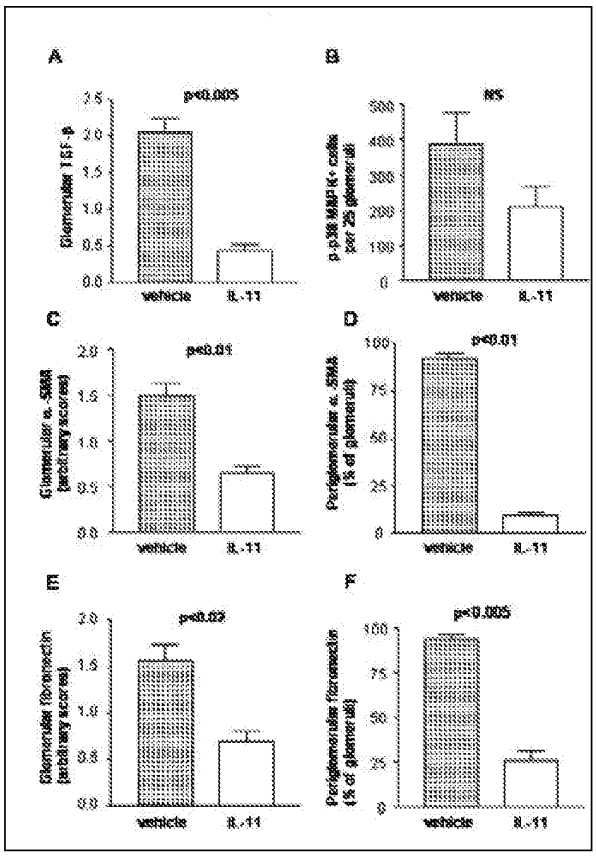

Fig. 3 - Treatment with rhIL-11 in nephrotoxic nephritis (NTN). Treatment with high-dose IL-11 reduced expression of glomerular TGF-β1 (A), glomerular α-SMA (C), periglomerular α-SMA (D), glomerular fibronectin (E) and periglomerular fibronectin (F) in comparison with vehicle-treated rats. The reduction in glomerular p-p38 MAPK (B) was not significant.

Discussion

In this study, we have shown that treatment with a high dose of IL-11 reduced glomerular expression of TGF-β1, α-SMA and fibronectin in NTN. To our knowledge, the present study is the first demonstration that administration of rhIL-11 may alleviate glomerular expression of TGF-β1 activation of myofibroblasts and extracellular matrix deposition in experimental glomerulonephritis.

In the kidney, myofibroblasts may derive from perivascular smooth muscle cells, pericytes and interstitial fibroblasts, after stimulation by cytokines such as TGF-β1 and IL-1β (15-17). Myofibroblasts are implicated in the development of sclerotic lesions (18, 19). Adenoviral gene transfer of soluble TGF-β1 receptor II reduced the number of α-SMA (+) cells and ameliorated interstitial fibrosis in NTN (20). In our study, α-SMA was expressed initially in the periglomerular area on day 4; however, its periglomerular as well as mesangial expression was increased on day 6, when the fibronectin expression was also increased. The periglomerular α-SMA up-regulation can be attributed to myofibroblast formation. Activated myofibroblasts become hypertrophic and secrete extracellular matrix proteins, and this may lead to glomerulosclerosis, fibrous crescent formation and tubulointerstitial fibrosis (18).

Phosphorylation and activation of p38 MAPK was noticed very early, only 5 hours after NTS induction, but most interestingly, its activation happened in a repeated way. P-p38 MAPK expression was increased 5 hours after induction of NTN, reduced to normal levels during days 2-4 and relapsed on day 6. Transient inactivation of p-p38 MAPK may be due to its interaction with MAPK phosphatases (MKPs). MKPs are a family of protein phosphatases, which are responsible for the dephosphorylation and inactivation of MAPKs. MKPs are activated simultaneously with MAPKs (20, 21), and they may be responsible for p-p38 MAPK inactivation. In the present study this inactivation seemed to be transient, because p38 MAPK was reactivated later, probably as a result of cytokine and growth factor production. To our knowledge, this dual activation of p38 MAPK has not been described previously; however, more specific studies are necessary to investigate it further.

There was only a small reduction in renal p-p38 MAPK expression in IL-11–treated rats. These results suggest that glomerular expression of TGF-β1 and infiltration/transformation of myofibroblasts may proceed independently of p-p38 MAPK, this is in accordance with a previous study, which showed that p-p38 MAPK was not the only downstream signalling intermediate in the pathway from TGF-β1 to α-SMA (22). Also, administration of a TGF-β1 receptor inhibitor (SD-208) resulted in the attenuation myofibroblast transformation of lung fibroblasts, an effect that could not be achieved by a p38 MAPK inhibitor(SD 282) (23).

In our previous report, both high and low doses of IL-11 reduced proteinuria and glomerular fibrinoid necrosis, but had a different effect on glomerular macrophages (10). Daily treatment with a high dose (1,360 μg) of rhIL-11 reduced the number of infiltrating macrophages; a low dose (800 μg) of rhIL-11 reduced only the number of activated macrophages, not the total number of macrophages. Based on this reduction, the anti-TGF effect of IL-11 could be attributed to its anti-inflammatory properties, a mechanism that has also been described for other agents (24). However,

the fact that low-dose rhIL-11 was enough to reduce IL-1β production, and macrophage activity, but not TGF-β1, α-SMA and fibronectin expression, makes the position more complicated. It seems that administration of rhIL-11 has the potential to reduce inflammation, even when given at lower doses, but this anti-inflammatory effect is not enough to justify its anti-TGF effect, which requires higher dosage, and is also independent from p38 MAPK dephosphorylation and inactivation. One of the speculations is that the reduction in the number of glomerular macrophages is needed to reduce the TGF-β1 expression. Further work is needed to investigate this possibility.

Findings of the present study suggest that IL-11 has a dose-dependent effect in glomerular expression of TGF-β1, myofibroblast differentiation and extracellular matrix deposition in NTN. This finding may be of relevance to development of possible new applications of IL-11 and also novel treatment strategies in patients with glomerulonephritis.

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References

1. Trepicchio WL, Dorner AJ. Interleukin-11: a gp130 cytokine. Ann N Y Acad Sci. 1998;856:12-21.
2. Müller-Newen G. The cytokine receptor gp130: faithfully promiscuous. Sci STKE. 2003;2003:PE40.
3. Heinrich PC, Behrmann I, Haan S, Hermanns HM, Muller-Newen G, Schaper F. Principles of interleukin (IL)-6-type cytokine signalling and its regulation. Biochem J. 2003;374:1-20.
4. Schwertschlag US, Trepicchio WL, Dykstra KH, Keith JC, Turner KJ, Dorner AJ. Hematopoietic, immunomodulatory and epithelial effects of interleukin-11. Leukemia. 1999;13:1307-1315.
5. Kurzrock R, Cortes J, Thomas DA, Jeha S, Pilat S, Talpaz M. Pilot study of low-dose interleukin-11 in patients with bone marrow failure. J Clin Oncol. 2001;19:4165-4172.
6. Ragni MV, Jankowitz RC, Chapman HL, et al. A phase II prospective open-label escalating dose trial of recombinant interleukin-11 in mild von Willebrand disease. Haemophilia. 2008;14:968-977.
7. Schmitz B, Thiele J, Witte O, Kaufmann R, Wickenhauser C, Fischer R. Influence of cytokines (IL-1 alpha, IL-3, IL-11, GM-CSF) on megakaryocyte-fibroblast interactions in normal human bone marrow. Eur J Haematol. 1995;55:24-32.
8. Peterson RL, Wang L, Albert L, Keith JC Jr, Dorner AJ. Molecular effects of recombinant human interleukin-11 in the HLA-B27 rat model of inflammatory bowel disease. Lab Invest. 1998;78:1503-1512.
9. Tang W, Geba GP, Zheng T, et al. Targeted expression of IL-11 in the murine airway causes lymphocytic inflammation, bronchial remodeling, and airways obstruction. J Clin Invest. 1996;98:2845-2853.
10. Lai PC, Cook HT, Smith J, Keith JC Jr, Pusey CD, Tam FW. Interleukin-11 attenuates nephrotoxic nephritis in Wistar Kyoto rats. J Am Soc Nephrol. 2001;12:2310-2320.
11. Lai PC, Smith J, Bhangal G, et al. Interleukin-11 reduces renal injury and glomerular NF-kappa B activity in murine experimental glomerulonephritis. Nephron Exp Nephrol. 2005;101:146-154.
12. Tam FW, Smith J, Morel D, et al. Development of scarring and renal failure in a rat model of crescentic glomerulonephritis. Nephrol Dial Transplant. 1999;14:1658-1666.
13. Martin MM, Buckenberger JA, Jiang J, et al. TGF-beta1 stimulates human AT1 receptor expression in lung fibroblasts by cross talk between the Smad, p38 MAPK, JNK, and PI3K signaling pathways. Am J Physiol Lung Cell Mol Physiol. 2007;293:790-799.
14. Kutz SM, Hordines J, McKeown-Longo PJ, Higgins PJ. TGF-

ß1-induced PAI-1 gene expression requires MEK activity and cell-to-substrate adhesion. J Cell Sci. 2001;114:3905-3914.
15. Vesey DA, Cheung CW, Cuttle L, Endre ZA, Gobe G, Johnson DW. Interleukin-1beta induces human proximal tubule cell injury, alpha-smooth muscle actin expression and fibronectin production. Kidney Int. 2002;62:31-40.
16. Kondo S, Kagami S, Urushihara M, et al. Transforming growth factor-beta1 stimulates collagen matrix remodeling through increased adhesive and contractive potential by human renal fibroblasts. Biochim Biophys Acta. 2004;1693:91-100.
17. Zeisberg EM, Potenta SE, Sugimoto H, Zeisberg M, Kalluri R. Fibroblasts in kidney fibrosis emerge via endothelial-to-mesenchymal transition. J Am Soc Nephrol. 2008;19:2282-2287.
18. Goumenos D, Tsomi K, Iatrou C, et al. Myofibroblasts and the progression of crescentic glomerulonephritis. Nephrol Dial Transplant. 1998;13:1652-1661.
19. Roberts IS, Burrows C, Shanks JH, Venning M, McWilliam LJ. Interstitial myofibroblasts: predictors of progression in membranous nephropathy. J Clin Pathol. 1997;50:123-127.
20. Zhou A, Ueno H, Shimomura M, et al. Blockade of TGF-beta action ameliorates renal dysfunction and histologic progression in anti-GBM nephritis. Kidney Int. 2003;64:92-101.
21. Dickinson RJ, Keyse SM. Diverse physiological functions for dual-specificity MAP kinase phosphatases. J Cell Sci. 2006;119:4607-4615.
22. Ding Q, Gladson CL, Wu H, Hayasaka H, Olman MA. Focal adhesion kinase (FAK)-related non-kinase inhibits myofibroblast differentiation through differential MAPK activation in a FAK-dependent manner. J Biol Chem. 2008;283:26839-26849.
23. Kapoun AM, Gaspar NJ, Wang Y, et al. Transforming growth factor-beta receptor type 1 (TGFbetaRI) kinase activity but not p38 activation is required for TGFbetaRI-induced myofibroblast differentiation and profibrotic gene expression. Mol Pharmacol. 2006;70:518-531.
24. Nagatoya K, Moriyama T, Kawada N, et al. Y-27632 prevents tubulointerstitial fibrosis in mouse kidneys with unilateral ureteral obstruction. Kidney Int. 2002;61:1684-1695.

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Transforming Growth Factor-β Stimulates Interleukin-11 Transcription via Complex Activating Protein-1-dependent Pathways*

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Studies were undertaken to characterize the mechanism by which transforming growth factor-β_1 (TGF-β_1) stimulates epithelial cell interleukin (IL)-11 production. Nuclear run-on studies demonstrated that TGF-β_1 is a potent stimulator of IL-11 gene transcription. TGF-β_1 also stimulated the luciferase activity in cells transfected with reporter gene constructs containing nucleotides −728 to +58 of the IL-11 promoter. Studies with progressive 5′ deletion constructs and site-specific mutations demonstrated that this stimulation was dependent on 2 AP-1 sites between nucleotides −100 and −82 in the IL-11 promoter. Mobility shift assays demonstrated that TGF-β_1 stimulated AP-1 protein-DNA binding to both AP-1 sites. Supershift analysis demonstrated that JunD was the major moiety contributing to AP-1-DNA binding in unstimulated cells and that c-Jun-, Fra-1-, and Fra-2-DNA binding were increased whereas JunD-DNA binding was decreased in TGF-β_1-stimulated cells. The sequence in the IL-11 promoter that contains the AP-1 sites also conferred TGF-β_1 responsiveness, in a position-independent fashion, on a heterologous minimal promoter. Thus, TGF-β_1 stimulates IL-11 gene transcription via a complex AP-1-dependent pathway that is dependent on 2 AP-1 motifs between nucleotides −100 and −82 that function as an enhancer in the IL-11 promoter.

Interleukin-11 (IL-11)[1] was originally discovered as a soluble factor in supernatants from transformed stromal cells that stimulated plasmacytoma cell proliferation (1). It has subsequently been shown to be a pleiotropic member of the IL-6-type cytokine family that mediates its biologic activities via binding to a multimeric receptor complex that contains the gp130 molecule (2–5). Among its many effects are the ability to regulate hematopoiesis, stimulate the production of acute phase proteins, induce the tissue inhibitor of metalloproteinase-1, regulate bone metabolism, and alter epithelial proliferation (2, 6–10). Studies from our laboratories and others have also demonstrated that IL-11 can induce tissue fibrosis, regulate tissue myocyte and myofibroblast accumulation, alter airway physiology, and confer protection in the context of mucosal injury of the respiratory and gastrointestinal tracts (11–14).

In keeping with the biologic importance of IL-11, a number of investigators have studied its sites of production and the regulation of these responses. These studies demonstrated that IL-11 is produced by a variety of stromal cells in response to a variety of stimuli, including cytokines, histamine, eosinophil major basic protein, and respiratory tropic viruses (7, 15–20). A prominent finding in our studies of fibroblasts (18), epithelial cells (19), and osteoblasts (20) and studies by others of chondrocytes and synoviocytes (7) has been the importance of TGF-β moieties in the stimulation of IL-11 production. These studies also demonstrated that TGF-β_1 stimulation of IL-11 protein production is associated with proportionate changes in IL-11 mRNA accumulation and, in our studies, IL-11 gene transcription (18).

The IL-11 promoter has been cloned and the cis-elements and trans-acting factors that regulate the levels of basal IL-11 production have been identified by our laboratories (21). Despite the demonstrated importance of gene transcription in the stimulation of IL-11 production, the cis-elements and trans-acting factors that mediate the transcriptional activation of IL-11 have not been investigated. To further our understanding of the regulation of IL-11, studies were undertaken to characterize the transcriptional elements utilized by TGF-β_1 in the stimulation of IL-11. These studies identify two activating protein-1 (AP-1) motifs between −100 and −82 in the IL-11 promoter that are essential for TGF-β-induced IL-11 transcriptional activation. They also demonstrate that this stimulation is associated with complex alterations in the composition of the AP-1 subunits that bind to these sites and that DNA which contains these AP-1 elements confers TGF-β_1 responsiveness on a heterologous promoter. Lastly they demonstrate that this mechanism is stimulus-specific since respiratory syncytial virus (RSV) stimulates IL-11 transcription via a different mechanism.

EXPERIMENTAL PROCEDURES

Cell Culture and TGF-β_1 Stimulation

A549 human alveolar epithelial-like cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and grown to confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (22). At confluence, varying concentrations of recombinant human TGF-β_1 (1–10 ng/ml) (R & D Systems, Minneapolis) or medium controls were added, and the cells were incubated for up to 48 h. At the desired points in time, supernatants were removed and stored at −20 °C, and nuclei were harvested for further usage (see below).

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[1] The abbreviations used are: IL, interleukin; TGF-β_1, transforming growth factor-β_1; AP-1, activating protein-1; RSV, respiratory syncytial virus; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assays; bp, base pair(s); tk, thymidine kinase.

Nuclear Run-on Assay

The relative rates of gene transcription were assessed using modifications of protocols previously described by our laboratory (22–25). A549 cells were incubated for 16 h under control conditions, with TGF-β_1 (10 ng/ml), or after infection with respiratory syncytial virus (RSV) at a multiplicity of infection of 3 as described previously (17–19). The cells (3×10^7 per condition) were then washed twice with ice-cold phosphate-buffered saline, pelleted, and resuspended in lysis buffer (10 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, 3 mM $CaCl_2$, 3 μM dithiothreitol (DTT), 300 mM sucrose, 0.5% Triton X-100). The nuclei were then harvested by centrifugation and resuspended in 100 μl of storage buffer (50 mM Tris-HCl, pH 8.3, 5 mM $MgCl_2$, 0.1 mM EDTA, 40% glycerol) and stored at −80 °C until further utilized. Nylon membranes were prepared carrying 20 μg each of isolated cDNA fragments encoding IL-11 (a gift of Dr. Paul Schendel, Genetics Institute, Cambridge, MA) and pUC18 without a cDNA insert (a control for nonspecific hybridization) using a slot-blotting apparatus (MINI-FOLD II, Schleicher & Schuell) and baked in a vacuum oven (80 °C for 2 h). When ready, nuclei were thawed on ice and pelleted in a microcentrifuge at 4 °C for 30 s, and *in vitro* transcription and RNA labeling were carried out in transcription buffer (20 mM Tris-HCl, pH 8.3, 100 mM KCl, 4.5 mM $MgCl_2$, 2 mM DTT, and 400 μM each of ATP, GTP, and CTP) in the presence of 200 μCi of [α-^{32}P]UTP (~3000 Ci/mmol, Amersham Corp.) and 20% glycerol at 30 °C for 30 min. The reaction was followed with a cold chase with 1 μl of 100 mM UTP for 10 min at 30 °C. The reaction was then terminated by incubating with stop buffer (50 mM Tris-HCl, pH 8.3, 500 mM NaCl, 5 mM EDTA) with 200 μg/ml RNase-free DNase I and 750 units/ml RNasin (Boehringer Mannheim) at 30 °C for 15 min. RNA was extracted with phenol/chloroform, precipitated, and washed with alcohol. Dried RNA pellets were dissolved in equal volumes of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.8), and radioactivity was determined by the mean of duplicate countings of 1-μl aliquots. Hybridization was performed by incubating each membrane with equal numbers of counts of radiolabeled RNA. The membranes were then washed at high stringency, and binding was evaluated using autoradiography.

Primer Extension Analysis

A549 cells were incubated for 16 h with TGF-β_1 (10 ng/ml). The supernatants were then removed, and poly(A)$^+$ RNA was isolated using oligo(dT) affinity based methodology as described (26, 27). Primer extension was then performed using a radiolabeled 20-base complementary synthetic oligonucleotide corresponding to oligonucleotides −11 to +9 with respect to the translation start site. The 5′ end of the resulting IL-1 mRNA was defined using the Moloney murine leukemia virus primer extension system (Promega, Madison, WI) as described by the manufacturer. In this system the 5′ end-labeled oligonucleotide hybridized with the IL-11 mRNA and was utilized as a primer by the Moloney murine leukemia virus reverse transcriptase which, in the presence of deoxynucleotides, synthesized cDNA until the 5′ end of the mRNA was reached. The extended product was then resolved on an 8% urea/polyacrylamide sequencing gel along with a known DNA sequence ladder.

Plasmid Construction

A 786-bp *Pvu*II fragment of the human IL-11 promoter was previously isolated and cloned in our laboratories (21). This promoter fragment contained the sequences between −728 and +58 relative to the transcription start site defined above. It was cloned into the *Sma*I site of the luciferase reporter gene vector pXP2-luc (ATCC) to generate the construct pXP2-IL-11-728.

Preparation of 5′ Deletion Constructs

Two techniques were used to generate a series of 5′ deletions of the pXP2-IL-11-728 parent construct. When appropriate restriction sites were present, they were utilized to generate deletion mutants. This approach was utilized with the *Ava*II site at −324 and the *Hin*fI site at −96. In both cases, the −728 to +58 fragment of the IL-11 promoter was subjected to enzyme digestion, and the desired fragment was recloned into the vector pXP2-luc using standard approaches. When appropriate restriction sites were not available Bal-31 exonuclease digestion was employed to introduce deletions. This technique takes advantage of the fact that Bal-31 degrades both the 5′ and 3′ ends of double-stranded DNA without inserting internal cleavages (28). Briefly, *Bam*HI-linearized parent construct pXP2-IL-11-324 was incubated with Bal-31 exonuclease for varying periods. *Bam*HI linkers (New England Biolabs, catalogue number 1071, Beverly, MA) were then added, and the DNA was subjected to *Bam*HI/*Xho*I double digestion. The DNA fragments with the various 5′ deletions were then separated by electrophoresis, electroeluted, and ligated into *Bam*HI/*Xho*I-linearized pXP2-luc vector. Clones from the subsequent transformation were screened for insert size, and DNA sequencing was used to verify junction sequences for all clones that were chosen for further utilization.

Through the combined efforts of both approaches, a series of constructs were prepared whose 5′ ends extended from −728 to −81. In all cases, the 3′ end was +58 relative to the transcription initiation site.

Site-directed Mutagenesis

Mutation of the AP-1 sites in the parent IL-11 promoter was performed using the Muta-Gene M13 *In Vitro* Mutagenesis Kit (Bio-Rad, catalog number 170-3580) based on Kunkel's method (29, 30). The 382-bp *Bam*HI/*Xho*I fragment of IL-11 promoter was excised from pXP2-IL-11-324 and subcloned into M13 phage. The recombinant phage DNA was then transformed into bacterial strain *Escherichia coli* CJ236 (dut−, ung−, thi−, and relA−) to generate uracil-containing single-stranded DNA. Such single-stranded DNA was allowed to anneal to mutagenic primer, and second strand DNA was synthesized with T7 DNA polymerase and T4 DNA ligase. When transformed into bacterial strain MV1190(dut+, ung+), uracil-containing single-stranded DNA template was degraded, and only newly synthesized mutation-bearing second strand DNA would propagate. The wild type and mutated AP-1 sequences are as follows: wild type 5′ (distal) AP-1, 5′-TGAGTCA-3′; mutated 5′ (distal) AP-1, 5′-TGAcgaA-3′; wild type 3′ (proximal) AP-1, 5′-TGTGTCA-3′; mutated 3′ (proximal) AP-1, 5′-TGTcgaA-3′. All of the AP-1 mutation constructs underwent DNA sequencing to verify the site and extent of the induced alterations.

Preparation of IL-11/tk/luc Constructs

A 156-bp *Bam*HI/*Xho*I fragment from the herpes simplex virus thymidine kinase (tk) minimal promoter/chloramphenicol acetyltransferase reporter gene construct ptk-CAT (31) was obtained from Dr. Anuradha Ray (Yale University, New Haven) and subcloned into the *Bam*HI and *Xho*I sites of the pXP2-luc reporter gene construct to generate ptk-XP2. Two oligonucleotides (5′-GAT CCG AGG GTG AGT CAG GAT GTG TCA GGC CGA AGC TT-3′ and 5′-GAT CAA GCT TCG GCC TGA CAC CTG ACT CAC CCT CG-3′) were then synthesized and annealed to form a 38-bp DNA duplex with sticky ends compatible with the *Bam*HI site (5′ of herpes simplex virus tk promoter) in ptk-XP2. This insert contains a 27-bp DNA sequence of the IL-11 promoter (−103 to −77 relative to transcription start site) that contains both the 5′ and 3′ AP-1 sites. This double-stranded DNA sequence was then cloned into ptk-XP2 in the correct (sense) (pIL11(+)tk-XP2) and reverse (antisense) (pIL11(−)tk-XP2) directions. DNA sequencing was performed to verify the sequence and orientation of DNA insertion.

Cell Transfection and Reporter Gene Assay

A549 cells were seeded at 40–50% confluence and incubated overnight in DMEM with high glucose and 10% fetal bovine serum. Transfections were performed using the DEAE-dextran method as described previously by our laboratory (22). The cells were then incubated for 24 h in serum-free DMEM alone or in DMEM supplemented with TGF-β_1 (10 ng/ml). In experiments where RSV was utilized the A549 cells were incubated for 90 min with RSV (multiplicity of infection = 3) or appropriate medium control, washed, and then incubated for 24 h in serum-free DMEM. At the end of these incubations, cell lysates were prepared, and luciferase activity was assessed on a Lumat luminometer using the Luciferase Assay System from Promega (Madison, WI). In all transfections the construct pCMV-β-gal (CLONTECH, Palo Alto, CA) was also included to control for transfection efficiency. The β-galactosidase activity in unstimulated and stimulated cell lysates was characterized using the CPRG method as described previously by this laboratory (22). The β-galactosidase levels were then used to standardize the measurements of luciferase activity.

Electrophoretic Mobility Shift Assay (EMSA)

Preparation of Nuclear Extracts—Nuclear extracts were prepared using modifications of the techniques of Schreiber *et al.* (32). Unstimulated, TGF-β_1-stimulated, and RSV-infected A549 cells were prepared as noted above. At the desired points in time, the cells (10^7 per condition) were mechanically detached, suspended in Tris-buffered saline freshly supplemented with protease inhibitors (1 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride), pelleted at 4 °C, and resuspended, and swollen in solution A (10 mM HEPES, pH

7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT with freshly added protease inhibitors as above) for 15 min on ice. Membrane lysis was accomplished by adding 25 μl of Nonidet P-40 followed by vigorous agitation. The nuclei were collected by centrifugation, resuspended in 50 μl of solution B (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and freshly added protease inhibitors as above), and agitated vigorously at 4 °C for 15 min. The membrane debris was discarded, and the nuclear extracts were snap-frozen in small aliquots and stored at −80 °C. The protein concentrations of the nuclear extracts were determined using the DC Protein Assay System (Bio-Rad).

Oligonucleotide Probes—Double-stranded oligonucleotide probes were used in these experiments. For the sake of simplicity, only the top strand DNA sequences are illustrated here. Four oligonucleotide probes were synthesized using the oligonucleotide synthesis facility at Yale University. They include the following: (i) wild type 5′ AP-1 sequence in the IL-11 promoter (5′ AP-1) (5′-GGGAGGGTGAGTCAGGATGTG-3′); (ii) mutated 5′ AP-1 (5′-GGGAGGGTGAcgaAGGATGTG-3′); (iii) wild type 3′ AP-1 sequence in the IL-11 promoter (3′ AP-1) (5′-AGTCAGGATGTGTCAGGCCGGCCC-3′); and (iv) mutated 3′ AP-1 (5′-AGTCAGGATGTcgaAGGCCGGCCC-3′).

Four other oligonucleotides were obtained from commercial sources (Stratagene, La Jolla, CA). They included the following: (i) a classic AP-1 oligonucleotide (5′-CTAGTGATGAGTCAGCCGGATC-3′); (ii) an AP-2 oligonucleotide (5′-GATCGAACTGACCGCCCGCGGCCCGT-3′); (iii) an AP-3 oligonucleotide (5′-CTAGTGGGACTTTCCACAGATC-3′); and (iv) an SP-1 oligonucleotide (5′-GATCGATCGGGGCGGGGCGAT-C-3′).

Electrophoresis—EMSAs were performed using the techniques of Schreiber *et al.* (32). Radiolabeled double-stranded oligonucleotide probes were prepared by annealing complementary oligonucleotides and end-labeling using [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs). The labeled probes were purified by push-column chromatography, diluted with TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) to the desired concentration, and incubated with equal aliquots of nuclear extract (2–5 μg) and 2 μg of poly[dI-dC]·poly[dI-dC] in a total volume of 20 μl at room temperature for 1 h. Resolution was accomplished by electrophoresing 10 μl of the reaction solution on vertical 6% native polyacrylamide gels containing 2% glycerol using 25% TBE buffer (22.3 mM Tris-HCl, 22.3 mM boric acid, 0.25 mM EDTA, pH 8.0). Binding was assessed via autoradiography.

Supershift EMSA—Supershift assays were used to determine which members of the AP-1 family were involved in TGFβ$_1$-stimulation of IL-11 gene transcription. In these studies EMSA were performed as described above except that isotype matched rabbit polyclonal antibodies against AP-1 proteins or control preimmune antiserum were included during the 1-h radiolabeled probe-extract incubation period. All of the antibodies that were used were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). They included antibodies that react with all Jun family members (Pan-Jun) (catalogue number SC-44X), JunB (catalogue number 46X), JunD (catalogue number SC-74X), c-Jun (catalogue number SC822X); all Fos family members (pan-Fos) (catalogue number SC-253X), c-Fos (catalogue number SC-52X), FosB (catalogue number SC-48X), Fra-1 (catalogue number SC-605X), and Fra-2 (catalogue number SC604X).

Respiratory Syncytial Virus (RSV) Preparation and Infection

RSV (A-2 strain) was obtained from the ATCC. Stock virus was prepared in permissive cell lines and titered, and A549 cells were infected with the virus as described previously by this laboratory (17, 19).

RESULTS

TGF-β$_1$ and RSV Stimulate IL-11 Gene Transcription—Previous studies from our laboratory demonstrated that TGF-β$_1$ is a potent stimulator of IL-11 gene transcription in lung fibroblasts (18) and that TGF-β$_1$ and RSV stimulate A549 alveolar epithelial cell IL-11 protein production and mRNA accumulation (17, 19). To determine if both stimuli augment IL-11 gene transcription in A549 cells, nuclear run-on assays were performed, and the levels of IL-11 gene transcription were evaluated at base line, after TGF-β$_1$ stimulation and after RSV infection. At base line, the levels of IL-11 gene transcription in A549 cells were near the limits of detection with our assay (Fig. 1). In contrast, both TGF-β$_1$ and RSV caused significant increases in IL-11 gene transcription (Fig. 1).

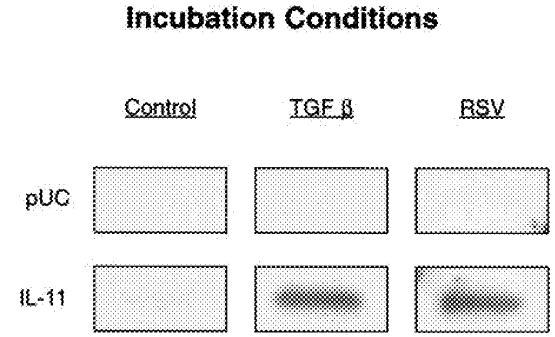

FIG. 1. **Demonstration of the effects of TGF-β$_1$ and RSV on IL-11 gene transcription.** A549 cells were incubated in medium alone (*Control*), stimulated with TGF-β$_1$ (*TGF-β*) (10 ng/ml), or infected with *RSV*. Twenty four hours later their nuclei were harvested, and gene transcription was assessed as described under "Experimental Procedures." The levels of IL-11 gene transcription are compared with the hybridization noted with pUC18 (*pUC*) without a cDNA insert which served as a negative control.

Characterization of the Transcription Start Site in the IL-11 Promoter—Prior to initiating studies designed to define the stimulation-responsive cis-elements in the IL-11 promoter, primer extension analysis was used to characterize the transcription initiation site in TGF-β$_1$-stimulated A549 cells. A single transcription initiation site was detected. This start site was 154 bp upstream of the ATG (data not shown) and is within 1–2 bases of the start site previously described in unstimulated PU-34 primate bone marrow fibroblasts by our laboratories (21).

TGF-β$_1$ Stimulates IL-11 Promoter Activity—To begin to characterize the mechanism by which TGF-β$_1$ stimulates IL-11 gene transcription, transient transfection assays were performed with a promoter-luciferase reporter gene construct containing IL-11 promoter elements between nucleotides −728 and +58 (relative to the transcription start site). The levels of luciferase activity in A549 cells were evaluated at base line and after TGF-β$_1$ stimulation. As can be seen in Fig. 2, only a modest level of luciferase activity was able to be detected in unstimulated A549 cells. In contrast, TGF-β$_1$ was an impressive, dose-dependent stimulator of the promoter activity of this construct (Fig. 2). This demonstrates that the −728 to +58 fragment of the IL-11 promoter contains TGF-β$_1$-responsive sequences.

Previous studies from our laboratory demonstrated that RSV also stimulates A549 cell IL-11 production and mRNA accumulation (17, 19). The studies noted above demonstrate that this stimulation is, at least in part, transcriptionally mediated. To determine if the cis-elements in the IL-11 promoter that respond to TGF-β$_1$ also respond to RSV, we compared the ability of these two stimuli to stimulate the −728 to +58 IL-11 promoter-luciferase construct. As noted above, TGF-β was a potent stimulator of this construct. In contrast, RSV, an equally potent stimulator of IL-11 transcription in the run-on assays, did not stimulate this construct in an impressive fashion (data not shown). Thus, the response elements between −728 and +58 in the IL-11 promoter are at least partially stimulus-specific since they respond vigorously to TGF-β$_1$ but not vigorously to RSV.

Definition of the TGF-β$_1$ Response Element in the IL-11 Promoter—To define further the cis-element(s) in the IL-11 promoter that responds to TGF-β$_1$, intrinsic restriction sites and Bal-31 digestion were employed to obtain IL-11 promoter fragments that contain progressively larger 5′ deletions. These promoter constructs were then cloned into our luciferase reporter construct, and their responsiveness to TGF-β$_1$ was as-

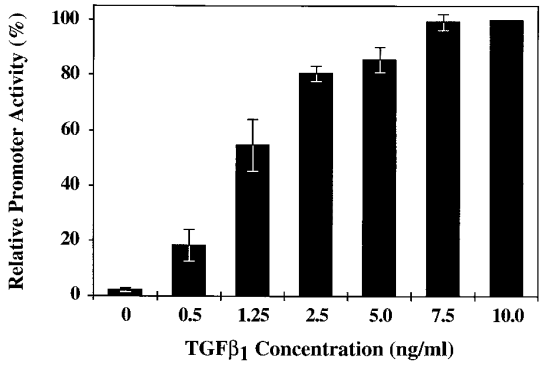

FIG. 2. **TGF-β_1 stimulation of IL-11 promoter-luciferase activity.** A549 cells were transfected with wild type IL-11 promoter-luciferase constructs that contained IL-11 sequences extending from nucleotides −728 to +58 relative to the transcription start site (pXP2-IL-11-728). The cells were then incubated for 24 h in the presence and/or absence of the noted concentrations of TGF-β_1; cell lysates were prepared, and luciferase activity was quantitated as described under "Experimental Procedures." The luciferase activities in the lysates from cells incubated with concentrations of TGF-β_1 <10 ng/ml are expressed as a percent of the activity in lysates from cells incubated with 10 ng/ml TGF-β_1. The noted values represent the mean ± S.E. of three separate determinations.

sessed. Constructs whose 5′ end extended from −728 to −81 were generated and tested. The parent (−728 to +58) and all of the 5′ deletion constructs did not express significant levels of luciferase activity in unstimulated A549 cells. TGF-β_1, however, was a potent stimulator of IL-11 promoter-driven luciferase activity in the parent construct (Fig. 3). Interestingly, 5′ deletions extending from −728 to −100 did not significantly alter TGF-β_1 responsiveness (Fig. 3). In contrast, deletions past −100 markedly diminished TGF-β_1 responsiveness. When stimulated with TGF-β_1, these constructs had ≤5% of the TGF-β_1 inducibility of the wild type −728 to +58 parent construct (Fig. 3). These studies demonstrate that elements that are essential for TGF-β_1 induction exist proximal to nucleotide −100 in the IL-11 promoter.

Importance of AP-1 Sites in TGF-β-mediated Transcriptional Activation—Inspection of the sequence immediately 3′ of −100 in the IL-11 promoter demonstrated two AP-1-like elements separated by 3 base pairs (Fig. 4). To define the role that these elements play in conferring TGF-β_1 responsiveness, constructs were prepared that contained point mutations at these sites, individually and in combination. Neither mutation caused a significant increase in the basal levels of IL-11 promoter activity (Fig. 5). Individual mutation in the 5′ (distal) or 3′ (proximal) sites caused an approximately 75% decrease in TGF-β_1 responsiveness (Fig. 5). Interestingly, the simultaneous mutation of both the 5′ and the 3′ AP-1-like sites abrogated TGF-β_1 responsiveness in this system. These constructs had ≤5% of the TGF-β_1 responsiveness of the wild type promoter-luciferase construct (Fig. 5). These studies demonstrate that both of these AP-1 sites play important roles in TGF-β_1-induced IL-11 activation.

Effect of TGF-β_1 on AP-1 Protein-DNA Binding—To gain additional insight into the trans-acting factors that bind to the AP-1 sites in the IL-11 promoter, electrophoretic mobility shift assays (EMSA) were performed using labeled oligonucleotides identical to the 5′ and 3′ AP-1-like sequences in the IL-11 promoter. At base line, protein-DNA binding was detected at the 5′ site but not the 3′ site (Fig. 6). TGF-β_1 stimulation caused an impressive increase in binding to the 5′ site. This stimulation was noted 2–6 h and peaked approximately 12–24 h after the addition of TGF-β_1 to the A549 cell cultures (Fig. 6). TGF-β_1 stimulation also induced protein-DNA binding at the 3′ AP-1 site in the IL-11 promoter. This induction had a slower kinetic than the induction at the 5′ site. It was, however, readily detected after 12–24 h of TGF-β_1-cell incubation (Fig. 6). In both cases, the protein-DNA binding was eliminated by the addition of excess unlabeled oligonucleotides encoding the 5′ AP-1 site, 3′ AP-1 site, or a consensus AP-1 sequence but not by oligonucleotides encoding NF-κB, AP-2, or SP-1 sequences (data not shown). When viewed in combination, these studies demonstrate that TGF-β_1 enhances AP-1 family protein binding to both the 5′ and 3′ AP-1 sites in the IL-11 promoter.

Effects of TGF-β_1 on AP-1 Subunit Composition—To understand further the trans-acting factors binding to the AP-1 sites in the IL-11 promoter, supershift EMSAs were performed using antibodies to a variety of AP-1 family proteins. At both the 5′ and 3′ sites the importance of AP-1 family members was confirmed since anti-pan Jun and anti-pan Fos antibodies (antibodies against all Jun proteins and all Fos family proteins, respectively) caused impressive supershifts in this assay (Fig. 7 and data not shown). In addition, characteristic patterns of AP-1 subunit usage were noted when selective antibodies were employed. At base line, the binding to the 5′ IL-11 AP-1 site was composed almost entirely of JunD AP-1 moieties (Fig. 7). Interestingly, TGF-β_1 increased the contribution of c-Jun, Fra-1, and Fra-2 proteins while simultaneously decreasing the contribution of JunD proteins to the 5′ AP-1-DNA binding. Significant alterations in c-Fos, FosB, and JunB were not noted (Fig. 7). Similar alterations in AP-1 subunit binding to the 3′ site were noted (data not shown). These studies demonstrate that TGF-β_1 stimulation of IL-11 gene transcription is associated with impressive and selective alterations in the composition of the AP-1 moieties that bind to the IL-11 promoter. These changes are characterized by an enhanced contribution from c-Jun, Fra-1, and Fra-2 at the 5′ and 3′ sites and a simultaneous decrease in the base line binding of JunD to the 5′ site.

TGF-β_1 Response Elements Confer Responsiveness on a Minimal Promoter Construct—To understand further the TGF-β_1 response elements in the IL-11 promoter, studies were undertaken to determine if they could confer TGF-β_1 responsiveness on a minimal promoter-reporter gene construct. This was done by generating constructs containing IL-11 promoter fragments between −103 and −77, in the sense and antisense direction, in series with the herpes simplex virus minimal promoter and luciferase reporter gene. The activity of these constructs in the presence and absence of TGF-β_1 was compared with the activity of the minimal promoter-luciferase construct under the same conditions. The parent minimal promoter-luciferase construct did not demonstrate significant levels of activity in the presence or absence of TGF-β_1 (Fig. 8). In contrast, TGF-β_1 was a potent stimulator of the sense and antisense IL-11 promoter-minimal promoter-Luc constructs (Fig. 8). These studies demonstrate that the sequences between −103 and −77 in the IL-11 promoter act as an enhancer and confer TGF-β_1 responsiveness on heterologous promoter elements.

DISCUSSION

Studies from a number of laboratories have demonstrated that TGF-β_1 is a potent stimulator of IL-11 production. To understand further the biology of IL-11, the mechanism of this stimulation was characterized. Nuclear run-on studies demonstrated that this stimulatory effect is, at least in part, transcriptionally mediated, and transient transfection assays demonstrated that TGF-β_1 is a potent stimulator of IL-11 promoter-reporter gene constructs. Two AP-1 motifs located between nucleotides −100 and −82 in the IL-11 promoter were noted to be necessary for this induction. In addition, a 25-base pair nucleotide sequence that contains these AP-1 sites was able to

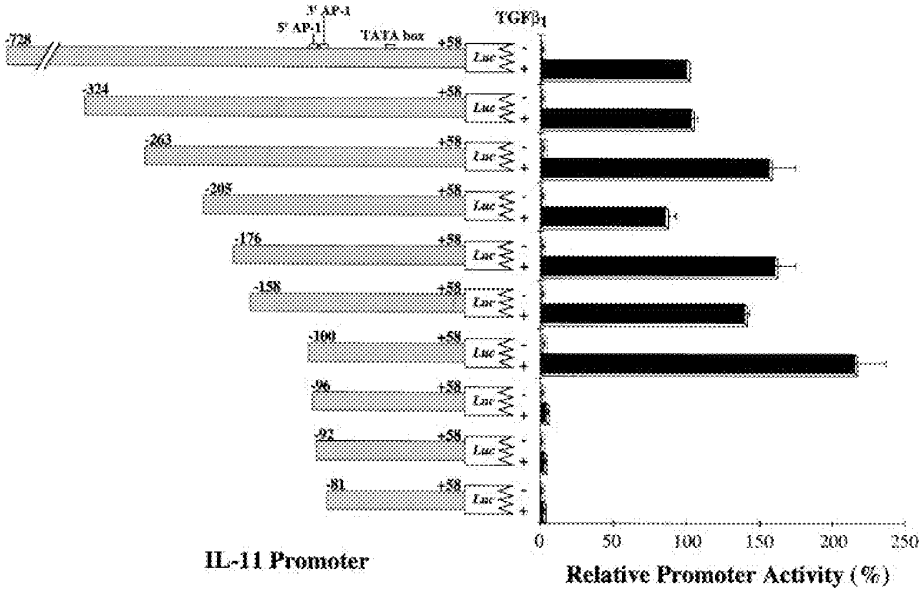

FIG. 3. **Effect of progressive 5′ deletion mutation on the TGF-β_1 responsiveness of pXP-2-IL-11-728.** Progressive 5′ deletion mutations were made in construct pXP-2-IL-11-728 using intrinsic restriction sites and Bal-31 digestion. These mutants are illustrated in the left half of the figure. They were then transfected into A549 cells, and the luciferase activities of these cells were quantitated after incubation for 24 h in medium alone (*open bars*) or with TGF-β_1 (*solid bars*) as described under "Experimental Procedures." The luciferase activities of the deletion mutants are expressed as a percent of the activities of cells transfected with the unaltered parent construct. The noted values represent the mean ± S.E. of three separate determinations.

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-331  CCAGCCGGAC CCCCACCCAC AGTCGGGCCC CAGCGCTTGA GCCTGAGTGT
-281  CTGCTCCGGC CCGTGGAGGT GGAGGGAGGG GACGCCAATG ACCTCACCAG
-231  CCCCTCTCCG ACCACCCCCC CCTTTCCCTT TTCAACTTTT CCAACTTTTC
-181  CTTCCGTGCC CTCCTCCGAG CGCGGCGGCG TGAGCCCTGC AAGGCAGCCG
                    5'AP-1          3'AP-1
-131  CTCCGTCTGA ATGGAAAAGG CAGGCAGGGA GGGTGAGTCA GGATGTGTCA
-081  GGCCGGCCCT CCCCTGCCGC CTGCCCCCCG CCCGCCCGCC CCAGGCCCCC
      TATA box                    Transcription Initiation Site
-031  TATATAACCC CCCAGGCGTC CACACTCCCT CACTGCCGCG GGCCCTGCTG
 020  CTCAGGGCAC ATGCCTCCCC TCCCCAGCCG CGGGCCCAGC TGACCCTCGG
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FIG. 4. **Sequence of the IL-11 promoter illustrating the transcription start site, the 5′ and 3′ AP-1 sites, and the TATA box.**

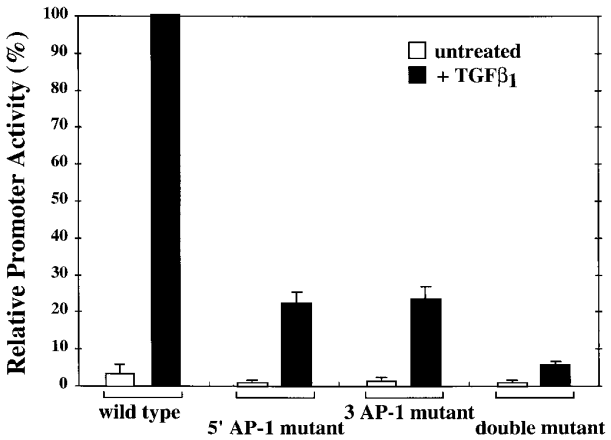

FIG. 5. **Effect of AP-1 mutations on TGF-β_1 responsiveness.** Luciferase reporter gene constructs were prepared that contained IL-11 promoter sequences extending from −324 to +58 relative to the transcription start site (pXP2-IL-11-324). They were then transfected into A549 cells, and luciferase activities were assessed in the presence (*solid bars*) and absence (*open bars*) of TGF-β_1 (10 ng/ml) treatment. Comparisons were made of wild type constructs and constructs with mutations in the 5′ AP-1 site, 3′ AP-1 site, and both AP-1 sites (double mutant). Luciferase activities are expressed as a percent of the activity in lysates from cells transfected with the wild type construct and stimulated with TGF-β_1. The noted values represent the mean ± S.E. of at least three separate determinations.

confer TGF-β_1 responsiveness on a heterologous minimal promoter. Supershift EMSA demonstrated that JunD is the major AP-1 protein binding promoter DNA in unstimulated cells. TGF-β_1 stimulation was associated with enhanced protein-DNA binding to both AP-1 motifs with an augmented contribution by c-Jun, Fra-1, and Fra-2 proteins and a decreased contribution by JunD proteins. These are the first studies to investigate, in depth, the mechanisms that stimulate IL-11 gene transcription. All in all, they demonstrate the importance in this inductive response of complex alterations in AP-1 transcription factors and a cis-enhancer element that contains two closely approximated AP-1 motifs.

TGF-β_1 is a pleiotropic cytokine with far reaching effects on tissue homeostasis. Prominent in this regard are its ability to stimulate tissue fibrosis, regulate matrix molecule elaboration, and inhibit tissue inflammation. In keeping with its biologic importance, a significant amount of effort has been directed at characterizing the mechanism(s) by which TGF-β mediates its biologic effects. Studies of TGF-β_1 regulation of cytokine production (33), cell proliferation (34), and retinoic acid receptor expression (35) have demonstrated that AP-1 activation can play a major role in these processes. Our studies add to this body of data by demonstrating that TGF-β_1 stimulation of IL-11 elaboration is also mediated via an AP-1-dependent pathway.

AP-1 was initially identified as a DNA binding activity in HeLa cell extracts that bound to cis-elements within the promoter and enhancer sequences of the human metallothionein IIA gene and simian virus 40 (36). It is now a term that is used to refer to dimeric proteins produced by the complex immediate-early gene family that couple a variety of extracellular stimuli from the cell surface to the nucleus to initiate alterations in gene expression and cell phenotype (37, 38). Investi-

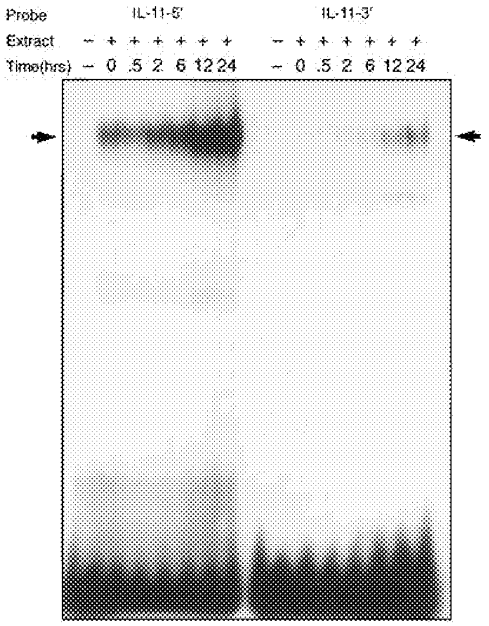

FIG. 6. **Electrophoretic mobility shift assay (EMSA) characterizing the protein binding to the 5′ and 3′ AP-1 sites in the IL-11 promoter.** A549 cells were incubated in medium alone or in the presence of TGF-β_1 for the noted periods. Nuclear lysates were prepared and EMSA were performed using oligonucleotides encoding the 5′ and 3′ AP-1 sites in the IL-11 promoter as described under "Experimental Procedures." The *arrows* highlight the shifted bands.

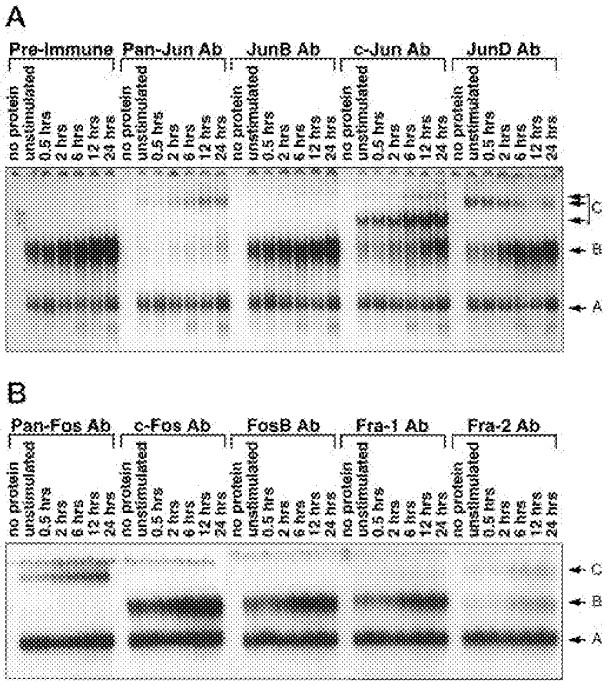

FIG. 7. **Characterization of the TGF-β_1-induced alterations in AP-1 subunit binding to the 5′ (distal) AP-1 site in the IL-11 promoter.** A549 cells were incubated in medium alone or with TGF-β_1 (10 ng/ml) for the noted periods. Nuclear lysates were then prepared, and supershift EMSA were performed as described under "Experimental Procedures." *A*, comparisons are made of the protein-DNA binding seen with preimmune antiserum (*Pre-immune*), antibody that binds all Jun moieties (*Pan-Jun Ab*), antibody to JunB proteins (*JunB Ab*), antibody to c-Jun proteins (*c-Jun Ab*), and antibody to JunD proteins (*JunD Ab*). *B*, comparisons are made of the effects of antibodies that bind to all Fos moieties (*Pan-Fos Ab*), antibody that binds c-Fos proteins (*c-Fos Ab*), antibody that binds FosB proteins (*FosB Ab*), antibody that binds Fra-1 proteins (*Fra-1 Ab*), and antibody that binds Fra-2 proteins (*Fra-2 Ab*). In this figure the nonspecific bands are highlighted by the *arrows* labeled *A*; the TGF-β_1-induced AP-1 binding reactions are highlighted by the *arrows* labeled *B*, and the supershifted bands are highlighted by the *arrows* labeled *C*.

gations of these moieties have revealed a consensus binding site for these dimers, the palindromic sequence, 5′-TGA(G/C)TCA-3′ (36). Our studies demonstrated that the sequence between −100 and −82 plays a crucial role in the transcriptional activation of the IL-11 promoter. Inspection of this region revealed a classic AP-1 binding site (the distal (5′) AP-1 sequence) and a binding site that differs at a single nucleotide (the proximal (3′) AP-1 sequence). Both sites were shown to bind AP-1 moieties. In addition, mutation of each AP-1 site significantly decreased and the simultaneous mutation of both AP-1 sites totally abrogated TGF-β_1-induced IL-11 promoter activation. Furthermore, oligonucleotides made up largely of these 2 AP-1 sites conferred TGF-β_1 responsiveness upon a minimal heterologous promoter. These studies demonstrate that both AP-1 sites play important roles in IL-11 transcriptional activation and the enhancer function of this crucial promoter region. This is in keeping with studies of other genes such as involucrin (39) and tissue factor (40) whose promoters contain multiple AP-1 sites that play important roles in gene activation.

TGF-β_1 is one of a number of stimuli that induce epithelial cell IL-11 production *in vitro*. Previous studies from our laboratory demonstrated that a variety of respiratory tropic viruses also stimulate A549 cell production of IL-11 protein and the accumulation of IL-11 mRNA (17, 19). The studies in this manuscript demonstrate that RSV stimulates A549 cell IL-11 production via a mechanism that is, at least in part, transcriptionally mediated. They also demonstrate that the magnitude of induction of IL-11 induced by RSV is comparable to that seen with TGF-β_1. At the start of these studies, we hypothesized that RSV and TGF-β_1 would activate the IL-11 promoter via similar mechanisms. This did not, however, prove to be true. Although RSV was as potent as TGF-β_1 in the induction of IL-11 protein production, mRNA accumulation, and gene transcription, it did not efficiently stimulate the IL-11 promoter-reporter gene constructs used in these studies. This demonstrates that TGF-β_1 and RSV activate the IL-11 promoter via different mechanisms. It also provides insight into the stimulus specificity of the pathways used to activate IL-11 gene transcription.

AP-1 proteins are made up of homo- and heterodimers composed of Fos, Jun, and activating transcription factor subunits (37). At least 24 different combinations have been described (36). This complexity is the result of important tissue-specific, stimulus-specific, and temporally regulated differences in AP-1 subunit expression (36, 40, 41). This results in AP-1 moieties that differ in their DNA binding capacities, transactivation capacities, and biologic effects (42–44). To gain insight into the AP-1-mediated events involved in TGF-β_1 stimulation of IL-11, we used supershift gel mobility shift assays to define the Fos and Jun proteins involved in this inductive response. These studies demonstrate that, in the absence of stimulation, JunD is the major AP-1 moiety that can be detected. This is in keeping with studies from a number of different laboratories demonstrating that JunD is constitutively expressed in a variety of cells and tissues (45) and studies from our laboratories that demonstrate that JunD plays an important role in the constitutive elaboration of IL-11 by PU34 cells (21). Our studies also demonstrate that TGF-β_1 stimulation is associated with an increase in c-Jun, Fra-1, and Fra-2 and a decrease in

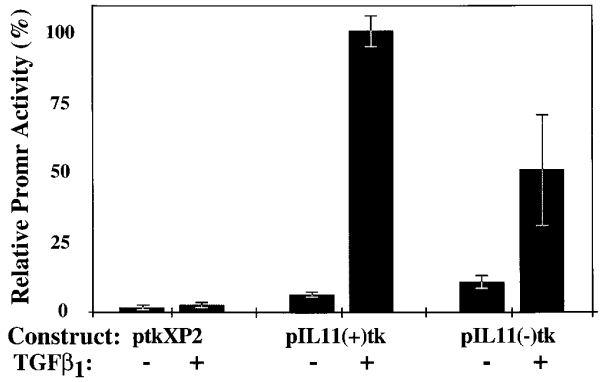

FIG. 8. **Demonstration that the double AP-1 sequence in the IL-11 promoter can confer TGF-β_1 responsiveness on a heterologous promoter.** Luciferase reporter constructs were prepared that contained the herpes simplex virus tk minimal promoter and the sequence in the IL-11 promoter from nucleotide −103 to −77 (a stretch that contains both AP-1 sites) inserted in a sense (*pIL11(+)tk*) and antisense (*pIL11(−)tk*) orientation. These constructs were transfected into A549 cells, and the luciferase activities in these cells were assessed after incubation for 24 h in medium alone or with TGF-β_1 (10 ng/ml). In these experiments the responses of the IL-11 minimal promoter-luciferase constructs were compared with the responses of the parent tk-minimal promoter-luciferase construct (ptkXP2). The luciferase activities in these experiments are expressed as a percent of the luciferase activities in cells transfected with *pIL-11(+)tk* and stimulated with TGF-β_1. The noted values represent the mean ± S.E. of a minimum of three separate experiments.

JunD-IL-11 promoter binding. These observations are in accord with the known inducibility of these AP-1 subunits and the well documented stimulus specificity of AP-1 subunit induction (36, 40, 43, 44, 46, 47). It is impossible to determine, from these studies, the degree to which the TGF-β_1-induced alterations in c-Jun, Fra-1, Fra-2, and/or JunD individually contribute to the induction of IL-11 studied in the present analysis. It is possible, however, to hypothesize that all may play an important role. JunD is a potent transactivator but, in contrast to many other AP-1 subunit moieties, is not induced in a major fashion by extracellular stimuli (21, 45). The JunD homodimers that would form in unstimulated cells under conditions of JunD excess would therefore play an important role in the regulation of basal IL-11 production but be unable to meet the enhanced transcriptional demands after TGF-β_1 stimulation. In contrast, TGF-β_1 stimulation enhances the contribution of c-Jun, Fra-1, and Fra-2 resulting in AP-1 dimers that are responsive to the conditions of stimulation. The entry of c-Jun, Fra-1, and Fra-2 into the AP-1-DNA binding complex can thus be speculated to be a key event underlying TGF-β_1-stimulated transcription of the IL-11 gene in A549 cells. A similar transcriptional activation paradigm has been proposed to explain the contribution of AP-1 in the stimulation of tissue factor by serum in mouse fibroblasts (40).

Our studies demonstrate that two closely approximated AP-1 sites in the IL-11 promoter play important roles in the stimulation of IL-11 gene transcription by TGF-β_1. It is important to keep in mind, however, that transcriptional activation is frequently a multi-factorial process that involves the concerted and coordinated interaction of a number of different transcription factors. This is well documented for the AP-1 moieties that are known to interact with a variety of other transcription factors including nuclear factor-κB (NF-κB), CREB, nuclear factor IL-6 (NF-IL-6), liver regeneration factor-1 (LRF-1), and polyoma virus enhancer A binding protein-3 (PEA_3) (36, 48). The transcriptional activities of AP-1 moieties are also regulated by a variety of other proteins including the Jun dimerizing protein (37) and the Jun activation domain binding protein 1 (JAB1) (37). In accord with this information, it is important to point out that the present studies, while implicating AP-1 in the regulation of TGF-β_1-stimulated IL-11 transcription, do not address the importance of each of these other moieties. It is likely that additional investigations will demonstrate that other cis- elements and/or trans-activating factors are involved in the coordinated production of IL-11 under a variety of circumstances.

In summary, the present studies demonstrate that TGF-β_1 stimulates IL-11 gene transcription in A549 cells and that this stimulation is mediated via a complex AP-1-dependent activation pathway. They also highlight two closely approximated AP-1 sites in the IL-11 promoter that are essential for this activation and demonstrate that DNA sequences that contain these two sites can confer TGF-β_1 responsiveness on a heterologous minimal promoter. Lastly, these studies demonstrate that, in the absence of TGF-β_1 stimulation, JunD is the major AP-1 subunit involved in IL-11 promoter-protein binding and that TGF-β_1 stimulation is associated with increased c-Jun, Fra-1, and Fra-2 and decreased JunD-DNA binding.

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REFERENCES

1. Paul, S. R., Bennett, F., Calvetti, J. A., Kelleher, K., Wood, C. R., Oharra, R. M. J., Leary, A. C., Sibley, B., Clark, S. C., Williams, D. A., and Yang, Y.-C. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 7512–7516
2. Du, X. X., and Williams, D. A. (1994) *Blood* **83,** 2023–2030
3. Quesniaux, V. F. J. (1992) *Res. Immunol.* **143,** 385–400
4. Ip, N. Y., Nye, S. H., Boulton, T. G., Davis, S., Taga, T., Li, Y., Birren, S. J., Yasukawa, K., Kishimoto, T., Anderson, D. J., Stahl, N., and Yancopoulos, G. D. (1992) *Cell* **69,** 1121–1132
5. Zhang, X.-G., Gu, J.-J., Lu, Z.-Y., Yasukawa, K., Yancopoulos, G. D., Turner, K., Shoyab, M., Taga, T., Kishimoto, T., Bataille, R., and Klein, B. (1994) *J. Exp. Med.* **177,** 1337–1342
6. Girasole, G., Passeri, G., Jilka, R. L., and Manolagas, S. C. (1994) *J. Clin. Invest.* **93,** 1516–1524
7. Maier, R., Ganu, V., and Lotz, M. (1993) *J. Biol. Chem.* **268,** 21527–21532
8. Potten, C. S. (1995) *Int. J. Cancer* **62,** 356–361
9. Quesniaux, V. F. J. (1994) *Leuk. & Lymphoma* **14,** 241–249
10. Quesniaux, V. F. J., Mayer, P., Liehl, E., Turner, K., Goldman, S. J., and Fagg, B. (1993) *Rev. Exp. Pathol.* **34,** 205–214
11. Tang, W., Geba, G. P., Zheng, T., Ray, P., Homer, R. J., Kuhn, C., III, Flavell, R. A., and Elias, J. A. (1996) *J. Clin. Invest.* **98,** 2845–2853
12. Redlich, A. A., Gao, X., Rockwell, S., Kelley, M., and Elias, J. A. (1996) *J. Immunol.* **157,** 1705–1710
13. Du, X. X., Doerschuk, C. M., Orazi, A., and Williams, D. A. (1994) *Blood* **83,** 33–37
14. Keith, J., James, C., Albert, L., Sonis, S. T., Pfeiffer, C. J., and Schaub, R. G. (1994) *Stem Cells* **12,** Suppl. 1, 79–90
15. Rochester, C. L., Ackerman, S. J., Zheng, T., and Elias, J. A. (1996) *J. Immunol.* **156,** 4449–4456
16. Zheng, T., Nathanson, M., and Elias, J. A. (1994) *J. Immunol.* **153,** 4742–4752
17. Einarsson, O., Geba, G. P., Zhu, Z., Landry, M., and Elias, J. A. (1996) *J. Clin. Invest.* **97,** 915–924
18. Elias, J. A., Zheng, T., Whiting, N. L., Trow, T. K., Merrill, W. W., Zitnik, R., Ray, P., and Alderman, E. M. (1994) *J. Immunol.* **152,** 2421–2429
19. Elias, J. A., Zheng, T., Einarsson, O., Landry, M., Trow, T., Rebert, N., and Panuska, J. (1994) *J. Biol. Chem.* **269,** 22261–22268
20. Elias, J. A., Tang, W., and Horowitz, M. C. (1995) *Endocrinology* **136,** 489–498
21. Yang, L., and Yang, Y.-C. (1994) *J. Biol. Chem.* **269,** 32732–32739
22. Zhu, Z., Tang, W., Ray, A., Wu, Y., Einarsson, O., Landry, M. L., Gwaltney, J., Jr., and Elias, J. A. (1996) *J. Clin. Invest.* **97,** 421–430
23. Elias, J. A., and Lentz, V. (1990) *J. Immunol.* **145,** 161–166
24. Elias, J. A., Lentz, V., and Cummings, P. J. (1991) *J. Immunol.* **146,** 3437–3443
25. Leng, S. X., and Elias, J. A. (1997) *J. Immunol.* **159,** 2161–2168
26. Aviv, H., and Leder, P. (1972) *Proc. Natl. Acad. Sci. U. S. A.* **69,** 1408–1412
27. Edmonds, M., Vaughan, M., Jr., and Nakazato, H. (1971) *Proc. Natl. Acad. Sci. U. S. A.* **68,** 1336–1340
28. Sambrook, J. (1989) in *Molecular Cloning: A Laboratory Manual* (Sambrook, J., Fritsch, E. F., and Maniatie, T., eds) 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
29. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) *Methods Enzymol.* **154,** 367–382
30. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 488–492
31. Ray, A., LaForge, K. S., and Sehgal, P. B. (1990) *Mol. Cell. Biol.* **10,** 5736–5746
32. Schreiber, E., Matthias, P., Muller, M. M., and Schaffner, W. (1989) *Nucleic Acids Res.* **17,** 6419
33. Takeshita, A., Chen, Y., Watanabe, A., Kitano, S., and Hanawawa, S. (1995) *J. Immunol.* **155,** 419–426

34. Mauviel, A., Chung, K.-Y., Agarwal, A., Tamai, K., and Uitto, J. (1996) *J. Biol. Chem.* **271,** 10917–10923
35. Chen, Y., Takeshita, A., Ozaki, K., Kitano, S., and Hanazawa, S. (1996) *J. Biol. Chem.* **271,** 31602–31606
36. Foletta, V. (1996) *Immunol. Cell Biol.* **74,** 121–133
37. Karin, M., Liu, Z.-G., and Zandi, E. (1997) *Curr. Opin. Cell Biol.* **9,** 240–246
38. Xanthoudakis, S., and Curran, T. (1996) *Adv. Exp. Med. Biol.* **387,** 69–75
39. Welter, J. F., Crish, J. F., Agarwal, C., and Eckert, R. L. (1995) *J. Biol. Chem.* **270,** 12614–12622
40. Felts, S. J., Stoflet, E. S., Eggers, C. T., and Getz, M. J. (1995) *Biochemistry* **34,** 12355–12362
41. Lebrun, C. J., Blume, A., Herdegen, T., Seifert, K., Bravo, R., and Unger, T. (1995) *Neuroscience* **65,** 93–99
42. Pfarr, C. M., Mechta, F., Spyrou, G., Lallemand, D., Carillo, S., and Yaniv, M. (1994) *Cell* **76,** 747–760
43. Musti, A. M., Treier, M., Peverali, F. A., and Bohmann, D. (1996) *Biol. Chem.* **377,** 619–624
44. Wang, H., Xie, Z., and Scott, R. E. (1996) *Oncogene* **13,** 2639–2647
45. Berger, I., and Shaul, Y. (1994) *DNA Cell Biol.* **13,** 249–255
46. Ding, W. Q., Larsson, C., and Alling, C. (1996) *Neuroreport* **7,** 2191–2195
47. Wang, H., Xie, Z., and Scott, R. E. (1996) *J. Cell Biol.* **135,** 1151–1162
48. Benbow, U., and Brinckerhoff, C. E. (1997) *Matrix Biol.* **15,** 519–526

CELL BIOLOGY AND METABOLISM:

Transforming Growth Factor-β Stimulates Interleukin-11 Transcription via Complex Activating Protein-1-dependent Pathways

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Targeted Expression of IL-11 in the Murine Airway Causes Lymphocytic Inflammation, Bronchial Remodeling, and Airways Obstruction

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Abstract

Interleukin-11 is a pleotropic cytokine produced by lung stromal cells in response to respiratory viruses, cytokines, and histamine. To further define its potential effector functions, the Clara cell 10-kD protein promoter was used to express IL-11 and the airways of the resulting transgene mice were characterized. In contrast to transgene (−) littermates, the airways of IL-11 transgene (+) animals manifest nodular peribronchiolar mononuclear cell infiltrates and impressive airways remodeling with subepithelial fibrosis. The inflammatory foci contained large numbers of B220(+) and MHC Class II(+) cells and lesser numbers of CD3(+), CD4(+), and CD8(+) cells. The fibrotic response contained increased amounts of types III and I collagen, increased numbers of α smooth muscle actin and desmin-containing cells and a spectrum of stromal elements including fibroblasts, myofibroblasts, and smooth muscle cells. Physiologic evaluation also demonstrated that 2-mo-old transgene (+) mice had increased airways resistance and non-specific airways hyperresponsiveness to methacholine when compared with their transgene (−) littermates. These studies demonstrate that the targeted expression of IL-11 in the mouse airway causes a B and T cell–predominant inflammatory response, airway remodeling with increased types III and I collagen, the local accumulation of fibroblasts, myofibroblasts, and myocytes, and obstructive physiologic dysregulation. IL-11 may play an important role in the inflammatory and fibrotic responses in viral and/or nonviral human airway disorders. (*J. Clin. Invest.* 1996. 98:2845–2853.) Key words: epithelial cell • fibrosis • cytokine • myofibroblast • collagen

Introduction

Obstructive airways disorders are a major cause of morbidity and mortality, with asthma affecting ~ 9–12 million people (1, 2), chronic obstructive pulmonary disease (COPD)[1] affecting 12–14 million people (3), and bronchiolitis and bronchiectasis affecting large numbers of people (4, 5) in the United States alone. Chronic airway inflammation and airway remodeling (defined as fibrosis, matrix alterations, and/or changes in structural or resident cells of the airway wall) are important features of these disorders (3–11). However, the pathogenetic mechanisms that generate these responses and the relationship between these responses and the physiologic dysregulation characteristic of these disorders are poorly understood.

Respiratory viruses play an important role in obstructive disorders of the human airway. Viruses are important precipitants of asthmatic exacerbations (6, 12–15) and may similarly exacerbate COPD (16). In addition, epidemiologic investigations have demonstrated important associations between infantile viral infections and the existence of asthma (12, 13, 17, 18), and pediatric infections and COPD (16, 19) in later life. These viral effects are felt to be mediated via a number of mechanisms, including the induction and modulation of local inflammation (10, 12, 20). Virus-stimulated cytokine production is increasingly understood to play a prominent role in the generation of these inflammatory abnormalities (6, 21–23). The contribution(s) that each virus-stimulated cytokine makes to the pathologic and physiologic abnormalities characteristic of viral infections in hosts with normal and obstructed airways has, however, been inadequately investigated. In addition, we know little about the mechanism(s) by which pediatric viral infections and virus-induced cytokines predispose to airways disorders in later life.

IL-11 was initially discovered as a plasmacytoma proliferation stimulating activity in supernatants from transformed marrow fibroblasts (24, 25). In accordance with this finding, most studies of IL-11 have focused on its roles in hematopoiesis (25). IL-11 has, however, been shown to have a variety of other bioactivities, including the ability to stimulate the acute phase response (25), augment the production of metalloproteinase inhibitors (26, 27), increase immunoglobulin production (25, 28), and alter neural phenotype (29). Previous studies from our laboratory demonstrated that human lung fibroblasts and epithelial cells produce IL-11 in response to cytokines (IL-1 and TGF-β_1), histamine, and viruses that have been epidemiologically associated with asthmatic exacerbations (rhinovirus, respiratory syncytial virus [RSV], and parainfluenza virus type 3, [PIV3]) (21, 30, 31). Our studies have also demonstrated that IL-11 can be found in the nasal secretions of children with viral upper respiratory tract infections and that IL-11 induces

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1. *Abbreviations used in this paper:* AHR, airways hyperresponsiveness; BAL, bronchoalveolar lavage; CC10, clara cell 10-kD protein; COPD, chronic obstructive pulmonary disease; MCh, methacholine; PIV3, parainfluenza virus type 3; RSV, respiratory synctial virus.

airways hyperresponsiveness (AHR) when inhaled in a transient fashion into murine lungs (23). As a result of these observations, we postulated that IL-11 plays an important role in the pathogenesis of human airway disorders, particularly those that are associated with viral infections.

In keeping with the chronic nature of disorders such as asthma, COPD, bronchiectasis, and bronchiolitis, we addressed this hypothesis by defining the respiratory tract manifestations of IL-11 when chronically present in the respiratory tract. This was done by generating and evaluating the airways of transgenic mice in which the Clara cell 10-kD protein (CC10) promoter was used to target IL-11 to the respiratory tree. These studies demonstrate that IL-11 causes impressive airway alterations with transgene (+) animals manifesting a nodular B and T cell–predominant peribronchiolar inflammatory response, bronchial remodeling with subepithelial fibrosis, and physiologic dysfunction characterized by airways obstruction and nonspecific AHR.

Methods

Production and identification of transgenic mice. To study the effector functions of IL-11 in the airway, we took advantage of the fact that the murine respiratory tract epithelium contains 50–60% clara cells (32, 33). As previously described (32), we used the promoter of the CC10 gene to target the expression of human IL-11 to airway tissues. The rat CC10 promoter was a gift of Drs. Barry Stripp and Jeffrey Whitsett (34). It was isolated as a 2.3-kb HindIII fragment and subcloned into the HindIII site of construct pKS-SV40, yielding construct pKS-CC10-SV40. pKS-SV40 had been previously prepared by inserting a 0.85-kb BglII/BamHI fragment containing SV40 intronic and polyadenylation sites into the BamHI site in construct pBluescript IIKS (Stratagene Inc., La Jolla, CA). The cDNA encoding human IL-11 was a generous gift of Dr. Paul Schendel (Genetics Institute, Cambridge, MA). It was isolated as a 1.2-kb EcoRI fragment, end filled with Klenow enzyme and subcloned into the EcoRV site in pKS-CC10-SV40 using standard techniques. All constructs were checked for correct orientation of the inserts by restriction enzyme digestion, and junction sequences were confirmed by sequencing. The resulting CC10-IL-11-SV40 construct was purified, digested with Asp 718 and BamHI to generate the CC10-IL-11-SV40 fragment (Fig. 1), separated by electrophoresis through 1% agarose, and isolated by electroelution into dialysis tubing. The DNA fragment was then purified through Elutip-D columns following the manufacturer's instructions (Schleicher and Schuell, Inc., Keene, NH) and dialyzed against injection buffer (0.5 mM Tris-HCl/25 mM EDTA, pH 7.5). Transgenic mice were prepared in (CBA × C57 BL/6) F_2 eggs using standard pronuclear injection as previously described (32, 35). The presence or absence of the transgene was evaluated in offspring animals using tail-derived DNA. This was initially done by Southern blot analysis using ^{32}P-labeled IL-11 cDNA as a probe. Similar results were obtained by PCR using 5′-CGACTGGACCGGCTGCTGC-3′ and 5′-CTAACTAGGGGGAGATAATGGCGGGGGGA-3′ as upper and lower primers, respectively. 35 cycles were performed. Each cycle was heated at 95°C for 1 min, annealed at 63°C for 1 min, and elongated at 72°C for 2 min.

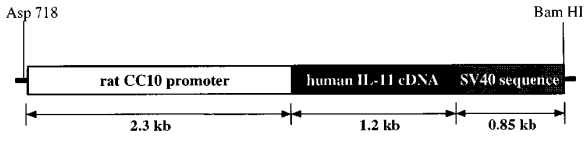

Figure 1. Schematic illustration of CC10-IL-11 construct used in the preparation of the transgenic mice described in this manuscript.

Bronchoalveolar lavage and quantification of IL-11 levels. Mice were killed via cervical dislocation, a median sternotomy was performed, blood was obtained via right heart puncture and aspiration, and serum was prepared. The trachea was then isolated via blunt dissection and small caliber tubing was inserted and secured in the airway. Three successive washes of 0.75 ml PBS with 0.1% BSA were then instilled and gently aspirated. Each bronchoalveolar lavage (BAL) aliquot was centrifuged and the supernatants were harvested and stored individually at −70°C until ready to be used. The levels of IL-11 in the BAL fluid and serum were quantitated immunologically via ELISA and biologically using the B9.11 plasmacytoma proliferation bioassay. The ELISA was performed as previously described by our laboratory (21, 30) using antibodies 11h3/15.6.1 and 11h3/19.6.1 provided by Dr. Edward Alderman (Genetics Institute). The bioassay was also performed as previously described by our laboratory (36, 37) using B9.11 cells also provided by Genetics Institute. Since both IL-6 and IL-11 can stimulate B9.11 cell proliferation, this assay was performed in the presence and absence of neutralizing antibodies against IL-11 (a gift of Dr. Alderman) and IL-6 (a gift of Dr. Pravin Sehgal, New York Medical College, Valhalla, NY) to assess the relative contribution of each of these moieties.

Northern analysis. Total cellular RNA from a variety of mouse tissues was obtained using guanidine isothiocyanate extraction and formaldehyde-agarose gel electrophoresis as previously described (21, 30). IL-11 gene expression was assessed by probing with ^{32}P-labeled IL-11 cDNA. Equality of sample loading and efficiency of transfer were assessed via ethidium bromide staining.

Histologic evaluation. Animals were killed via cervical dislocation, median stenotomies were performed, and right heart perfusion was accomplished with calcium and magnesium-free PBS to clear the intravascular space. The heart and lungs were then removed en bloc, inflated with 1 cc neutral buffered 10% formalin, fixed overnight in 10% formalin, embedded in paraffin, and sectioned and stained. Hematoxylin and eosin, and Mallory's trichrome stains were performed.

Morphometric analysis. Morphometric study was carried out on mice aged 15 d, 1 mo, and 2 mo. The thickness of the walls of small airways from the base of the columnar epithelium to the outer limit of the adventitia was measured using an eye-piece reticle. Bronchioles < 250 μm in diameter that presented a closed circular or oval profile were selected and all measurements were made at 400 magnification to the nearest whole micrometer. The wall thickness was routinely evaluated at two points on opposite sides of the short axis of the elliptical profiles and measurements were made at locations where cell borders appeared sharp to minimize tangential sectioning. 5–12 airways were measured per mouse, mean 8.4. The presence or absence of lymphoid nodules was recorded for each bronchiolar profile, whether or not it was considered appropriate for measuring wall thickness. Statistical evaluations of the morphometric results were performed by the Bonferonni multiple comparisons test using Instat software for the Macintosh.

Immunohistochemistry. Animals were killed, the vascular tree was perfused, and the heart and lungs were removed en bloc as described above. The tissues were then processed using a number of approaches. For evaluations of cell surface markers and subepithelial airway cellularity, lungs were inflated with 1 × PBS/33% (vol/vol) OCT tissue-tek compound (Miles Laboratories, Inc., Elkhart, IN) and snap frozen in OCT by submersion into 2-methylbutane cooled with dry ice. Tissue sections were cut, transferred onto silane-treated glass slides, fixed with acetone for 15 min, and stained with various antibody reagents as previously described (32). Sections were blocked with avidin-blocking kit (Vector Laboratories, Inc., Burlingame, CA) and BSA before reaction with the desired biotinylated primary antibody. The slides were then washed three times (in 0.1 M Tris buffer, pH 7.5) and the tissue sections were incubated with a prediluted streptavidin-alkaline phosphatase solution (Vector Laboratories, Inc.) for 1 h. The sections were washed and developed using Vector red staining (Vector Laboratories, Inc.) in accordance with the manufacturer's instructions. The slides were counter stained with

Meyer's hematoxylin, and then mounted with aqueous histologic mounting medium (Zymed Laboratories, Inc., So. San Francisco, CA).

For types I and III collagen immunostaining, mouse lungs were chilled in acetone containing protease inhibitors (20 mM iodoacetamide and 2 mM phenylmethyl sulfonyl fluoride) at −20°C for 16–20 h. The lungs were then chopped into ~ 2 × 5 × 6 mm^3 pieces and immersed three times in glycol methacrylate (GMA) monomer at 4°C for 6 h. The samples were then embedded in GMA according to the manufacturer's instructions (JB4 Embedding Kit; Polysciences Inc., Warrington, PA) and 2-μm sections were cut and transferred onto silane-treated glass slides. Immunohistochemical staining was undertaken as described above except that sections were treated with 1 M citric acid (pH 3.0) for 2 h before staining, and primary antibody incubation took place at 4°C overnight.

The antibodies that were employed and their sources are listed below. They included antibodies to CD3, CD4, CD8 (Gibco Laboratories, Grand Island, NY), Mac-1 (Pharmingen, San Diego, CA), B220 (Pharmingen), MHC Class II (a gift from Dr. Kim Bottomly, Yale University), type I collagen (Chemicon International, Inc., Temecula, CA), type III collagen (a gift from Dr. J. Madri, Yale University), α-smooth muscle actin (Sigma Chemical Co., St. Louis, MO), and desmin (Sigma Chemical Co.).

Electron microscopy. Fragments of lung from three age- and sex-matched littermate pairs were fixed in 3% gluteraldehyde, postfixed in osmium tetroxide, and embedded in Epox 812 (Ernest F. Fulham, Inc., Latham, NY). Tissues were then cut onto grids, stained with uranyl acetate and lead nitrate, and examined in a Philips 300 microscope (Philips Electronic Instruments, Inc., Mahwah, NJ).

Physiologic evaluation of transgenic mice. Age, sex, and weight matched littermate mice were anesthetized with pentobarbital (90 mg/kg) and tracheostomized with an 18-gauge angiocatheter. Airways resistance was then measured using a modification of the techniques described by Martin et al. (38) as previously described (32). With these techniques, the changes in the lung volumes of anesthetized and tracheostomized mice were measured plethysmographically by determining the pressure in a Plexiglass chamber using an inline microswitch pressure transducer. Flow was measured by differentiation of the volume signal and transpulmonary pressure was determined via a second Microswitch pressure transducer placed in line with the plethysmograph and animal ventilator. Resistance was then calculated using the method of Amdur and Mead (39). The resistance of the tracheostomy catheter was routinely eliminated. Baseline measurements of pulmonary resistance were obtained by ventilating the mouse in the plethysmograph at volumes of 0.4 ml at a rate of 150 breaths per minute (settings previously shown to produce normal arterial blood gases in this species) (38). Bronchial reactivity was also assessed using noncumulative methacholine challenge procedures as previously described by our laboratory (32). In this procedure, increasing concentrations of methacholine (MCh) in PBS were administered by nebulization (20 one-ml breaths) using a Devilbiss Aerosonic nebulizer (Model 5000; DeVilbiss Health Care, Somerset, PA) that produces particles 1–3 μM in diameter. Pulmonary resistance was calculated precisely 1 min later. Stepwise increases in MCh dose were then given until the pulmonary resistance, in comparison with the baseline level, had at least doubled. All animals received serial threefold increases in MCh from 1 to 100 mg/ml. The data are expressed as the PC_{100} (provocative challenge 100), the dose at which pulmonary resistance was 100% above the baseline level as calculated by linear regression analysis.

Statistical analysis. Values are expressed as means±SEM. Unless otherwise noted, group means are compared with the Student's two tailed unpaired *t* test using the StatView software for the Macintosh.

Results

Generation of transgenic mice. To generate transgenic mice in which IL-11 is overproduced in a lung-specific/selective fash-

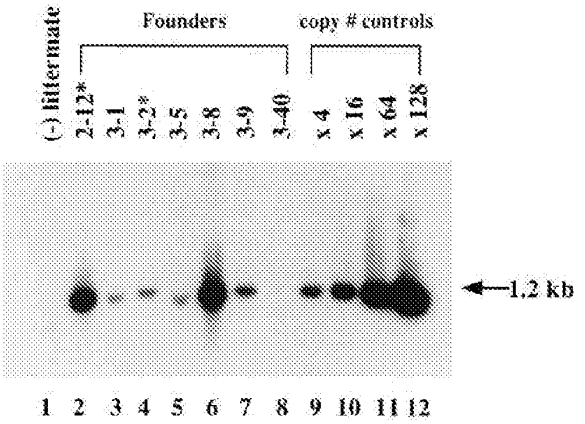

Figure 2. Southern blot analysis of CC10-IL-11 mice. Tail DNA was obtained and the presence or absence of the CC10-IL-11 construct was determined using Southern blot analysis as described in Methods. The results obtained using tail DNA from transgene (+) founder animals (lanes *2–8*) are compared with a transgene (−) littermate (lane *1*) and copy number control (lanes *9–12*).

ion, pronuclear microinjections of the CC10-IL-11-SV40 construct were performed on two separate occasions. From these microinjections, seven animals with transgene copy numbers varying between 1 and 70 were obtained (Fig. 2). These founder animals were bred with C57 BL/6 mice and the transgene status of these offspring were similarly analyzed. This analysis demonstrated that the transgenes passed on to the off-

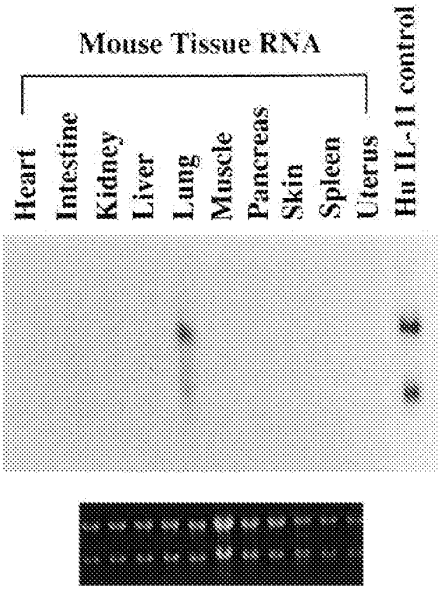

Figure 3. Northern blot analysis of IL-11 mRNA expression in mouse organs. Total cellular RNA was isolated from the noted organs of transgene (+) CC10-IL-11 mice and the levels of IL-11 mRNA characterized using Northern blot analysis as described in Methods. The IL-11 mRNA in total cellular RNA from the different tissues is compared with the IL-11 mRNA in TGF-β_1 (10 ng/ml)–stimulated human lung fibroblasts (*Hu IL-11 control*). Ethidium bromide controls are in bottom panel.

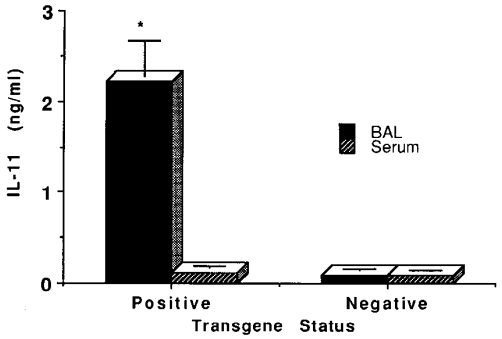

Figure 4. Levels of immunoreactive IL-11 in BAL fluid and serum of transgene (+) and (−) littermates. The noted values represent the mean±SEM of the evaluations of four separate pairs of transgene (+) and (−) littermates (*$P < 0.01$ vs. serum of transgene (+) animals and BAL and serum of transgene (−) animals; paired t test).

spring of these founder animals in a Mendelian fashion. Of these founders, lines 2-12 and 3-2 were chosen for more extensive analysis. Since they manifest similar pathologic, immunologic, and physiologic abnormalities, they will be discussed in a unified fashion.

Organ specificity and intensity of IL-11 gene expression and protein production. To determine if the CC10-IL-11 transgene was appropriately expressed, Northern analysis was used to compare the levels of IL-11 mRNA in the lungs and extrapulmonary organs of transgene (+) and (−) littermates. IL-11 mRNA was readily detected in the lungs of transgene (+) animals, but could not be appreciated in the lungs of transgene (−) animals (Fig. 3 and data not shown). In accordance with in vitro studies using fibroblasts and epithelial cells (21, 30), this IL-11 mRNA appeared to have one major and one minor transcript. IL-11 gene expression also appeared to be appropriately targeted to the lung since human IL-11 mRNA was not detectable in the RNA from a variety of extrapulmonary organs (Fig. 3). In all cases, IL-11 mRNA appeared to be appropriately translated since IL-11 was easily detected immunologically and biologically in the BAL fluid of the transgene (+) animals, but not in the serum of transgene (+) animals or the serum or BAL fluid of transgene (−) littermates (Fig. 4 and Table I).

Table I. IL-11 Bioactivity in BAL Fluids From Transgene (+) and (−) Animals

	[^{3}H]-Tdr incorporation‡	
Incubation conditions*	No antibody	+ Anti–IL-11
BAL (−)	3,178±3,708	1,705±166
BAL (+)	57,383±5,351	5,568±378
BAL (+)	52,960±4,717	6,189±5,507
Negative control	1,896±101	—

*BAL were performed on transgene (+) and (−) littermate F_2 progeny of IL-11 transgenic mice. ‡B9.11 plasmacytoma [^{3}H]-Tdr incorporation assessed in the presence and absence of anti–IL-11. BAL plasmacytoma-stimulating activities are compared with the proliferation ([^{3}H]-Tdr incorporation) of B9.11 cells incubated in medium alone (negative control). Values represent the mean±SEM of triplicate determinations.

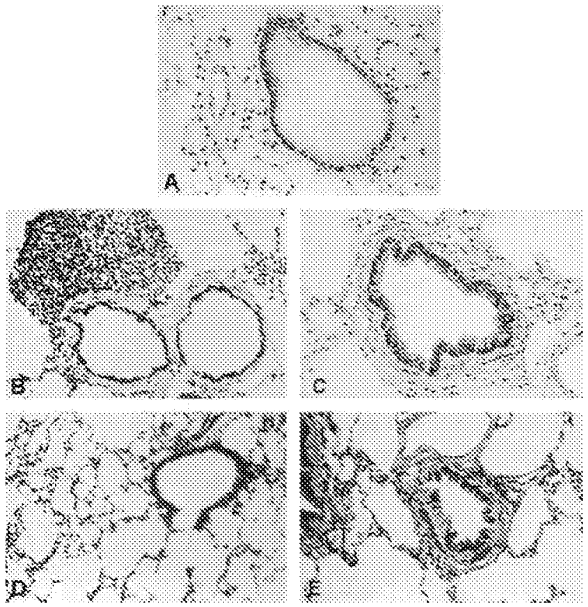

Figure 5. Histologic abnormalities in airways of CC10-IL-11 transgenic mice. The lungs of transgene (+) and (−) animals were removed, fixed, and evaluated using hematoxylin and eosin and trichrome stains. The histologic appearance of the transgene (−) mouse lung (*A*) is compared with the peribronchiolar lymphocytic infiltrates and bronchiolar thickening in the transgene (+) animals (*B* and *C*). The collagen content of the lungs of transgene (−) animals appears in green in *D*. This contrasts with the impressive subepithelial fibrosis seen in the airways of transgene (+) animals (*E*). (Original magnification 67.5×.)

Effect of IL-11 on murine airways. Progeny mice were killed at various intervals between 0.5 and 2 mo of age, and the airways of transgene (+) and (−) littermates were compared. A total of 78 age and sex matched littermate pairs were evaluated. In contrast with their transgene (−) littermates, the transgene (+) animals manifest an impressive airway phenotype composed of: (*a*) nodular collections of lymphocyte-like cells next to bronchi and bronchioles, and (*b*) airway wall thickening and remodeling (Fig. 5, *A–C*). The collections of lymphocytes were appreciated less often in the 0.5-mo-old animals, but were prominently noted in the 1- and 2-mo-old animals (data not shown). The impressive and progressive effects of IL-11 on the thickness of the airway wall were easily seen in the morphometric evaluations (Fig. 6). Insight into the cause of this thickening and remodeling was obtained from the trichrome evaluations. These stains demonstrated only small amounts of collagen in the lungs of transgene (−) animals. This contrasted with the extensive subepithelial fibrosis seen in the airways of the IL-11 transgene (+) animals (Fig. 5, *D* and *E*).

Composition of peribronchiolar nodules. The results noted above demonstrate that IL-11 overexpression in the murine airway generates nodular collections of lymphocyte-like mononuclear cells. The phenotype of these cells was, therefore, analyzed by immunohistochemistry using frozen lung sections. These studies demonstrated that the majority of the cells in these nodules were MHC Class II (+) and B220 (+) (Fig. 7). Collections of CD3(+), CD4(+), and CD8(+) cells were also noted (Fig. 6). Significant Mac-1 immunoreactivity was not

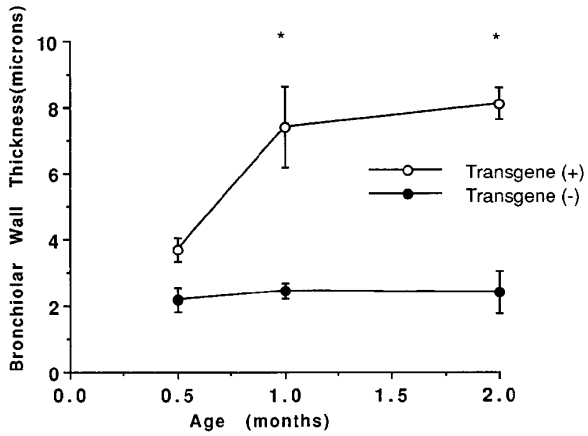

Figure 6. Morphometric analysis of airway wall thickening of CC10-IL-11 animals. The thickness of the bronchioles of 0.5-, 1-, and 2-mo-old transgene (+) and (−) littermates were measured as described in Methods. Values represent the mean±SEM of at least three pairs of animals at each time point (*$P < 0.001$ Bonferonni Multiple comparisons test).

appreciated with only a rare cell staining with this antibody (Fig. 7). None of the antibodies that were used reacted with the airways in sections of lung from transgene (−) mice, in great extent because of the lack of airway inflammation in these animals (data not shown). These observations demonstrate that these nodules are composed of large numbers of B lymphocytes and lesser numbers of CD4(+) and CD8(+) T cells.

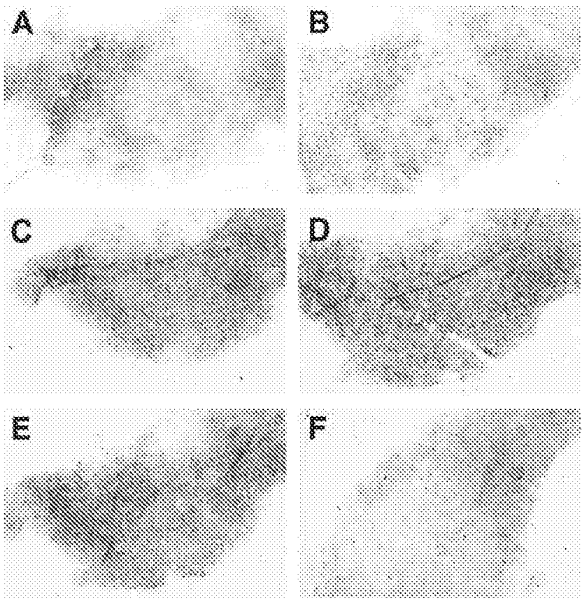

Figure 7. Immunohistochemistry of peribronchiolar nodular infiltrates. Immunohistochemical techniques were used to evaluate the cellular composition of the peribronchiolar infiltrates seen in the transgene (+) CC10-IL-11 animals. Antibodies against B220 (*A*), MHC class II (*B*), CD3 (*C*), CD4 (*D*), CD8 (*E*), and Mac-1 (*F*) were employed. (Original magnification 50×.)

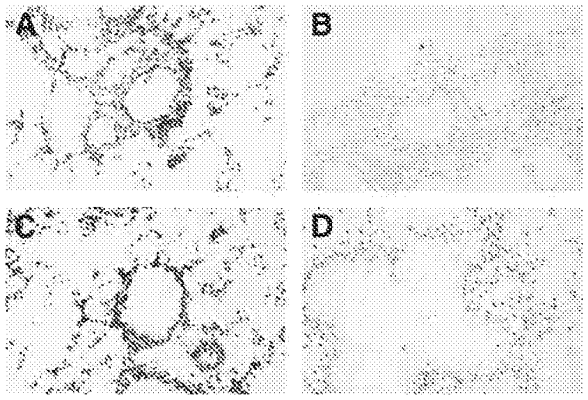

Figure 8. Immunohistochemical analysis of fibrotic response in CC10-IL-11 transgenic mice. Immunohistochemistry was used to evaluate the type I collagen in the airways of transgene (+) (*A*) and (−) (*C*) animals and the type III collagen in the airways of transgene (+) (*B*) and (−) (*D*) animals. (Original magnification 50×.)

Composition of the subepithelial fibrotic response. Studies were undertaken to determine if types I or III collagen were increased in the airways of the transgene (+) animals. These immunohistochemical evaluations demonstrated modest increases in type I collagen and impressive increases in type III collagen in transgene (+) vs. (−) animals (Fig. 8). Thus, the subepithelial fibrosis seen in CC10-IL-11 transgenic animals results, at least in part, from the increased accumulation of type III and, to a lesser extent, type I collagens.

Structural characterization of the transgenic airway. Immunohistochemistry and electron microscopy were used to further characterize the cellular and structural alterations in the airways of the CC10-IL-11 transgene (+) animals. The immunohistochemical evaluations demonstrated an increase in the

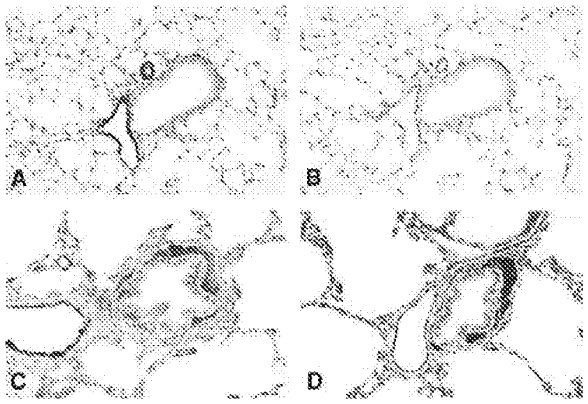

Figure 9. Immunohistochemical evaluation of subepithelial cellularity in CC10-IL-11 mice. Immunohistochemistry was used to evaluate the cellular components of the subepithelial fibrotic response in CC10-IL-11 transgene (+) and (−) animals. *A* and *C* represent the α–smooth muscle actin immunoreactivity in transgene (−) and (+) animals, respectively. *B* and *D* represent the desmin immunoreactivity in transgene (−) and (+) animals, respectively. (Original magnification 50×.)

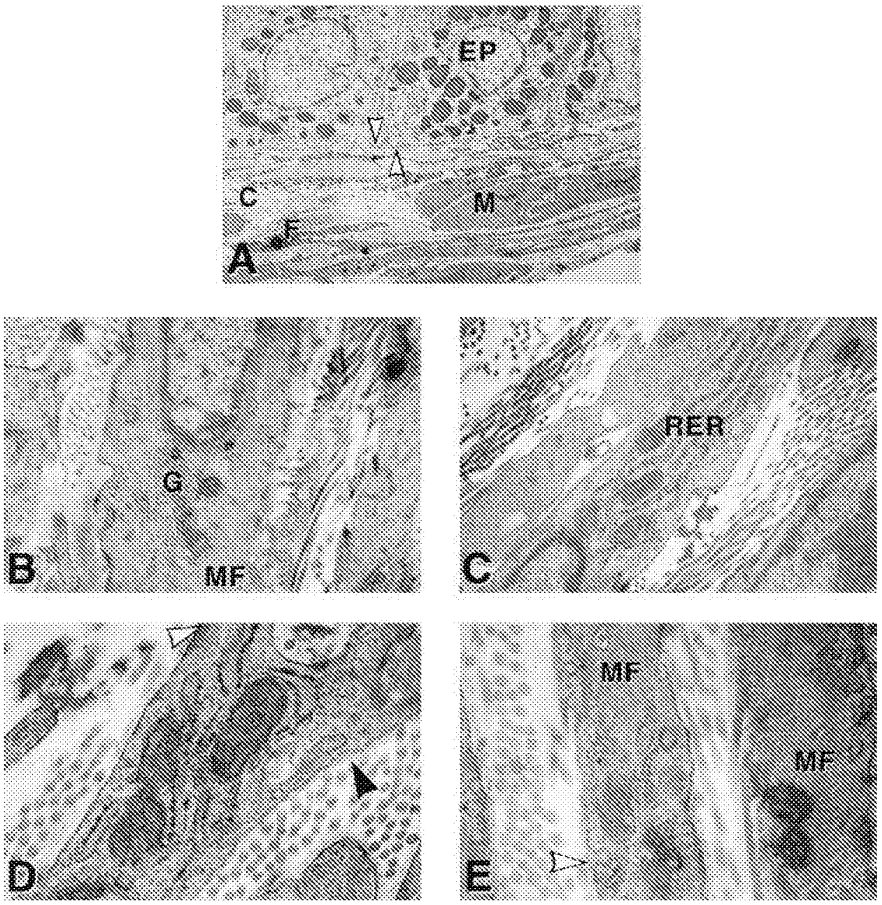

Figure 10. Electron microscopic appearance of peripheral airway of CC10-IL-11 mice. (*A*) Low power view of airway wall with epithelial cells (*EP*), myocytes (*M*), and fibroblastic cells (*F*). Note intact basement membrane (*open arrows*) and collagen deposition (*C*) (4,000×). (*B*) Smooth muscle cell with well formed Golgi (*G*). Note myofilaments (*MF*) (18,000×). (*C*) Fibroblast with rough endoplasmic reticulum (*RER*) (18,000×). (*D*) Myofibroblast with subplasmalemmal dense body (*open arrow*) and basal lamina (*closed arrow*) (36,000×). (*E*) Myofibroblast with myofilaments (*MF*) and subplasmalemmal vesicles (*open arrow*) (36,000×).

number of α smooth muscle actin and desmin staining cells in the walls of the transgene (+) vs. (−) animals (Fig. 9). This staining was not uniform, however, with some stromal cells failing to manifest either of these markers. The electron micrographs demonstrated that the CC10-IL-11 transgene (+) animals had normal basement membranes and enhanced striated collagen deposition (Fig. 10). They also demonstrated the accumulation of a variety of stromal cells in these fibrotic locations. Ultrastructurally, many cells appeared to be fibroblasts. Others appeared to be myofibroblasts based on their fibroblast-like appearance and the presence of myofilaments, dense bodies, subplasmalemmal vesicles, and/or identifiable basal lamina (Fig. 10 and data not shown). Smooth muscle cells with abundant myofilaments were also appreciated. Some of these cells showed morphologic evidence of increased synthetic activity with increased rough endoplasmic reticulum and prominent Golgi (Fig. 10). Thus, the subepithelial response in the airways of the CC10-IL-11 transgene (+) animals occurs in the absence of basement membrane thickening and is characterized by increased interstitial collagen deposition and heightened stromal cellularity with the local accumulation of fibroblasts, myofibroblasts, and smooth muscle cells.

Effect of IL-11 on airway physiology. Airways obstruction and hyperresponsiveness to nonspecific stimuli such as MCh are prominent features of asthma, COPD, and a variety of diseases characterized by chronic airways inflammation and/or fibrosis. Thus, studies were undertaken to characterize the physiologic profile of IL-11 transgene (+) and (−) animals. Overall, 18 age, sex, and weight matched littermate pairs were evaluated. The baseline airways resistance of IL-11 transgene (+) animals greatly exceeded that of IL-11 transgene (−) animals. At 2 mo of age, the airways resistance of the transgene (+) animals was approximately threefold greater than the resistance of the transgene (−) animals (745.3±227.5 vs. 227.5±6.4 cm H_2O/liters per s; $P < 0.05$). In addition, 1.5–2-mo-old transgene (+) animals manifest exaggerated sensitivity to methacholine since they achieved a 100% increase in airways resistance at 1/10 to 1/100 the dose of methacholine required by their transgene (−) littermate controls (Fig. 11). When viewed in combination, these studies demonstrate that IL-11 transgene (+) animals manifest increased airways resistance and airways hyperresponsiveness to MCh when compared with transgene (−) littermate controls.

Discussion

Airway inflammation and fibrosis are prominent features of a variety of disorders including asthma, COPD, bronchiectasis, and bronchiolitis (3–11). In these disorders, the relationship(s) between inflammation, fibrosis, and physiologic dysregulation, and the contribution that individual mediators make to the pathogenesis of these abnormalities are poorly understood.

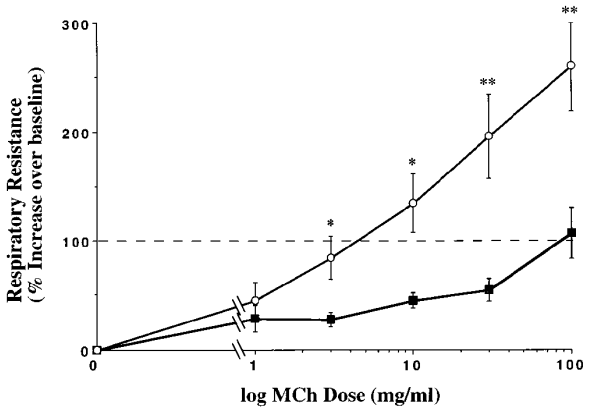

Figure 11. Methacholine sensitivity of transgene (−) and (+) mice. The effect of varying concentrations of methacholine on the airways resistance of paired transgene (+) (○) and transgene (−) (■) littermates were evaluated as described in Methods. Values represent the mean±SEM of six age and sex matched littermates evaluated on the same day (*$P < 0.05$, **$P < 0.01$, *t* test).

This is due, in great extent, to the complexity of the inflammatory and fibrotic responses in these disorders, which precludes the clear attribution of cause and effect. It is also the result of our need to rely on in vitro and acute challenge in vivo protocols in our modeling of these disorders. The limitations of our present approach are nicely illustrated with IL-11. In vitro studies from our laboratory and others have clearly demonstrated that this cytokine is produced by a variety of stromal cells, including airway and alveolar epithelial cells, after incubation with cytokines (21, 30), histamine (31), and respiratory viruses (21, 23). These studies have also demonstrated that IL-11 can be found in abnormal quantities at sites of human viral respiratory tract infection, and acute studies have demonstrated that IL-11 causes AHR in vivo (23). In contrast, the chronic effects of IL-11 in the lung or other visceral organs have not been characterized. To gain insight into the role that IL-11 might play in chronic inflammatory airway disorders such as asthma, COPD, bronchiectasis, and bronchiolitis, we used the CC10 promoter to chronically express IL-11 in the murine airway. These studies demonstrate that IL-11 induces a nodular lymphocytic peribronchial and peribronchiolar inflammatory response. They also demonstrate, for the first time, that IL-11 is a fibrogenic cytokine since it caused impressive airway remodeling with subepithelial fibrosis and the local accumulation of fibroblasts, myofibroblasts, and smooth muscle cells. Lastly, these studies demonstrate that these pathologic abnormalities are associated with prominent physiologic dysfunction with airways obstruction and AHR to methacholine.

IL-11 was initially discovered as an IL-6–like plasmacytoma proliferation stimulating activity in fibroblast-conditioned medium that could not be neutralized with antibodies against IL-6 (24). It has subsequently been shown to be an important hematopoietic growth factor (25), stimulate the acute phase response (25), augment immunoglobulin production (25, 28), induce the expression of metalloproteinase inhibitors (26, 27), regulate neural phenotype (29), regulate bone metabolism (40), and protect against the combined effects of radiation and chemotherapy (41). IL-11 and IL-6 are now grouped together as IL-6–type cytokines based on their overlapping functional profiles and shared use of the gp130 molecule in their multimeric receptor complexes (42). In keeping with these findings, a comparison of the phenotype of the CC10-IL-11 animals described in this report and CC10-IL-6 mice described previously by our laboratories (32), shows interesting similarities and differences. The inflammatory response seen in both the IL-11 and IL-6 transgenic lines was almost exclusively lymphocytic and contained significant numbers of B cells. This is in keeping with the known B cell stimulatory activities of both of these cytokine moieties (28, 42, 43). Interestingly, however, inflammation were more prominent in the CC10-IL-6 animals and airway remodeling and subepithelial fibrosis was more prominent in CC10-IL-11 animals. In addition, marked physiologic differences were noted with CC10-IL-6 animals manifesting normal baseline airways resistance and airways hyporesponsiveness to methacholine, while CC10-IL-11 animals demonstrated increased baseline airways resistance and AHR to methacholine. These findings clearly support the contention that the effects that are seen in these transgenic animals are cytokine specific and not merely the result of a murine immune response to transgenically expressed human protein. They also support, in a powerful fashion, the contention that different components of the inflammatory response, even cytokines in the same cytokine family with common receptor components, can have markedly different effects when chronically expressed in vivo. The power of this approach is also evident in this comparison since it allows correlations to be made between physiology and pathology. Specifically, these observations simultaneously demonstrate an association between airway remodeling and airways obstruction and hyperresponsiveness to methacholine, and a relative dissociation between inflammation and these physiologic abnormalities. These observations suggest that the remodeling process may be quite important and that the type of airway inflammation induced by IL-6 is less important in the pathogenesis of these obstructive physiologic abnormalities.

Remodeling responses in the human airway, characterized by tissue fibrosis, matrix alterations, and quantitative and/or qualitative alterations of the structural and other resident cells that make up the airway wall are well documented in a variety of pulmonary pathologies (3–5, 8, 9, 11, 44, 45). These responses have been extensively studied in asthma where smooth muscle hyperplasia and hypertrophy are well documented (44, 45), and the "basement membrane thickening" that has been appreciated in asthmatic airway biopsies for decades is known to be due to the subepithelial deposition of type III collagen and other matrix molecules by activated airway myofibroblasts (7, 9). In contrast with other fibrotic pulmonary disorders, animal models of the airway fibrotic response are poorly developed and our knowledge of the role(s) that mediators play in the generation of airway scarring is rudimentary. This study demonstrates that IL-11 is a fibrogenic molecule and describes, for the first time, an animal model that reproduces many of the important features of the remodeled asthmatic airway. Specifically, it demonstrates that the targeted expression of IL-11 in the murine airway causes prominent subepithelial fibrosis with impressive increases in type III and to a lesser extent type I collagen. In addition, it demonstrates that this response occurs in the absence of overt basement membrane pathology and contains increased numbers of a variety of stromal cells, including airway myofibroblasts. This

study did not address the mechanism(s) responsible for this fibrotic response. There are, however, a number of possible pathways that deserve comment. First, IL-11 is known to stimulate the production of tissue inhibitor of metalloproteinase–1 (26, 27). Tissue inhibitor of metalloproteinase–1 decreases collagen degradation and thus could contribute to collagen accumulation and tissue fibrosis. Second, studies from this and other laboratories have demonstrated that TGF-β is a potent stimulator of IL-11 production (26, 30). This raises the possibility that fibrogenic effector functions of TGF-β, such as its stimulation of collagen production and α-smooth muscle actin accumulation (46), may also be mediated by IL-11. Additional investigation will be required to sort out these possibilities.

In previous years, an intrinsic abnormality of the airway myocyte was felt to be at the heart of the asthmatic diathesis. More recent studies have revised this concept and led to a renewed appreciation of the role of inflammation and tissue fibrosis in this disorder (6–9). As a result of these studies it is now known that a chronic eosinophilic and mononuclear cell–predominant inflammatory response, epithelial injury and desquamation, and subepithelial fibrosis are prominent features of the pathology of asthma. In addition, it is now assumed that the inflammation and epithelial injury are largely responsible for the physiologic abnormalities characteristic of this disorder. Our studies, however, add an additional layer of complexity to these concepts. First, they raise questions about the link between inflammation and AHR since prominent physiologic dysregulation was seen in the CC10-IL-11 animals that manifest only a mild nodular peribronchiolar lymphocytic response. Second, they raise questions about the importance of eosinophils since they demonstrate that AHR and airways obstruction are seen in CC10-IL-11 animals in the absence of prominent tissue eosinophilia. Lastly, they raise questions about the importance of epithelial desquamation in this disorder since CC10-IL-11 animals manifest prominent physiologic dysregulation in the absence of this abnormality. These questions are, however, not without precedent since a growing body of data from this and other laboratories has shown a dissociation between inflammation, eosinophil infiltration, epithelial desquamation, and physiologic dysregulation in human asthma and animal models of the asthmatic diathesis (32, 47–50). They are also in accord with the well established observation that airways obstruction and/or AHR are prominent features of airways disorders such as bronchitis, bronchiectasis, and endobronchial sarcoidosis, which are not characterized by eosinophilia or epithelial sloughing (10, 51, 52). When viewed in combination, these observations suggest that a variety of different pathogenic processes can lead to airways obstruction and AHR and that different processes may be operative in different disorders.

Viruses are being increasingly appreciated to play an important role in the pathogenesis of asthma and other airway disorders. RSV is the most common cause of infantile bronchiolitis (10), RSV, PIV3, and rhinovirus are major inducers of asthmatic exacerbations in children and adults (12–15), and infantile viral infections are strongly associated with the development of asthma and COPD in later life (12, 13, 17, 19). The mechanisms behind these associations are, however, poorly understood. Previous data from our laboratory demonstrated that rhinovirus, RSV, and PIV3 are potent inducers of IL-11 production in vitro (21, 23). This contrasts with the inability of other pneumotropic infectious agents to similarly induce the production of this cytokine, and suggests that IL-11 plays an important role in the pathogenesis of these viral disorders. The demonstration in these studies that IL-11 causes lymphocytic infiltration, airway remodeling, and physiologic dysfunction has obvious relevance to the pathogenesis of viral airways disorders. The impressive similarities between some of the airway and physiologic alterations seen in the CC10-IL-11 transgenic mice and neonatal rats with experimental PIV-1 infection (53) further supports the contention that IL-11 is involved in mediating these abnormalities. It allows for the tantalizing speculation that neonatal viral infections predispose to chronic airways dysfunction at least in part via the induction of IL-11 production, which causes lymphocytic infiltration and airway remodeling. When coupled with the observation that IL-11 inhibits human macrophage IL-12 production (S.X. Leng, Department of Internal Medicine, Yale University; and J.A. Elias, unpublished observations), one is left with the interesting possibility that IL-11 production during neonatal and/or pediatric viral infections can also predispose to the development of the Th_2 lymphocytic response that is felt to play a key role in the asthmatic diathesis (54).

In conclusion, our studies demonstrate that the transgenic expression of IL-11 in the mouse airway results in a nodular lymphocytic infiltrate, airway remodeling, and physiologic dysregulation that mimics in important ways the pathologic and physiologic features of viral and other airways disorders. These findings suggest that IL-11 may play an important role in the pathogenesis of the airways abnormalities seen in these often times devastating disorders. In this respect, CC10-IL-11 transgenic mice represent an excellent animal model of airway remodeling and inflammation that can be used to explore therapies directed at these processes and the contribution that these processes make to the physiologic abnormalities seen in a variety of human airways disorders.

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References

1. Weiss, K.B., P.J. Gergen, and T.A. Hodgson. 1992. An economic evaluation of asthma in the United States. *N. Engl. J. Med.* 326:862–866.

2. Lenfant, C., and S.S. Hurd. 1990. National asthma education program. *Chest.* 98:226–227.

3. Snider, G.L., J. Faling, and S.I. Rennard. 1994. Chronic bronchitis and emphysema. *In* Textbook of Respiratory Medicine. 2nd ed. J.F. Murray and J.A. Nadel, editors. W.B. Saunders Co., Philadelphia. 1331–1397.

4. Lazarus, S.C. 1994. Disorders of the intrathoracic airways. *In* Textbook of Respiratory Medicine. 2nd ed. J.F. Murray and J.A. Nadel, editors. W.B. Saunders Co., Philadelphia. 1471–1483.

5. Luce, J.M. 1994. Bronchiectasis. *In* Textbook of Respiratory Medicine. 2nd ed. J.F. Murray and J.A. Nadel, editors. W.B. Saunders Co., Philadelphia. 1398–1417.

6. Shelhamer, J.H., S.J. Levine, T. Wu, D.B. Jacoby, M.A. Kaliner, and S.I. Rennard. 1995. Airway inflammation. *Ann. Intern. Med.* 123:288–304.

7. Holgate, S.T., J.R. Wilson, and P.H. Howarth. 1992. New insights into airway inflammation by endobronchial biopsy. *Am. Rev. Respir. Dis.* 145:S2–S6.

8. Arm, J.P., and T.K. Lee. 1992. The pathology of bronchial asthma. *Adv. Immunol.* 51:323–382.

9. Brewster, C.E.P., P.H. Howarth, R. Djukanovic, J. Wilson, S.T. Holgate,

and W.R. Roche. 1990. Myofibroblasts and subepithelial fibrosis in bronchial asthma. *Am. J. Respir. Cell Mol. Biol.* 3:507–511.

10. McIntosh, K., and R.M. Chanock. 1990. Respiratory syncytial virus. *In* Virology. 2nd ed. B.M. Fields and D.M. Knipe, editors. Raven Press, Ltd., New York. 1045–1072.

11. Gross, N.J. 1995. Airway inflammation in COPD, reality or myth? *Chest.* 107:210s–213s.

12. Stark, J.M., and F.M. Graziano. 1995. Lower airway response to viruses. *In* Asthma and Rhinitis. W.W. Busse and S.T. Holgate, editors. Blackwell Scientific Publications, Boston. 1229–1243.

13. Pattemore, P.K., S.L. Johnston, and P.G. Bardin. 1992. Viruses as precipitants of asthma symptoms. I. Epidemiology. *Clin. Exp. Allergy.* 22:325–336.

14. Johnston, S.L., P.K. Pattemore, G. Sanderson, S. Smith, F. Lampe, L. Josephs, P. Symington, S. O'Toole, S.H. Myint, D.A.J. Tyrrell, and S.T. Holgate. 1995. Community study of role of viral infections in exacerbations of asthma in 9–11 year old children. *Brit. Med. J.* 310:1225–1228.

15. Nicholson, K.G., J. Kent, and D.C. Ireland. 1993. Respiratory viruses and exacerbations of asthma in adults. *Brit. Med. J.* 307:982–986.

16. Monto, A.S. 1995. Epidemiology of respiratory viruses in persons with and without asthma and COPD. *Am. J. Respir. Crit. Care Med.* 151:1653–1658.

17. Rooney, J.C., and H.E. Williams. 1971. The relationship between proved viral bronchiolitis and subsequent wheezing. *J. Pediatr.* 79:744–747.

18. Sigurs, N., R. Bjarnason, F. Sigurbergsson, B. Kjellman, and B. Bjorksten. 1995. Asthma and immunoglobulin antibodies after respiratory syncytial virus bronchiolitis: a prospective cohort study with matched controls. *Pediatrics.* 95:500–505.

19. Krzyzanowski, M., D.L. Sherrill, and D. Lebowitz. 1990. Longitudinal analysis of the effects of acute lower respiratory illnesses on pulmonary function in an adult population. *Am. J. Epidemiol.* 131:412–422.

20. Alwan, W.H., W.J. Kozlowska, and P.J.M. Openshaw. 1994. Distinct types of lung disease caused by functional subsets of antiviral T cells. *J. Exp. Med.* 179:81–89.

21. Elias, J.A., T. Zheng, O. Einarsson, M. Landry, T.K. Trow, N. Rebert, and J. Panuska. 1994. Epithelial interleukin-11: regulation by cytokines, respiratory syncytial virus and retinoic acid. *J. Biol. Chem.* 169:22261–22268.

22. Noah, T.L., and S. Becker. 1993. Respiratory syncytial virus–induced cytokine production by a human bronchial epithelial cell line. *Am. J. Physiol. (Lung Cell. Mol. Physiol.)* 265:L472–L478.

23. Einarsson, O., G.P. Geba, Z. Zhu, M. Landry, and J.A. Elias. 1996. Interleukin-11: stimulation in vivo and in vitro by respiratory viruses and induction of airways hyperresponsiveness. *J. Clin. Invest.* 97:915–924.

24. Paul, S.R., F. Bennett, J.A. Calvetti, K. Kelleher, C.R. Wood, R.M.J. Oharra, A.C. Leary, B. Sibley, S.C. Clark, D.A. Williams, and Y.-C. Yang. 1990. Molecular cloning of a cDNA encoding interluekin-11, a stromal cell–derived lymphopoietic and hematopoietic cytokine. *Proc. Natl. Acad. Sci. USA.* 87:7512–7516.

25. Du, X.X., and D.A. Williams. 1994. Interleukin-11: a multifunctional growth factor derived from the hematopoietic microenvironment. *Blood.* 83: 2023–2030.

26. Maier, R., V. Ganu, and M. Lotz. 1993. Interleukin-11, an inducible cytokine in human articular chondrocytes and synoviocytes, stimulates the production of the tissue inhibitor of metalloproteinases. *J. Biol. Chem.* 268:21527–21532.

27. Roeb, E., L. Graeve, R. Hoffmann, K. Decker, D.R. Edwards, and P.C. Heinrich. 1993. Regulation of tissue inhibitor of metalloproteinases-1 gene expression by cytokines and dexamethasone in rat hepatocyte primary cultures. *Hepatology.* 18:1437–1442.

28. Yin, T., P. Schendel, and Y.-C. Yang. 1992. Enhancement of in vitro and in vivo antigen-specific antibody responses by IL-11. *J. Exp. Med.* 175:211–216.

29. Fann, M.-J., and P.H. Patterson. 1994. Neuropoietic cytokines and activin A differentially regulate the phenotype of cultured sympathetic neurons. *Proc. Natl. Acad. Sci. USA.* 91:43–47.

30. Elias, J.A., T. Zheng, N.L. Whiting, T.K. Trow, W.W. Merrill, R. Zitnik, P. Ray, and E.M. Alderman. 1994. Interleukin-1 and transforming growth factor β regulation of fibroblast-derived interleukin-11. *J. Immunol.* 152:2421–2429.

31. Zheng, T., M. Nathanson, and J.A. Elias. 1994. Histamine augments cytokine-stimulated interleukin-11 production by human lung fibroblasts. *J. Immunol.* 153:4742–4752.

32. DiCosmo, B.F., G.G. Geba, D. Picarella, J.A. Elias, J.A. Rankin, B.R. Stripp, J.A. Whitsett, and R.A. Flavell. 1994. Airway targeted interleukin-6 in transgenic mice: uncoupling of airway inflammation and bronchial hyperreactivity. *J. Clin. Invest.* 94:2028–2035.

33. Pack, R.J., L.H. Al-Ugaily, and G. Morris. 1981. The cells of the tracheo-bronchial epithelium of the mouse: a quantitative light and electron microscope study. *J. Anat.* 132:71–84.

34. Stripp, B.R., P.L. Sawaya, D.S. Luse, K.A. Wikenheiser, S.E. Wert, J.A. Huffman, D.L. Lattier, G. Singh, S.L. Katyal, and J.A. Whitsett. 1992. Cis-acting elements that confer lung epithelial cell expression of the CC10 gene. *J. Biol. Chem.* 267:14703–14712.

35. Hogan, B., F. Constantini, and E. Lacy. 1986. Manipulating the mouse embryo: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

36. Elias, J.A., W. Tang, and M.C. Horowitz. 1995. Cytokine and hormonal stimulation of osteosarcoma interleukin-11 production. *Endocrinology.* 136: 489–498.

37. Zhu, Z., W. Tang, A. Ray, Y. Wu, O. Einarsson, M. Landry, J. Gwaltney, and J.A. Elias. 1996. Rhinovirus stimulation of interleukin-6 in vivo and in vitro: evidence for NF-κB-dependent transcriptional activation. *J. Clin. Invest.* 97:421–430.

38. Martin, T., N. Gerard, S. Galli, and J. Drazen. 1988. Pulmonary responses to bronchoconstrictor agonists in the mouse. *J. Appl. Physiol.* 64:2318–2323.

39. Amdur, M.O., and J. Mead. 1958. Mechanics of respiration in unanesthetized guinea pigs. *Am. J. Physiol.* 192:364–368.

40. Girasole, G., G. Passeri, R.L. Jilka, and S.C. Manolagas. 1994. Interleukin-11: a new cytokine critical for osteoclast development. *J. Clin. Invest.* 93: 1516–1524.

41. Du, X.X., C.M. Doerschuk, A. Orazi, and D.A. Williams. 1994. A bone marrow stromal-derived growth factor, interleukin-11, stimulates recovery of small intestinal mucosal cells after cytoablative therapy. *Blood.* 83:33–37.

42. Zhang, X.-G., J.-J. Gu, K. Yasukawa, G.D. Yancopoulos, K. Turner, M. Shoyab, T. Taga, T. Kishimoto, R. Bataile, and B. Klein. 1994. Ciliary neurotropic factor, interleukin 11, leukemia inhibitory factor, and oncostatin M are growth factors for human myeloma cell lines using the interleukin 6 signal transducer gp130. *J. Exp. Med.* 177:1337–1342.

43. Suematsu, S., T. Matsuda, K. Aozasa, S. Akira, N. Nakano, S. Ohno, J.-I. Miyazaki, K.-I. Yamamura, T. Hirano, and T. Kishimoto. 1989. IgG1 plasmacytosis in interleukin 6 transgenic mice. *Proc. Natl. Acad. Sci. USA.* 86:7547–7551.

44. Hossain, S. 1973. Quantitative measurement of bronchial muscle in men with asthma. *Am. Rev. Respir. Dis.* 107:99–109.

45. Ebina, M., T. Takahashi, T. Chiba, and M. Motomiya. 1993. Cellular hypertrophy and hyperplasia of airway smooth muscles underlying bronchial asthma. *Am. Rev. Respir. Dis.* 148:720–726.

46. Desmouliere, A., A. Geinoz, F. Gabbiani, and G. Gabbiani. 1993. Transforming growth factor-β_1 induces α-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. *J. Cell Biol.* 122:103–111.

47. Brugman, S.M., G.L. Larsen, P.M. Henson, J. Honor, and C.G. Irvin. 1993. Increased lower airways responsiveness associated with sinusitis in a rabbit model. *Am. Rev. Respir. Dis.* 147:314–320.

48. Corry, D.B., H.G. Folkesson, M.L. Warnock, D.J. Erle, M.A. Matthay, J.P. Weiner-Kronish, and R.M. Locksley. 1996. Interleukin-4, but not interleukin 5 or eosinophils, is required in a murine model of acute airway hyperreactivity. *J. Exp. Med.* 183:109–117.

49. Pattemore, P.K., M.I. Asher, A.C. Harrison, E.A. Mitchell, H.H. Rea, and A.W. Stewart. 1990. The interrelationship among bronchial hyperresponsiveness, the diagnosis of asthma, and asthma symptoms. *Am. Rev. Respir. Dis.* 142:549–554.

50. Swiecichowski, A.L., K.J. Long, M.L. Miller, and G.D. Leikauf. 1995. Formaldehyde-induced airway hyperreactivity in vivo and ex vivo in guinea pigs. *Environ. Res.* 310:1229–1231.

51. Pang, J., H.S. Chan, and J.Y. Sung. 1989. Prevalence of asthma, atopy, and bronchial hyperactivity in bronchiectasis: a controlled study. *Thorax.* 44: 948–951.

52. Levinson, R.S., L.F. Metzger, N.N. Stanley, S.G. Kelsen, M.D. Altose, N.S. Cherniack, and J.S. Brody. 1977. Airway function in sarcoidosis. *Am. J. Med.* 62:51–59.

53. Castleman, W.L., R.L. Sorkness, R.F. Lemanske, G. Grasee, and M.M. Suyemoto. 1988. Neonatal viral bronchiolitis and pneumonia induces bronchiolar hypoplasia and alveolar dysplasia in rats. *Lab. Invest.* 59:387–396.

54. Robinson, D.S., Q. Hamid, S. Ying, A. Tsicopoulos, J. Barkans, A.M. Bentley, C. Corrigan, S.R. Durham, and A.B. Kay. 1992. Predominant Th2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N. Engl. J. Med.* 326:298–304.

Polarized in vivo expression of IL-11 and IL-17 between acute and chronic skin lesions

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Background: In atopic dermatitis (AD) there is evidence of tissue fibrosis involving a number of structural changes, including papillary dermal fibrosis and epidermal hyperplasia. These changes are suggested to be the result of chronic inflammation of the skin. Several remodeling-associated cytokines, including transforming growth factor (TGF) β1, IL-11, and IL-17, have been shown to be increased in allergic diseases, including asthma.

Objective: We investigated TGF-β1, IL-11, and IL-17 expression in skin biopsy specimens recovered from acute and chronic skin lesions from patients with AD, as well as from uninvolved skin of patients with AD and skin from healthy volunteers. We also examined the correlation between the expression of these cytokines and the extent of total, type I, and type III collagen deposition.

Methods: We evaluated the expression of TGF-β1, IL-11, and IL-17 by means of immunohistochemistry. Collagen deposition was assessed by means of immunohistochemistry and van Gieson staining.

Results: TGF-β1 expression was markedly enhanced in both acute and particularly chronic lesions ($P < .001$). Although IL-11 expression was significantly increased only in chronic lesions ($P < .0001$), IL-17 was preferentially associated with acute lesions ($P < .005$). Although collagen type III deposition was not significantly different among the groups, type I collagen deposition was significantly increased in chronic AD lesions ($P < .0005$). There was a significant correlation between IL-11 and type I collagen deposition, as well as the number of eosinophils in skin specimens from patients with AD ($r^2 = 0.527$, and $r^2 = 0.622$, respectively; $P < .0001$).

Conclusion: These results suggest that TGF-β1, IL-11, and IL-17 are involved in the remodeling of skin lesions in patients with AD. However, IL-11 and IL-17 are preferentially expressed at different stages of the disease. Type I collagen appeared to be the major subtype involved in this repair process. (J Allergy Clin Immunol 2003;111:875-81.)

Key words: Atopic dermatitis, skin biopsy, fibrosis, cytokines, collagen, remodeling

Tissue remodeling has been well documented in asthmatic airways and is characterized by various structural changes, including airway wall thickening, subepithelial fibrosis, mucus overproduction, smooth muscle hyperplasia-hypertrophy, and epithelial hypertrophy.[1,2] Airway remodeling is a consequence of chronic inflammatory episodes,[3] which are associated predominantly with T_H2-type cytokines (IL-4, IL-13, and IL-5). Subepithelial fibrosis is described as the result of an enhanced deposition in the lamina reticularis of extracellular matrix (ECM) components, such as type I, type III, and type V collagens,[4-6] that might be regulated by fibrogenic cytokines (eg, transforming growth factor [TGF] β).

Numerous reports demonstrated that among all 3 TGF-β isoforms synthesized by mammalian cells, TGF-β1 is predominantly associated with fibrosis. TGF-β is the major fibrogenic cytokine that has been shown to act on fibroblasts for the synthesis and secretion of collagens, as well as other mediators.[7-12] TGF-β has been reported to participate in collagen accumulation within the ECM[9] and plays a critical role in wound healing and tissue remodeling. Various cell types, including eosinophils,[13-15] T lymphocytes, fibroblasts, macrophages, and epithelial cells,[16,17] have all been shown to produce TGF-β.

IL-11 is a hematopoietic cytokine that is produced by a variety of stromal cells, including epithelial cells, fibroblasts, and myocytes.[18-21] Studies performed in transgenic mice revealed that IL-11 induces some features of remodeling, such as enhanced deposition of type I and type III collagens.[22,23] IL-17 is a recently described cytokine mainly released by $CD4^+$ T cells that induces the release of proinflammatory mediators from macrophages and fibroblasts.[24-26] We recently demonstrated that IL-17 expression was increased in asthmatic airways, particularly in patients with severe asthma.[27,28] We also showed that recombinant IL-17 increased the release of IL-6 and IL-11 by fibroblasts isolated from bronchial biopsy specimens in asthmatic subjects.[27]

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Abbreviations used
AD: Atopic dermatitis
ECM: Extracellular matrix
HPF: High-power field
TGF: Transforming growth factor

Atopic dermatitis (AD) is a chronic inflammatory skin disease with increased serum IgE levels and tissue eosinophilia.[29,30] The expression of profibrotic cytokines has yet to be analyzed in AD. In this study we speculate that TGF-β, IL-11, and IL-17 contribute to tissue remodeling in the skin of patients with AD. We therefore assessed the expression of these cytokines and the extent of collagen deposition in skin biopsy specimens recovered from acute and chronic skin lesions, and we compared these results with results from uninvolved skin of patients with AD and skin from healthy volunteers. Furthermore, we investigated whether a particular subtype of collagen might be preferentially expressed or increased at various stages of the disease.

METHODS

Skin biopsy specimens

Twenty-four skin punch biopsy specimens (2 mm each) were obtained from 8 patients fulfilling the diagnostic criteria for AD.[31] Three specimens were recovered from each patient, one from acute erythematous AD lesions less than 3 days from onset, one from chronic lichenified AD lesions greater than 2 weeks from onset, and the last from an uninvolved skin area. Punch skin biopsy specimens were also obtained from 8 nonatopic healthy volunteers. Specimens were fixed immediately in 4% paraformaldehyde, embedded in OCT medium and snap-frozen in liquid nitrogen–cooled isopentane for immunohistochemistry.

The age of patients with AD ranged from 19 to 49 years (median, 28 years). All patients had a history of AD dating back to early infancy and had associated respiratory allergy (allergic rhinitis or asthma). Serum IgE levels ranged from 269 to 10,700 IU/mL (median, 5300 IU/mL). None of these patients had other skin pathologies, and none had been previously treated with oral corticosteroids. Topical steroids were withheld for at least 2 weeks before biopsy specimens were taken. The National Jewish Medical and Research Center Institutional Review Board approved the study, and all subjects provided informed written consent before entering the protocol.

Immunohistochemistry

As previously described,[32] we used the alkaline phosphatase–antialkaline phosphatase method to identify cytokines, collagen subtypes, and inflammatory cell phenotypes within the skin biopsy sections (5-mm tissue sections). Specific antibodies were used to identify eosinophils (anti-EG2; Kabi Pharmacia Diagnostics AB, Uppsala, Sweden), T cells (anti-CD3; Becton-Dickinson Canada, Mississauga, Ontario, Canada), type I and type III collagens (Medicorp, Montreal, Quebec, Canada), TGF-β1 (Cedarlane Laboratories, Hornby, Ontario, Canada), and IL-11 and IL-17 (R&D systems, Minneapolis, Minn). For negative control preparations, the primary antibody was replaced with either an irrelevant isotype-matched control immunoglobulin or TRIS-buffered saline solution.

Histochemistry

Total collagen was identified on skin biopsy sections by using van Gieson staining, which allowed collagen fibers to appear as red fibers.

Quantification

Slides were coded, and positive cells were counted blindly under a 200× magnification with an eyepiece graticule. Two to 6 fields were counted per section, depending on the size of the biopsy specimens and the pattern of alignment of the grid covering an intact area of epithelial and subepithelial areas. Immunoreactive cells were quantified as the mean cell count expressing the appropriate positive marker per high-power field (HPF; 0.202 cm^2). The severity of dermal fibrosis was scored on a 1 to 4 scale according to the extent of fibrosis within the dermis, with a score of 4 representing extensive fibrosis that extended to the whole papillary dermis. The within-observer coefficient of variation for repeated measures was less than 5%. Data are expressed in the text as means ± SD.

Statistical analysis

Statistical analysis of the results was performed by using Kruskal-Wallis nonparametric ANOVA, and subsequently, post hoc comparisons were made by using the Scheffe F and Dunn multiple comparison tests. The coefficients of correlation were calculated from the Pearson moment coefficient and corrected for multiple comparisons by using the Bonferroni correction factor (Systat version 8.0; SASS, Chicago, Ill). Results were considered as statistically significant when the P value was less than .05.

RESULTS

Phenotype of inflammatory cells

Skin biopsy specimens from acute AD lesions characteristically showed epidermal hyperplasia with intracellular edema and inflammatory cell infiltration in the epidermis. Mononuclear cell infiltrates were prominent in perivascular areas. In particular, $CD3^+$ T cells were markedly accumulated in acute lesions compared with in chronic lesions, uninvolved skin from patients with AD, and normal skin ($P < .0001$, Table I). Although the number of $CD3^+$ T cells was also significantly greater in chronic lesions than in uninvolved skin from patients with AD and normal skin ($P < .0001$), chronic lichenified lesions showed hyperkeratosis with minimal spongiosis. Epidermal and particularly dermal infiltrates showed increased numbers of eosinophils. The $EG2^+$ cell population was significantly greater in chronic lesions than in acute lesions, uninvolved skin from patients with AD, and normal skin ($P < .0001$). No significant difference was seen in the number of eosinophils in normal skin versus that in uninvolved skin from patients with AD.

TGF-β1, IL-11, and IL-17 expression

TGF-β1 expression was significantly increased in chronic and acute lesions (13.5 ± 1.7 and 9.0 ± 3.1 cells/HPF, respectively) compared with that in uninvolved areas (3.8 ± 2.4 cells/HPF; $P < .0001$ and $P < .001$ vs chronic and acute lesions, respectively) and normal skin (1.5 ± 1.4 cells/HPF; $P < .0001$ and $P < .005$ vs chronic and acute lesions, respectively; Fig 1). Moreover, the number of TGF-$\beta1^+$ cells was higher in chronic than

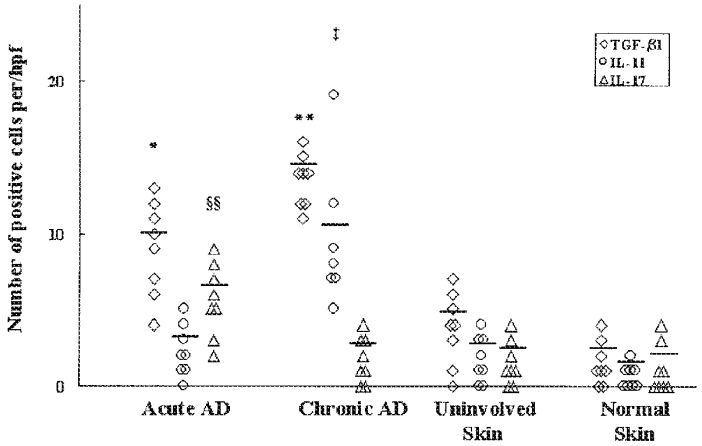

FIG 1. TGF-β1, IL-11, and IL-17 immunostainings in acute chronic lesions and uninvolved skin biopsy specimens of patients with AD and in skin of healthy volunteers. *Bars* represent means. $*P < .001$ and $P < .0001$ compared with uninvolved skin from patients with AD and normal skin, respectively; §§$P < .005$, $P < .001$, and $P < .0005$ compared with chronic lesions, uninvolved skin from patients with AD, and normal skin, respectively; $**P < .005$, $P < .0001$, and $P < .0001$ compared with acute lesions, uninvolved skin of patients with AD, and normal skin, respectively; ‡$P < .0001$ compared with acute lesions, uninvolved skin from patients with AD, and normal skin.

TABLE I. Phenotype of inflammatory cells accumulated in skin biopsy specimens

	Acute lesions	Chronic lesions	Uninvolved skin	Normal skin
$CD3^+$ cells (/HPF)	99.4 ± 22.4*	60.1 ± 8.3†	15.0 ± 7.1	9.8 ± 4.3
$EG2^+$ cells (/HPF)	3.9 ± 2.4	12.4 ± 5.1‡	0.1 ± 0.4	0.1 ± 0.4

Values are presented as means ± SD.
*$P < .0001$ compared with chronic lesions, uninvolved skin of patients with AD, and normal skin.
†$P < .0001$ compared with uninvolved skin of patients with AD and normal skin.
‡$P < .0001$ compared with acute lesions, uninvolved skin of patients with AD, and normal skin.

in acute lesions ($P < .005$). No significant difference was seen between uninvolved and normal skin.

IL-11 expression was only significantly increased in chronic lesions (9.6 ± 4.7 cells/HPF, $P < .0001$), whereas it reached similarly low levels in acute lesions and in uninvolved and normal skin (2.3 ± 1.7, 1.8 ± 1.5, and 0.6 ± 0.7 cells/HPF, respectively; Fig 1). In contrast, a significant increase in IL-17 expression was only detectable in acute lesions (5.6 ± 2.4 cells/HPF) compared with in the 3 other groups, which had low levels of IL-17 expression (1.8 ± 1.5 [$P < .005$], 1.5 ± 1.4 [$P < .001$], and 1.1 ± 1.6 cells/HPF [$P < .0005$] in chronic lesions, uninvolved skin, and normal skin, respectively; Fig 1).

Deposition of total, type I, and type III collagens

Total collagen deposition, as assessed by means of van Gieson staining, was not different among acute and chronic skin lesions (2.5 ± 1.2 and 2.4 ± 1.3, respectively), uninvolved skin and normal skin (1.5 ± 0.9 and 1.3 ± 1.0, respectively; Fig 2). However, the score of type I collagen, as assessed by means of immunostaining, was significantly higher in chronic skin lesions (3.1 ± 0.8) compared with that in acute lesions (1.3 ± 0.7, $P < .001$), uninvolved skin (1.3 ± 0.7, $P < .001$), and normal skin (0.6 ± 0.7, $P < .0001$). Collagen type I deposition was observed particularly in the upper dermis of skin lesions (Fig 3). Regarding type III collagen expression, no significant difference was seen among the 4 groups (1.6 ± 1.1, 2.0 ± 1.3, 2.1 ± 1.6, and 1.3 ± 0.7 in acute lesions, chronic lesions, uninvolved skin, and normal skin, respectively).

Correlation studies between eosinophils, fibrogenic cytokines, and type I collagen deposition

The results obtained in acute and chronic AD lesions and uninvolved skin specimens were analyzed together. We found a significant correlation among the number of $EG2^+$ cells (Fig 4, *A*), the number of $IL\text{-}11^+$ cells (Fig 4, *B*), and the deposition of type I collagen ($r^2 = 0.622$ [$P < .0001$] and $r^2 = 0.527$ [$P < .0001$], respectively).

DISCUSSION

Structural changes, including papillary dermal fibrosis, epidermal hyperplasia, and diffuse intercellular and

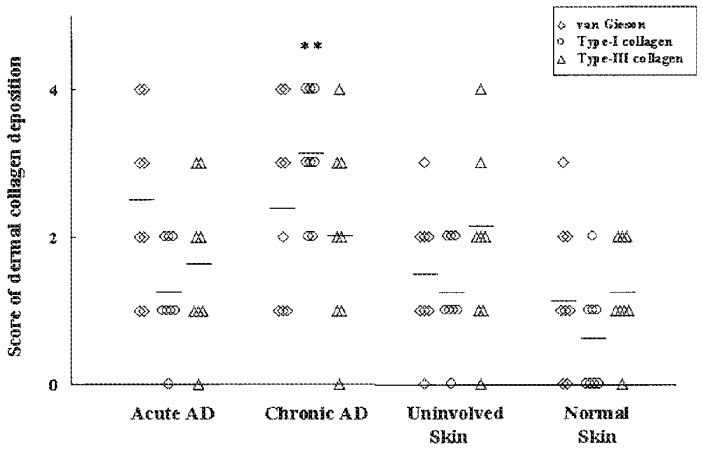

FIG 2. Papillary dermal fibrosis, as assessed by the extent of collagen deposition in acute chronic lesions and uninvolved skin biopsy specimens of patients with AD and in skin of healthy volunteers. $^{**}P < .0005$, $P < .0005$, and $P < .0001$ compared with acute lesions, uninvolved skin of patients with AD, and normal skin, respectively.

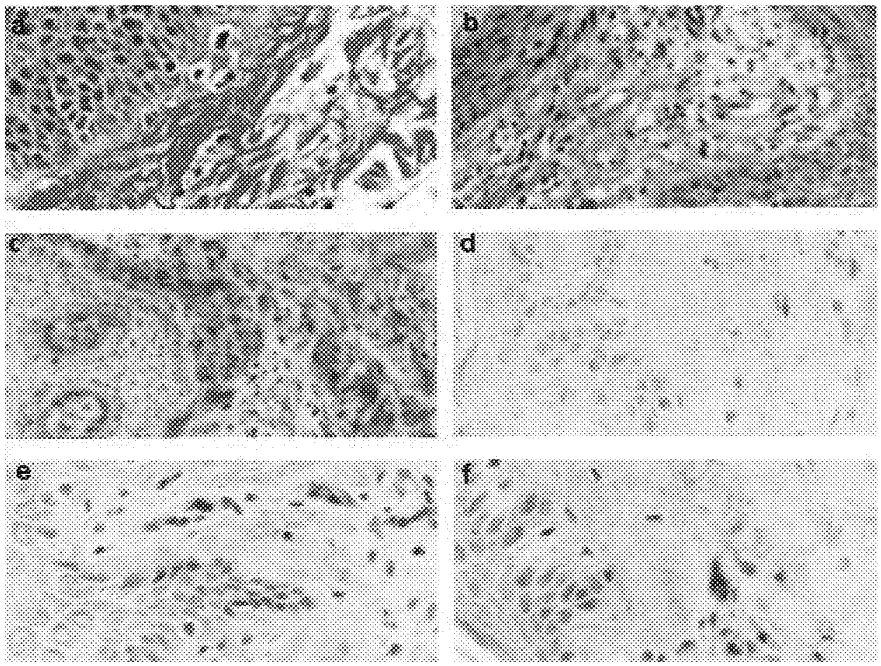

FIG 3. Representative examples of total collagen deposition, as detected by using van Gieson staining in chronic **(a)** and acute **(b)** AD; immunostaining for type I collagen in chronic AD **(c)** and in uninvolved skin from patients with AD **(d)**; and immunostaining for type III collagen in chronic **(e)** and acute **(f)** AD.

intracellular edema, are features of AD.[30,33] Previous studies have shown that matrix metalloproteinases and their specific inhibitors contribute to inflammation-induced tissue destruction and subsequent remodeling in AD.[34] We have established that remodeled asthmatic airways are associated with increased numbers of TGF-β1$^+$, IL-11$^+$, and IL-17$^+$ cells.[18,23,27,28] As such, we hypothesized that the expression of fibrogenic and fibrosis-associated cytokines, such as TGF-β1, IL-11, and IL-17, might be enhanced within AD skin lesions.

In this study we investigated the expression of TGF-β1, IL-11, and IL-17 within acute and chronic AD skin lesions by means of immunohistochemistry. To our knowledge, this is the first report demonstrating that the

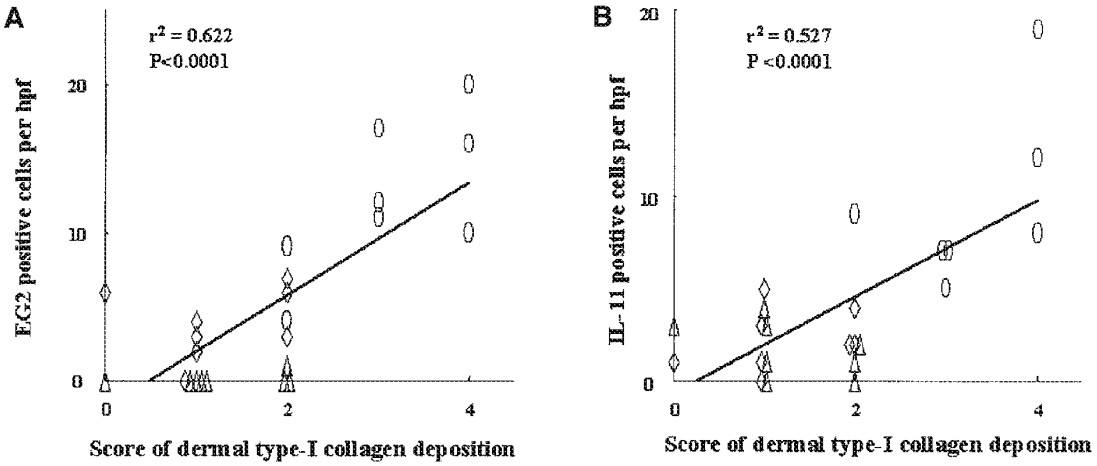

FIG 4. Correlation analysis. Significant correlation was found between type I collagen deposition and the number of $EG2^+$ eosinophils ($r^2 = 0.622$, $P < .001$; **A**) and the number of $IL\text{-}11^+$ cells ($r^2 = 0.527$, $P < .0001$; **B**) in patients with AD. Acute and chronic lesions and uninvolved skin of patients with AD are represented by *diamonds*, *circles*, and *triangles*, respectively.

number of cells expressing TGF-β1, IL-11, and IL-17 are increased in AD lesions compared with in uninvolved skin from the same individual and from normal control subjects. Moreover, acute skin lesions are associated predominantly with the expression of IL-17 and, to a lesser extent, with TGF-β1. In contrast, chronic lesions are predominantly associated with TGF-β1 and IL-11 expression. Morphologic analysis of our tissue sections revealed that the majority of IL-17 was T cells and, to a lesser extent, eosinophils. However, the main cellular source of IL-11 and TGF-β was eosinophils and, to a lesser extent, T cells.

TGF-β is a potent fibrogenic cytokine that stimulates fibroblasts to produce ECM components and protease inhibitors, to inhibit protease synthesis and release, and to promote fibroblast chemoattraction.[10,35,36] Among TGF-β1, TGF-β2, and TGF-β3 isoforms, TGF-β1 is predominantly expressed during fibrosis development.[19] Our data provide evidence for the first time that the expression of TGF-β1 occurs in the skin of patients with AD. The fact that this expression was particularly increased in chronic lesions suggests that this cytokine might be associated with more persistent lesions. Moreover, when all the AD biopsy specimens were examined, there was a significant correlation between the number of accumulated eosinophils and the number of $TGF\text{-}\beta1^+$ cells, which is consistent with our previous study in asthma.[18] Although we previously demonstrated that TGF-β1 expression was related to the severity of asthma,[18] its precise relationship in AD remains to be clarified.

The assumption that IL-11 plays a role in the development of subepithelial fibrosis and airway remodeling comes from studies done in IL-11 transgenic mice.[22] In vitro studies have also demonstrated that TGF-β1 is a potent stimulator of IL-11 production, accumulation, and gene transcription in human fibroblasts, alveolar and bronchial epithelial cells, and smooth muscle cells.[37-40] Our results are in agreement with these data because both TGF-β1 and IL-11 expression were increased in chronic lesions. In contrast, the presence of TGF-β1 and the absence of IL-11 in acute lesions might suggest that TGF-β1 initiates the upregulation of IL-11 observed in chronic stages of the disease. In addition, we have recently demonstrated that IL-11 mRNA expression was more dramatic as asthma became increasingly severe.[23] Moreover, this expression was related to increased type I and type III collagen deposition.

Because its expression was enhanced only in chronic skin AD lesions, we speculate that IL-11 is associated with the persistence of AD. In support of this is the significant correlation we found in our previous study[23] between the number of eosinophils and $IL\text{-}11^+$ cells. These results suggest that the accumulated eosinophils at the site of chronic lesions might be the major producers of IL-11 through TGF-β1 stimulation in an autocrine or paracrine manner. In addition, eosinophil-derived major basic protein has also been described to participate in the production of IL-11 by lung fibroblasts.[41] However, other IL-11–producing cells should be considered, such as structural cells, including stromal cells, fibroblasts, endothelial cells, and epithelial cells.[1,21] The evidence that TGF-β1 and IL-11 are expressed by eosinophils further supports the eventuality that these cells might be involved in the development of structural remodeling in the injured skin of patients with AD.

IL-17 is a novel cytokine apparently secreted preferentially by $CD4^+$ activated memory T cells.[24-26] Although we found no significant correlation between the expression of IL-17 and the number of T cells in our study, the increase in IL-17 in acute lesions paralleled the

augmentation of T cells. Our previous study showed that IL-17 expression was increased in bronchoalveolar lavage fluid, sputum, bronchial biopsy specimens, and peripheral blood eosinophils recovered from asthmatic patients.[27,28] Moreover, the pattern of IL-17 expression in asthmatic airways was predominantly associated with subepithelial fibrosis lesions.[28] Thus we hypothesized that IL-17 might be expressed and associated with dermal fibrosis in AD skin lesions. In fact, the increase in IL-17 expression was observed only in acute, but not in chronic, AD lesions. This observation is of interest because we have demonstrated that IL-17 increased the basal production of IL-11 by normal and asthmatic bronchial fibroblasts.[27] Therefore we might hypothesize that IL-17 initiates the development of tissue fibrosis in AD skin lesions indirectly through the release of IL-11. To further support our hypothesis, IL-17 stimulates epithelial and endothelial cells, fibroblasts, and macrophages for the release of IL-6, IL-1β, TNF-α, and GM-CSF,[24-26] a number of which are increased in AD skin lesions. Studies done in transgenic mice demonstrated that IL-6 is a potent fibrogenic cytokine, leading to the development of a subepithelial fibrosis in the airways.[42] IL-1, TNF-α, and GM-CSF also stimulate fibroblasts to induce the proliferation and synthesis of ECM components. We have shown also that IL-17 enhanced in vitro the basal production of IL-6 by fibroblasts isolated from asthmatic bronchial biopsy specimens.[27] Consequently, IL-17 might play a role in skin remodeling in AD and especially in dermal fibrosis but more probably in an indirect manner through the release of profibrotic cytokines, such as IL-11 and IL-6.

Regarding collagen, papillary dermal fibrosis corresponding to lichenified lesions has been well accepted as a characteristic feature of chronic AD.[30] Our results showed that type I collagen deposition was increased in chronic AD lesions, whereas total and type III collagen accumulation was not modulated among the groups. Of interest, when all the AD biopsy specimens were examined, the extent of type I collagen deposition was also found to be significantly correlated with the number of eosinophils and IL-11 expression. The lack of correlation between type I collagen and IL-17 expression was not surprising because our previous in vitro studies demonstrated that IL-17 was not able to stimulate bronchial fibroblasts to upregulate their production of type I and type III collagens.[27] On the other hand, type I collagen deposition and TGF-β expression did correlate; however, this failed to achieve statistical significance. Although we expected to find a significant relationship between these 2 parameters, this observation is in agreement with reports suggesting that some of the effects attributed to TGF-β1 might be in fact due to IL-11 because we do have a significant correlation between type I collagen and TGF-β1 expression. Because type I collagen is the predominant ECM component in fibrotic diseases,[43] our results can be regarded as reasonable findings. Although several studies showed a simultaneous increase in the deposition of type I and type III collagens in lesions in asthma[4,23] and in scleroderma,[44] we did not find any modulation in the extent of total and type III collagen in any of the skin biopsy specimens used in this study. Nevertheless, according to our results, we might hypothesize that the evaluation of type I collagen deposition is the most sensitive parameter for detection of dermal fibrosis in AD. In asthma, airway remodeling changes, such as airway wall thickening, might lead to a persistent reduction in airway lumen diameter and thus to bronchial hyperresponsiveness.[18,45,46] Indeed, several studies showed a link between asthma severity and subepithelial fibrosis,[18,23,45,46] as assessed by the thickness of basement membrane. Whether the extent of type I collagen deposition in the dermis might account for the severity of AD is not known and should be further investigated.

In summary, the increase in IL-17 and IL-11 expression was polarized toward acute and chronic AD lesions, respectively, whereas TGF-β1 expression was increased in both acute and chronic skin lesions, although at higher levels in the chronic situation. The finding that chronic AD skin lesions are associated with a predominance of TGF-β1– and IL-11–expressing cells and with type I collagen accumulation is also a new and interesting finding. On the basis of these results, we can speculate that in addition to TGF-β1, IL-17 initiates the development of tissue fibrosis in skin lesions in AD, whereas IL-11 is involved in the development and maintenance of this process. This process of tissue remodeling can have many clinical consequences, such as depigmentation or diminished responsiveness to topical agents. Studies, however, need to be done to investigate this further. According to the literature, T cells and macrophages contribute to the release of TGF-β1, IL-11, and IL-17 in the acute initial phase of the fibrosis process, whereas eosinophils are the major cellular source of these cytokines involved in the progression and perpetuation of fibrosis. Whether these cytokines are responsible for the characteristic lichenification and dermal fibrosis that accompanies chronic AD skin lesions requires further investigation.

REFERENCES

1. Elias JA, Zhu Z, Chupp G, Homer RJ. Airway remodeling in asthma. J Clin Invest 1999;104:1001-6.
2. Wilson JW, Li X. Inflammation and cytokines in airway wall remodeling. In: Stewart AG, ed. Airway wall remodeling in asthma. Boca Raton (FL): CRC Press; 1997. p. 65-109.
3. NIH/NHLBI. Expert panel report 2: guidelines for the diagnosis and management of asthma; Bethesda: National Institutes of Health; 1997. NIH publication no. 97-4051.
4. Roche WR, Beasley R, Williams JH, Holgate ST. Subepithelial fibrosis in the bronchi of asthmatics. Lancet 1999;1:520-4.
5. Bradding P, Redington AE, Holgate ST. Airway wall remodeling in the pathogenesis of asthma: cytokine expression in the airway. In: Stewart AG, ed. Airway wall remodeling in asthma. Boca Raton (FL): CRC Press; 1997. p. 29-63.
6. Jeffery PK, Godfrey RW, Adelroth E, Nelson F, Rogers A, Johansson SA. Effects of treatment on airway inflammation and thickening of basement membrane reticular collagen in asthma. A quantitative light and electron microscopic study. Am Rev Respir Dis 1992;145:890-9.
7. Leof EB, Proper JA, Goustin AS, Shipley GD, DiCorleto PE, Moses HL. Induction of c sis mRNA and activity similar to platelet-derived growth factor by transforming growth factor β: a proposed model for indirect

mitogenesis involving autocrine activity. Proc Natl Acad Sci U S A 1986;83:2453-7.
8. Pledger WJ, Howe PH, Leof EB. The regulation of cell proliferation by serum growth factors. Ann N Y Acad Sci 1982;397:1-10.
9. Derynck R, Choy L. Transforming growth factor-β and its receptors. In: Thomson A, ed. The cytokine handbook. 3rd ed. San Diego: Academic Press; 1982. p. 593-636.
10. Raghu G, Masta S, Meyers D, Narayanan AS. Collagen synthesis by normal and fibrotic human lung fibroblasts and the effect of transforming growth factor-β. Am Rev Respir Dis 1989;140:95-100.
11. Raghow R. Role of transforming growth factor-β in repair and fibrosis. Chest 1989;99(suppl 3):61S-5S.
12. Levi-Schaffer F, Garbuzenko E, Rubin A, Reich R, Pickholz D, Gillery P. Human eosinophils regulate human lung- and skin-derived fibroblast properties in vitro: a role for transforming growth factor β (TGF-β). Proc Natl Acad Sci U S A 1991;96:9660-5.
13. Ohno I, Lea RG, Flanders KC, Clark DA, Banwatt D, Dolovich J. Eosinophils in chronically inflamed human upper airway tissues express transforming growth factor β1 gene (TGF β1). J Clin Invest 1992; 89:1662-8.
14. Elovic A, Wong DT, Weller PF, Matossian K, Galli SJ. Expression of transforming growth factors-α and β1 messenger RNA and product by eosinophils in nasal polyps. J Allergy Clin Immunol 1994;93:864-9.
15. Wong DT, Elovic A, Matossian K, Nagura N, McBride J, Chou MY. Eosinophils from patients with blood eosinophilia express transforming growth factor β 1. Blood 1991;78:2702-7.
16. Khalil N, O'Connor RN, Flanders KC, Unruh H. TGF-β1, but not TGF-β2 or TGF-β3, is differentially present in epithelial cells of advanced pulmonary fibrosis: an immunohistochemical study. Am J Respir Cell Mol Biol 1996;14:131-8.
17. Zhang K, Flanders KC, Phan SH. Cellular localization of transforming growth factor-β expression in bleomycin-induced pulmonary fibrosis. Am J Pathol 1994;147:352-61.
18. Minshall EM, Leung DY, Martin RJ, Song YL, Cameron L, Ernst P, et al. Eosinophil-associated TGF-β1 mRNA expression and airways fibrosis in bronchial asthma. Am J Respir Cell Mol Biol 1997;17:326-33.
19. Coker RK, Laurent GJ, Shahzeidi S, Lympany PA, du Bois RM, Jeffery PK, et al. Transforming growth factors-β1, -β2, and -β3 stimulate fibroblast procollagen production in vitro but are differentially expressed during bleomycin-induced lung fibrosis. Am J Pathol 1997;150:981-91.
20. Leng SX, Elias JA. Interleukin-11. Int J Biochem Cell Biol 1997; 29:1059-62.
21. Einarsson O, Geba GP, Zhu Z, Landry M, Elias JA. Interleukin-11: stimulation in vivo and in vitro by respiratory viruses and induction of airways hyperresponsiveness. J Clin Invest 1996;97:915-24.
22. Tang W, Geba GP, Zheng T, Ray P, Homer RJ, Kuhn C. Targeted expression of IL-11 in the murine airway causes lymphocytic inflammation, bronchial remodeling, and airways obstruction. J Clin Invest 1996;98:2845-53.
23. Minshall E, Chakir J, Laviolette M, Molet S, Zhu Z, Olivenstein R. IL-11 expression is increased in severe asthma: association with epithelial cells and eosinophils. J Allergy Clin Immunol 2000;105:232-8.
24. Yao Z, Painter SL, Fanslow WC, Ulrich D, Macduff BM, Spriggs MK. Human IL-17: a novel cytokine derived from T cells. J Immunol 1995;155:5483-6.
25. Fossiez F, Djossou O, Chomarat P, Flores-Romo L, Ait-Yahia S, Maat C. T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. J Exp Med 1996;183:2593-603.
26. Jovanovic DV, Di Battista JA, Martel-Pelletier J, Jolicoeur FC, He Y, Zhang M. IL-17 stimulates the production and expression of proinflammatory cytokines, IL-β and TNF-α, by human macrophages. J Immunol 1989;160:3513-21.
27. Molet S, Hamid Q, Page N, Taha R, Nutku E, Davoine F. Interleukin-17, a new eosinophil-associated cytokine increased in asthmatic airways. Eur Respir J 1999;14(suppl 30):1939.
28. Molet S, Chakir J, Laviolette M, Zhu Z, Elias JA, Hamid Q. Response of remodeling-associated cytokines to steroids in bronchial biopsies of asthmatic patients. Am J Respir Crit Care Med 2000;161:742.
29. Leung DYM. Atopic dermatitis: new insights and opportunities for therapeutic intervention. J Allergy Clin Immunol 2000;105:860-76.
30. Leung DYM, Hamid Q. The immunology of atopic dermatitis. In: Leung DYM, ed. Atopic dermatitis: from pathogenesis to treatment. Georgetown: R.G. Landes Company; 1996. p. 113-44.
31. Beltrani VS. The clinical spectrum of atopic dermatitis. J Allergy Clin Immunol 1999;104(suppl):S87-98.
32. Giaid A, Michel RP, Stewart DJ, Sheppard M, Corrin B, Hamid Q. Expression of endothelin-1 in lungs of patients with cryptogenic fibrosing alveolitis. Lancet 1993;341:1550-4.
33. Leung DY, Bhan AK, Schneeberger EE, Geha RS. Characterization of the mononuclear cell infiltrate in atopic dermatitis using monoclonal antibodies. J Allergy Clin Immunol 1983;71:47-56.
34. Katoh N, Hirano S, Suehiro M, Ikenaga K, Yasuno H. Increased levels of serum tissue inhibitor of metalloproteinase-1 but not metalloproteinase-3 in atopic dermatitis. Clin Exp Immunol 2002;127:283-8.
35. Postlethwaite AE, Keski-Oja J, Moses HL, Kang AH. Stimulation of the chemotactic migration of human fibroblasts by transforming growth factor β. J Exp Med 1987;165:251-6.
36. Overall CM, Wrana JL, Sodek J. Independent regulation of collagenase, 72-kDa progelatinase, and metalloendoproteinase inhibitor expression in human fibroblasts by transforming growth factor-β. J Biol Chem 1989;264:1860-9.
37. Elias JA, Zheng T, Whiting NL, Trow TK, Merrill WW, Zitnik R. IL-1 and transforming growth factor-β regulation of fibroblast-derived IL-11. J Immunol 1994;152:2421-9.
38. Elias JA, Zheng T, Einarsson O, Landry M, Trow T, Rebert N, et al. Epithelial interleukin-11. Regulation by cytokines, respiratory syncytial virus, and retinoic acid. J Biol Chem 1994;269:22261-8.
39. Zheng T, Nathanson MH, Elias JA. Histamine augments cytokine-stimulated IL-11 production by human lung fibroblasts. J Immunol 1994;153:4742-52.
40. Elias JA, Wu Y, Zheng T, Panettieri R. Cytokine- and virus-stimulated airway smooth muscle cells produce IL-11 and other IL-6-type cytokines. Am J Physiol 1997;273:L648-55.
41. Rochester CL, Ackerman SJ, Zheng T, Elias JA. Eosinophil-fibroblast interactions. Granule major basic protein interacts with IL-1 and transforming growth factor-β in the stimulation of lung fibroblast IL-6-type cytokine production. J Immunol 1996;156:4449-56.
42. DiCosmo BF, Geba GP, Picarella D, Elias JA, Rankin JA, Stripp BR. Airway epithelial cell expression of interleukin-6 in transgenic mice. Uncoupling of airway inflammation and bronchial hyperreactivity. J Clin Invest 1994;94:2028-35.
43. Trojanowska M, LeRoy EC, Eckes B, Krieg T. Pathogenesis of fibrosis: type I collagen and the skin. J Mol Med 1998;76:266-74.
44. Uitto J, Bauer EA, Eisen AZ. Scleroderma: increased biosynthesis of triple-helical type I and type III procollagens associated with unaltered expression of collagenase by skin fibroblasts in culture. J Clin Invest 1979;64:921-30.
45. James AL, Pare PD, Hogg JC. The mechanics of airway narrowing in asthma. Am Rev Respir Dis 1989;139:242-6.
46. Van Schayck CP, Dompeling E, Van Herwaarden CL, Wever AM, Van Weel C. Interacting effects of atopy and bronchial hyperresponsiveness on the annual decline in lung function and the exacerbation rate in asthma. Am Rev Respir Dis 1990;144:1297-301.

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Transforming growth factor-β stimulates Interleukin-11 production by human periodontal ligament and gingival fibroblasts

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Abstract

Background: Transforming growth factor (TGF)-β is a potent multifunctional polypeptide, abundant in the bone matrix. Interleukin (IL)-11 is a pleiotropic cytokine with effects on multiple cell types. The present study was performed to evaluate the regulatory effects of TGF-β on IL-11 production by human periodontal ligament cells (PDL) and human gingival fibroblasts (HGF).

Material and Methods: The expression of TGF-β receptor in PDL and HGF were observed using flow cytometry. PDL and HGF were stimulated with TGF-β with or without protein kinase C (PKC) inhibitors and activator. IL-11, bone morphogenetic protein-2 (BMP-2) and TGF-β mRNA expression was quantified by real-time polymerase chain reaction (PCR). IL-11 production was measured using enzyme-linked immunosorbent assay.

Results: PDL and HGF expressed both TGF-β receptor I and TGF-β receptor II on the cell surfaces. IL-11 mRNA expression and IL-11 production were augmented by TGF-β in both PDL and HGF, with higher values in PDL. PKC inhibitors partially suppressed TGF-β-induced IL-11 production in PDL and HGF, whereas activator enhanced it. TGF-β mRNA and BMP-2 mRNA expression were up-regulated by TGF-β in PDL.

Conclusion: These results suggest that PDL produce IL-11 in response to TGF-β.

Key words: interleukin-11; periodontal fibroblasts; protein kinase C

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Interleukin-11 (IL-11) is a pleiotropic member of the IL-6-type cytokine family that mediates its biological activities via binding to a multimeric receptor complex that contains gp130 molecules (Kishimoto et al. 1995). Cellular and molecular components involved in the destruction of periodontal tissues are predominantly host derived. The balance of T-helper (Th)1 cytokines and Th2 cytokines is important in disease progression. IL-6 is a Th2 cytokine elevated in the periodontitis tissue, but the roles of IL-11 in periodotitis is still poorly understood (Berglundh & Donati 2005). IL-11 has many biological activities and has roles in haematopoiesis, immune responses, the nervous system and bone metabolism (Paul et al. 1990, Du & Williams 1997). IL-11 induces bone resorption by enhancing osteoclast formation in vitro (Girasole et al. 1994). In contrast, IL-11 also stimulates osteoblasts in vitro and bone formation in vivo. Overexpression of human IL-11 gene in transgenic mice results in the stimulation of bone formation (Takeuchi et al. 2002). IL-11 acts synergistically with bone morphogenetic protein-2 (BMP-2) to accelerate bone formation (Suga et al. 2001, 2003, Suga et al. 2004). IL-11 produced in human gingival fibroblasts (HGF) stimulated with butyric acid is involved in the attenuation of T-cell apoptosis by HGF (Kurita-Ochiai et al. 2002). IL-1α and tumour necrosis factor (TNF)-α stimulate HGF to produce IL-11 and the production is mainly mediated by internal prostaglandin E_2 (PGE_2) synthesis (He et al. 2004). IL-11 is a candidate molecule for therapeutic modulation of the host response in the management of periodontal diseases (Trepicchio et al. 1996, Martuscelli et al. 2000, Kinane & Attstrom 2005, Salvi & Lang 2005). IL-11 production by periodontal ligament cells (PDL) and HGF might be important for the homeostasis of alveolar bone, but so far the molecular mechanisms of IL-11 production by these cells have not been elucidated.

Bone homeostasis is regulated by osteoclastic bone resorption and osteoblastic bone formation. Osteoclasts and

osteoblasts are tightly regulated by each other (Rodan & Martin 1981, Suda et al. 1992). Osteoblasts are fibroblastic cells of mesenchymal origin, and osteoclasts differentiate from the monocyte/macrophage lineage cells (Katagiri & Takahashi 2002). Recently, receptor activator of nuclear factor κ B ligand (RANKL) was identified as an important factor involved in osteoclast development by osteoblasts (Lacey et al. 1998, Yasuda et al. 1998b). Expression of RANKL enhances osteoclast differentiation from osteoclast precursors, and its decoy receptor osteoprotegerin (OPG) suppresses osteoclast differentiation through the inhibition of cognate interaction between osteoblasts and osteoclast precursors (Simonet et al. 1997, Yasuda et al. 1998a). Periodontal ligament is the unique tissue connecting alveolar bone and tooth cementum. PDL and gingival fibroblasts (HGF) are two major fibroblasts of mesenchymal origin in periodontal tissue. PDL have similar functions as osteoblasts, as they regulate osteoclast differentiation through the expression of RANKL and OPG (Kanzaki et al. 2001). On the contrary, HGF produce large amounts of OPG, but rarely express RANKL (Nagasawa et al. 2002). Although the regulation of osteoclast differentiation by osteoblastic cells has been extensively studied, little is known about the regulation of osteoblast function by osteoclasts or macrophage lineage cells.

Transforming growth factor β (TGF-β) is produced by various cells including macrophages, as well as non-immune cells like gingival fibroblasts, in the periodontal tissue (Wahl et al. 1993, Cotrim et al. 2002). TGF-β is abundant in the bone matrix, and is released from the matrix during bone resorption (Seyedin et al. 1986). Macrophage cell lines secrete TGF-β and enhance alkaline phosphatase activity in osteoblastic cells (Champagne et al. 2002). TGF-β might be a candidate molecule for monocyte/macrophage lineage cells to regulate osteoblast functions (Champagne et al. 2002).

The aim of the present study was to investigate the regulatory effects of TGF-β on IL-11 production by both PDL and HGF.

Material and Methods

Reagents

Recombinant human TGF-β1 was purchased from Techne (Minneapolis, MN, USA). Stock solution of TGF-β1 (2 μg/ml) was reconstituted in 4 mM HCl containing 1 mg/ml bovine serum albumin according to the manufacturer's instructions and stored at -20°C until use. Flourosene isothiocyanate (FITC)-conjugated rabbit polyclonal antibody against human TGF-β receptor I, receptor II and FITC-conjugated normal rabbit Immunoglobulin G (IgG) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA). Staurosporine streptomyces (ST) was purchased from Sigma Chemicals (St Louis, MO, USA). Myristoylated protein kinase C (PKC) (Myr) peptide inhibitor and phorbol 12-myristate 13-acetate (PMA) were purchased from Promega (Madison, WI, USA). Stock solutions of ST (400 mM), Myr (4 mM) and PMA (30 μM) were prepared in dimethyl sulphoxide and stored at -20°C until use.

Cell culture

HGF were isolated from six systemically healthy patients (three females and three males, aged 21–29 years old, mean 30.5 ± 8.5) who had no sites with clinical attachment loss greater than 2 mm and no radiographic bone loss. Six healthy gingival samples were collected during routine crown lengthening surgery. The washed explants were placed in a sterile dish and minced into smaller pieces with a sterile scalpel blade. Attempts were made to remove the epithelium and leave only connective tissue. PDL were isolated from the teeth of six systemically healthy patients (four females and two males, 17–29 years old, mean 21.8 ± 4.7). These clinically healthy pre-molar teeth were extracted for orthodontic reasons. The mid-root surfaces of the teeth were scraped lightly with a sterile scalpel blade. Before starting, the study protocol was approved by the Ethics Committee of Tokyo Medical and Dental University. Informed consent was obtained from all 12 subjects, after verbal and written explanation regarding the nature of the study. All cell lines were prepared as described previously (Hayashi et al. 1994). Fibroblastic cells were allowed to grow out from the explant at 37°C in a humidified atmosphere with 5% CO_2 in the air until they formed a confluent layer, at which point they were subcultured. Cells subcultured to the fifth passage were used for these experiments.

Cell stimulation

PDL and HGF were seeded in 24-well culture plates at 1×10^5 cells per well, and were grown to confluence. Once confluent, the fibroblasts were cultured with or without 2 ng/ml TGF-β1 or an equal volume of vehicle. RNA was extracted from the cultured cells.

In addition, PDL and HGF were seeded in 96-well flat-bottomed culture plates at 1×10^5 cells per well, and were grown to confluence. Once confluent, the fibroblasts were stimulated with TGF-β1 with or without PKC inhibitors, 400 nM ST and 4 μM Myr or an equal volume of vehicle. In some experiments, PDL and HGF were stimulated with PKC activator, 30 nM PMA. After 24 h, the supernatants were collected.

Enzyme-linked immunosorbent assay (ELISA)

IL-11 in the cultured supernatants was measured using commercially available ELISA kits (IL-11, R&D Systems Inc., Minneapolis, MN, USA), according to the manufacturer's instructions.

RNA extraction and real-time reverse transcriptase polymerase chain reaction (RT-PCR)

Cultured HGF and PDL were washed three times with PBS, and RNA-Bee solution (Tel-Test, Inc. Friendswood, TX, USA) was added to the culture dish. RNA was extracted with phenol and chloroform, precipitated with isopropanol, washed with 75% ethanol and suspended in diethylpyrocarbonate-treated distilled water. Samples containing 5 μg of RNA were used for RT-PCR. First-strand cDNA was synthesized using a kit (Super-Script II First-Strand cDNA Synthesis Kit, Invitrogen, Grand Island, NY, USA). Real-time PCR analyses were performed in a fluorescent temperature cycler (LightCycler, Roche Molecular Biochemicals, Mannheim, Germany). For IL-11 and BMP-2, LightCycler-Primer Sets (Roche Molecular Biochemicals) were used according to the manufacturer's instructions. For TGF-β the primers were designed on the basis of previously described sequences (Champagne et al. 2002). The following primers were used:

TGF-β1 primer R, 5′-GCCCTGGACACCAACTATTGCT;

TGF-β1 primer F, 5-AGGCTCCAAATGTAGGGGCAGG.

One-tenth (2 μl) of each RT reaction served as template in a 20 μl PCR reaction mixture containing 1.5 mM $MgCl_2$, 0.5 μM of each primer and 1 U LightCycler DNA Master SYBR Green I mix (Roche Molecular Biochemicals). After initial denaturation at 94°C for 30 s, reactions were cycled 35 times using the following parameters for detection: 95°C for 1 s, primer annealing at 61°C for 7 s and primer extension at 72°C for 11 s. SYBR Green I fluorescence was detected at the end of each cycle to monitor the amount of PCR product formed during that cycle. At the end of each run, melting curve profiles were analysed (cooling the sample to 68°C and heating slowly to 95°C with continuous measurement of fluorescence) to confirm amplification of specific cDNA.

Detection of TGF-β receptor in PDL and HGF

In our preliminary study, we could not detect cell surface receptors on cells detached by using trypsin-EDTA. Confluent cultures of PDL and HGF were mechanically detached by using cell scrapers so as to avoid possible proteolytic destruction of cell surface antigens. The cells were then centrifuged and fixed with 1% para-formaldehyde in phophate-buffered solution (PBS) for 30 min. They were washed with PBS supplemented with 2% fetal bovine serum (FBS) (PBS/FBS) and centrifuged at 400 × *g* for 10 min. after each step. The cells were incubated for 30 min. with an FITC-conjugated rabbit polyclonal antibody against human TGF-β receptor I and receptor II (Santa Cruz Biotechnology Inc.). FITC-conjugated normal rabbit IgG (Santa Cruz Biotechnology Inc.) was used as a negative control. The expression of TGF-β receptors was observed using flow cytometry (Nagasawa et al. 2002).

Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA) using StatView. Fisher's protected least significance test was used for the post hoc comparison of specific groups.

Results

Expression of TGF-β receptors on PDL and HGF

4.1 ± 1.1% of PDL expressed TGF-β receptor I and 5.3 ± 0.2% of PDL expressed TGF-β receptor II on the cell surfaces. 5.6 ± 0.2% of HGF expressed TGF-β receptor I and 4.8 ± 2.3% of HGF expressed TGF-β receptor II on the cell surfaces.

Time course of IL-11 mRNA and BMP-2 mRNA expression in PDL stimulated with TGF-β

In the preliminary experiments, different concentrations of TGF-β were used to stimulate PDL and HGF (0.02, 0.2, 2 and 10 ng/ml TGF-β), and optimal response was observed at ≥ 2 ng/ml of TGF-β (data not shown). An increase of IL-11 mRNA in PDL was apparent in 6 h, with the addition of 2 ng/ml TGF-β. The level of IL-11 mRNA peaked between 6 and 12 h and then returned to a baseline level by 24 h (Fig. 1). Similarly, an increase of BMP-2 mRNA was apparent in 6 h with the addition of TGF-β and the level of BMP-2 mRNA peaked between 6 and 12 h and then returned to a baseline level by 24 h (Fig. 1).

Effect of TGF-β on IL-11 mRNA expression in PDL and HGF

Real-time PCR analysis showed that IL-11 mRNA expression was significantly up-regulated by TGF-β in both PDL and HGF (6 h after stimulation). The IL-11 mRNA was significantly higher in PDL than in HGF in both stimulated and non-stimulated conditions (Fig. 2a).

Effect of TGF-β on IL-11 production in PDL and HGF

PDL produced significantly higher amounts of IL-11 than HGF in either stimulated or non-stimulated conditions (Fig. 2b).

Effect of TGF-β1 on TGF-β1 mRNA expression in PDL and HGF

TGF-β mRNA expression was significantly up-regulated by TGF-β in PDL (Fig. 3).

The up-regulation of TGF-β mRNA was not statistically significant in HGF (Fig. 3).

Effect of PKC inhibitors on IL-11 production in PDL and HGF stimulated with TGF-β1

Staurosporine streptomyces (ST) and myristoylated (Myr) PKC significantly suppressed IL-11 production in PDL stimulated with TGF-β1 (Fig. 4a). The effect of the inhibitors was 31.9% and 37.4%, respectively. Staurosporine streptomyces (ST) and (Myr) PKC significantly suppressed IL-11 production by HGF stimulated with TGF-β (Fig. 4a). The effect of the inhibitors was 65.4% and 53.4%, respectively.

Effect of PKC activator on IL-11 production in PDL and HGF

PMA significantly enhanced IL-11 production in PDL, but the amount of IL-11

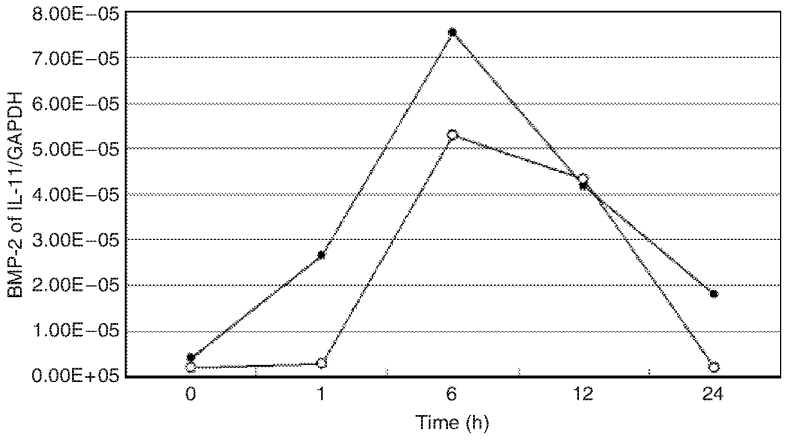

Fig. 1. Time course of Interleukin (IL)-11 mRNA and bone morphogenetic protein-2 (BMP-2) mRNA expression in periodontal ligament cell (PDL) stimulated with transforming growth factor (TGF)-β PDL was cultured in the presence of 2 ng/ml TGF-β. RNA harvested at 0, 1, 6, 12, 24 h, and reverse transcriptase polymerase chain reaction (RT-PCR) was performed by real-time PCR to evaluate IL-11 and BMP-2 mRNA expression. An increase of IL-11 mRNA and BMP-2 mRNA was apparent in 6 h after the addition of TGF-β. Open and closed circles indicate the increase of IL-11 mRNA (•) and BMP-2 mRNA (○), respectively. Data are representative of three separate experiments.

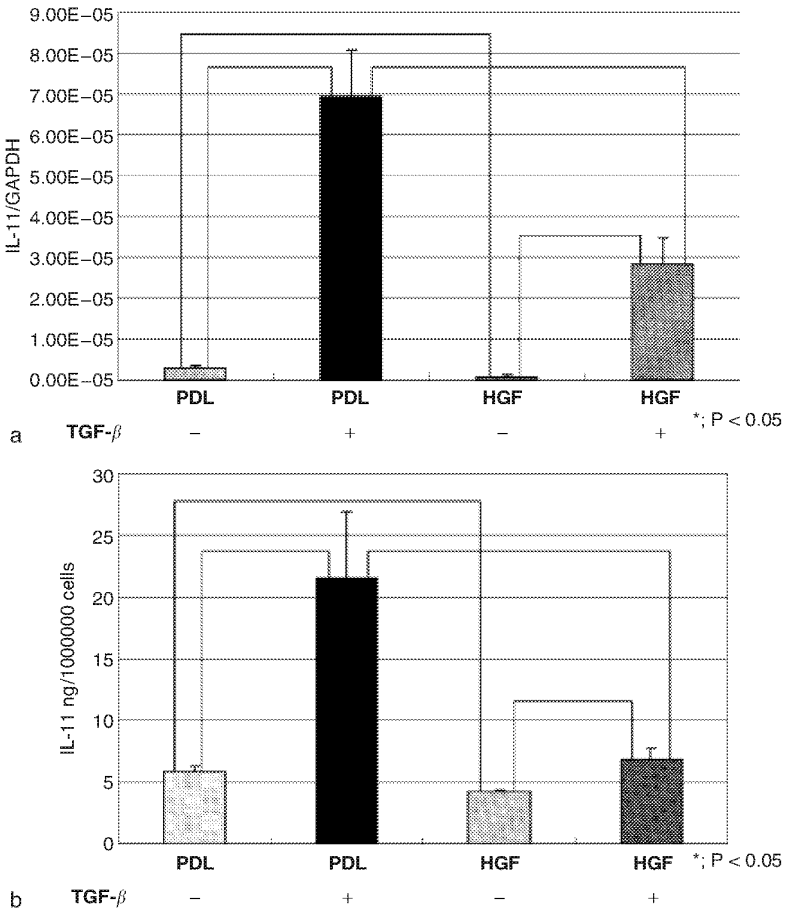

Fig. 2. (A) Effect of transforming growth factor (TGF)-β on Interleukin (IL)-11 mRNA expression in PDL and human gingival fibroblasts (HGF). The cells were stimulated with 2 ng/ml TGF-β for 6 h. After incubation, RNA was extracted and reverse transcriptase polymerase chain reaction (RT-PCR) was performed. IL-11 mRNA expression was evaluated by real-time PCR, as described in Material and Methods. Values shown are mean ± SD. Data are representative of three separate experiments. *Significantly different from control ($p<0.05$). (B) Effect of TGF-β on IL-11 production in PDL and HGF. The cells were stimulated with 2 ng/ml TGF-β for 24 h. After incubation, IL-11 levels in the culture media were determined by enzyme-linked immunosorbent assay, as described in Materials and Methods. Values shown are mean ± SD. Data are representative of three separate experiments. *Significantly different from control ($p<0.05$).

was less than that after TGF-β stimulation (Fig. 4b). The percent of the TGF-β response obtained with PMA alone was 45.3%. PMA-enhanced IL-11 production in HGF. The amount of IL-11 was also less than that after TGF-β stimulation (Fig. 4b). The percent of the TGF-β response obtained with PMA alone was 29.1%.

Discussion

In this study, TGF-β augmented IL-11 mRNA expression, resulting in the enhancement of IL-11 production. IL-11 mRNA was constitutively expressed in PDL and HGF, and it was significantly higher in PDL.

TGF-β is an important local regulator of metabolism (Centrella et al. 1994), and is produced by various kinds of cells including macrophages, as well as mesenchymal fibroblastic cells. Two classes of receptors (type I and type II) have been identified as signal-transducing receptors for TGF-β (Yamashita et al. 1996). We confirmed that TGF-β RI and RII were expressed on both PDL and HGF. Expression of TGF-β receptors on HGF and PDL suggests that PDL and HGF might be regulated by TGF-β released from the matrix during bone resorption or produced by various cells including macrophages in the periodontal tissue. It has been demonstrated using flow cytometry that both PDL and HGF express TGF-β RI and TGF-β RII, although in only a portion of the total cells (Parkar et al. 2001). In our results, percentage of the positive cells was lower than their report. It might be because of the different antibodies used. Cells expressing a low amount of TGF-β receptors might be counted as negative cells in our study.

Although a large amount of TGF-β is released from the bone matrix or produced by various cells including macrophages in the periodontal tissue, it is difficult to determine the physiological TGF-β concentration in periodontal tissue. It has been reported in vivo that 0.05 μg of TGF-β plus bone marrow cells induced bone formation in rabbit skull defect (Tabata et al. 2000). Our dose of TGF-β (2 ng/ml) is lower than their study, and the concentration might be closer to the physiological concentration.

IL-11 mRNA expression was significantly higher in PDL than in HGF in both TGF-β-stimulated and non-stimulated conditions (Fig. 2a). The production of IL-11 was also significantly higher in PDL than in HGF in both TGF-β-stimulated and non-stimulated conditions (Fig. 2b), suggesting that the production of IL-11 by PDL was transcriptionally regulated.

Somerman et al. (1988) compared PDL and HGF derived from the same patient, same passage, in vitro. They found greater protein and collagen production and higher alkaline phosphatase levels in PDL than in HGF. Although all the six lines showed similar responses in the present study, we should take into consideration that the PDL and HGF were derived from different individual. There is considerable heterogeneity within each group of gingival or periodontal ligament fibroblasts cell lines (Hassell & Stanek 1983, Dongari-Bagtzoglou et al. 1997).

Earlier reports indicate that IL-11 augments osteoclastogenesis in vitro (Hill et al. 1998, Yasuda et al. 1998b). It has been repoted that IL1-α and TNF-α stimulate HGF to produceIL-11 (He et al. 2004). IL-11 produced by IL-1-stimulated HGF might augment osteoclastogenesis. However, gingival concentrations of IL-11 were highest within gingiva adjacent to

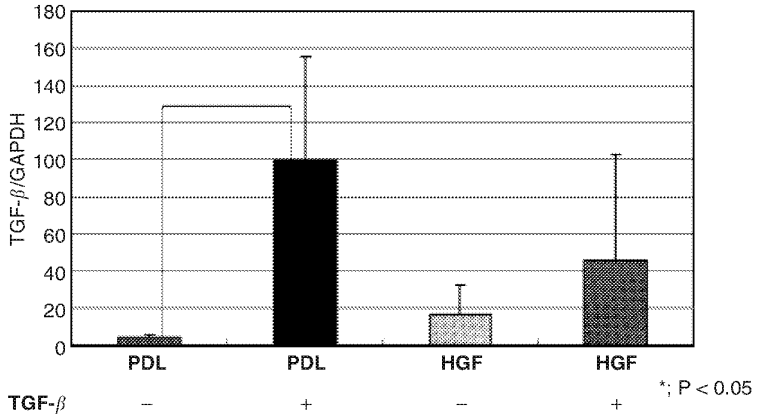

Fig. 3. Effect of transforming growth factor (TGF)-β on the expression of TGF-β mRNA in PDL and human gingival fibroblasts (HGF). The cells were stimulated with 2 ng/ml TGF-β for 6 h. After incubation, RNA was extracted and reverse transcriptase polymerase chain reaction (RT-PCR) was performed. TGF-β mRNA expression was evaluated by real-time PCR, as described in Material and Methods. Values shown are mean ± SD. Data are representative of three separate experiments. *Significantly different from control ($p < 0.05$).

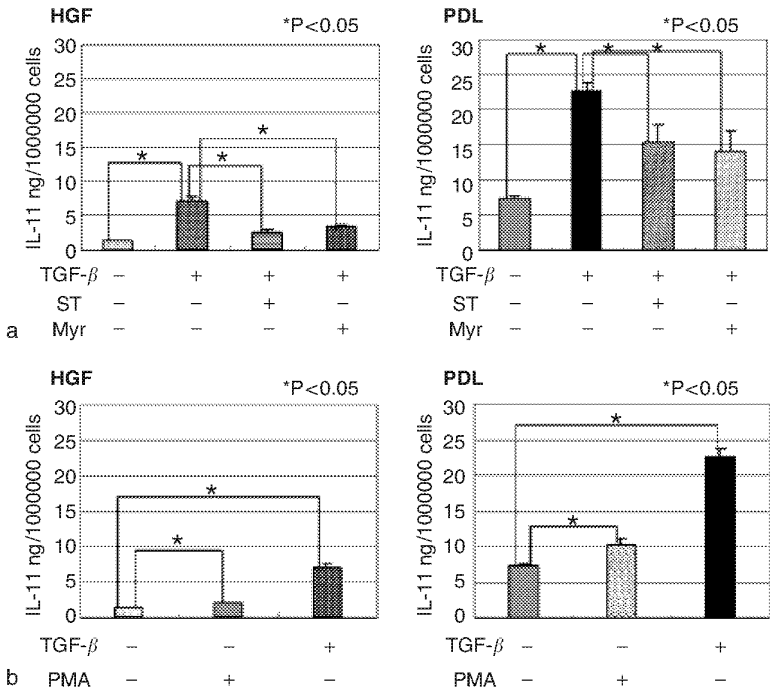

Fig. 4. (a) Effect of protein kinase C (PKC) inhibitors staurosporine streptomyces (ST) and myristoylated PKC (Myr) on transforming growth factor (TGF)-β-stimulated Interleukin (IL)-11 production in periodontal ligament cells (PDL) and human gingival fibroblasts (HGF). PDL and HGF were cultured with or without 2 ng/ml TGF-β1 and PKC inhibitors (400 nM ST and 4 μM Myr) were simultaneously added. After 24 h, supernatants were collected and enzyme-linked immunosorbent assay was performed. Values shown are mean ± SD. Data are representative of three separate experiments. *Significantly different from control ($p < 0.05$). (b) Effect of PKC activator phorbol 12-myristate 13-acetate (PMA) on TGF-β-stimulated IL-11 production in PDL and HGF. PDL and HGF were cultured with or without 2 ng/ml TGF-β or PKC activator (30 nM PMA). After 24 h, supernatants were collected and enzyme-linked immunosorbent assay was performed. Values shown are mean ± SD. Data are representative of three separate experiments. *Significantly different from control ($p < 0.05$).

3 mm diseased pockets and gingival concentrations of IL-11 were significantly lower in gingiva adjacent to 6 mm pockets (Johnson et al. 2004). They suggest that the elevated concentration of IL-11 is a consequence of changes in the gingival microenvironment during early stages of gingival inflammation. The lower IL-11 concentration within gingiva adjacent to 6 mm pockets could minimize the potential protective effect of IL-11. IL-11 has been reported to down-regulate synthesis of TNF-α and IL-1 β in vitro, which are potent proinflammatory cytokines (Trepicchio et al. 1996). In a ligature-induced beagle dog model, subcutaneous injection of IL-11 slowed down the progression of attachment and radiographic alveolar bone loss (Martuscelli et al. 2000), suggesting that IL-11 might ameliorate inflammatory response in the gingival tissue.

Several investigators reported the involvement of IL-11 in osteoblast differentiation and bone formation in vivo and in vitro. Transgenic mice expressing the IL-11 gene exhibited enhanced ratios of bone volume/tissue volume and increased bone formation rates, indicating that IL-11 positively affects bone formation (Takeuchi et al. 2002). IL-11 has been observed to induce osteoblastic differentiation of C3H10T1/2 mouse mesenchymal progenitor cells, to act synergistically with BMP-2, and to be an early acting mediator of bone formation (Suga et al. 2001). These cytokines acted synergistically on bone formation in vivo, in a rat ectopic model (Suga et al. 2003) and rabbit model (Suga et al. 2004).

In this study, increase of BMP-2 mRNA was apparent at 6 h with the addition of TGF-β in PDL, and this was similar to the increase of IL-11 mRNA stimulated with TGF-β1 (Fig. 1). PDL might augment bone formation through the combined production of IL-11 and BMP-2. IL-11 and BMP-2 might act synergistically on osteoblasts. Furthermore, TGF-β mRNA expression was up-regulated in PDL stimulated with TGF-β (Fig. 3). This suggests that autocrine TGF-β production may further augment IL-11 induction.

PKC-dependent activities have been found to participate in TGF-β1-induced gene expression (Halstead et al. 1995, Mucsi et al. 1996). For example, TGF-β activates PKC to induce collagen I expression in mesangial cells (Runyan et al. 2003). In PDL stimulated with TGF-β, both PKC inhibitors ST and Myr suppressed IL-11 production

(Fig. 4a), suggesting that the production of IL-11 was ,at least in part, mediated by PKC. The PKC activator PMA enhanced IL-11 production, suggesting that PKC activation directly augmented IL-11 production (Fig. 4b) in PDL. However, other pathways might regulate IL-11 production, as the inhibitors did not completely inhibit IL-11 production, nor did the PDL stimulated with PKC activators secrete comparable amounts of IL-11 to TGF-β stimulation.

In conclusion, PDL produced higher levels of IL-11 than HGF in response to TGF-β. Simultaneous production of IL-11 and BMP-2 by PDL might be a pathway for bone formation by periodontal ligament fibroblasts stimulated with TGF-β.

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References

Berglundh, T. & Donati, M. (2005) Aspects of adaptive host response in periodontitis, **32** (Suppl. 6), 87–107.

Centrella, M., Horowitz, M. C., Wozney, J. M. & McCarthy, T. L. (1994) Transforming growth factor-beta gene family members and bone, **15**, 27–39.

Champagne, C. M., Takebe, J., Offenbacher, S. & Cooper, L. F. (2002) Macrophage cell lines produce osteoinductive signals that include bone morphogenetic protein-2. *Bone* **30**, 26–31.

Cotrim, P., de Andrade, C. R., Martelli-Junior, H., Graner, E., Sauk, J. J. & Coletta, R. D. (2002) Expression of matrix metalloproteinases in cyclosporin-treated gingival fibroblasts is regulated by transforming growth factor (TGF)-beta1 autocrine stimulation. *Journal of Periodontology* **73**, 1313–1322.

Dongari-Bagtzoglou, A. I., Warren, W. D., Berton, M. T. & Ebersole, J. L. (1997) CD40 expression by gingival fibroblasts: correlation of phenotype with function. *International Immunology* **9**, 1233–1241.

Du, X. & Williams, D. A. (1997) Interleukin-11: review of molecular, cell biology and clinical use. *Blood* **89**, 3897–3908.

Girasole, G., Passeri, G., Jilka, R. L. & Manolagas, S. C. (1994) Interleukin-11: a new cytokine critical for osteoclast development, **93**, 1516–1524.

Halstead, J., Kemp, K. & Ignotz, R. A. (1995) Evidence for involvement of phosphatidylcholine-phospholipase C and protein kinase C in transforming growth factor-beta signaling, **270**, 13600–13603.

Hassell, T. M. & Stanek, E. J. 3rd (1983) Evidence that healthy human gingiva contains functionally heterogeneous fibroblast subpopulations, **28**, 617–625.

Hayashi, J., Saito, I., Ishikawa, I. & Miyasaka, N. (1994) Effects of cytokines and periodontopathic bacteria on the leukocyte function-associated antigen 1/intercellular adhesion molecule 1 pathway in gingival fibroblasts in adult periodontitis, **62**, 5205–5212.

He, L., Nagasawa, T. & Ishikawa, I. (2004) Production of interleukin-11 in human gingival fibroblasts stimulated with IL-1alpha and tumor necrosis factor-alpha. *Zhonghua Kou Qiang Yi Xue Za Zhi* **39**, 488–491.

Hill, P. A., Tumber, A., Papaioannou, S. & Meikle, M. C. (1998) The cellular actions of interleukin-11 on bone resorption in vitro. *Endocrinology* **139**, 1564–1572.

Johnson, R. B., Wood, N. & Serio, F. G. (2004) Interleukin-11 and IL-17 and the pathogenesis of periodontal disease, **75**, 37–43.

Kanzaki, H., Chiba, M., Shimizu, Y. & Mitani, H. (2001) Dual regulation of osteoclast differentiation by periodontal ligament cells through RANKL stimulation and OPG inhibition, **80**, 887–891.

Katagiri, T. & Takahashi, N. (2002) Regulatory mechanisms of osteoblast and osteoclast differentiation, **8**, 147–159.

Kinane, D. F. & Attstrom, R. (2005) Advances in the pathogenesis of periodontitis. Group B consensus report of the fifth European workshop in periodontology, **32** (Suppl. 6), 130–131.

Kishimoto, T., Akira, S., Narazaki, M. & Taga, T. (1995) Interleukin-6 family of cytokines and gp130. *Blood* **86**, 1243–1254.

Kurita-Ochiai, T., Ochiai, K., Suzuki, N., Otsuka, K. & Fukushima, K. (2002) Human gingival fibroblasts rescue butyric acid-induced T-cell apoptosis. *Infection and Immunity* **70**, 2361–2367.

Lacey, D. L., Timms, E., Tan, H. L., Kelley, M. J., Dunstan, C. R., Burgess, T., Elliott, R., Colombero, A., Elliott, G., Scully, S., Hsu, H., Sullivan, J., Hawkins, N., Davy, E., Capparelli, C., Eli, A., Qian, Y. X., Kaufman, S., Sarosi, I., Shalhoub, V., Senaldi, G., Guo, J., Delaney, J. & Boyle, W. J. (1998) Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* **93**, 165–176.

Martuscelli, G., Fiorellini, J. P., Crohin, C. C. & Howell, T. H. (2000) The effect of interleukin-11 on the progression of ligature-induced periodontal disease in the beagle dog. *Journal of Periodontology* **71**, 573–578.

Mucsi, I., Skorecki, K. L. & Goldberg, H. J. (1996) Extracellular signal-regulated kinase and the small GTP-binding protein, Rac, contribute to the effects of transforming growth factor-beta1 on gene expression, **271**, 16567–16572.

Nagasawa, T., Kobayashi, H., Kiji, M., Aramaki, M., Mahanonda, R., Kojima, T., Murakami, Y., Saito, M., Morotome, Y. & Ishikawa, I. (2002) LPS-stimulated human gingival fibroblasts inhibit the differentiation of monocytes into osteoclasts through the production of osteoprotegerin, **130**, 338–344.

Parkar, M. H., Kuru, L., Giouzeli, M. & Olsen, I. (2001) Expression of growth-factor receptors in normal and regenerating human periodontal cells, **46**, 275–284.

Paul, S. R., Bennett, F., Calvetti, J. A., Kelleher, K., Wood, C. R., O'Hara, R. M. Jr, Leary, A. C., Sibley, B., Clark, S. C., Williams, D. A. & Yang, Y. (1990) Molecular cloning of a cDNA encoding interleukin 11, a stromal cell-derived lymphopoietic and hematopoietic cytokine. *Proceedings of the National Academy of Sciences USA* **87**, 7512–7516.

Rodan, G. A. & Martin, T. J. (1981) Role of osteoblasts in hormonal control of bone resorption–a hypothesis. *Calciferous Tissue International* **33**, 349–351.

Runyan, C. E., Schnaper, H. W. & Poncelet, A. C. (2003) Smad3 and PKCdelta mediate TGF-beta1-induced collagen I expression in human mesangial cells. *American Journal of Physiology and Renal Physiology* **285**, F413–F422.

Salvi, G. E. & Lang, N. P. (2005) Host response modulation in the management of periodontal diseases. *Journal of Clinical Periodontology* **32** (Suppl. 6), 108–129.

Seyedin, S. M., Thompson, A. Y., Bentz, H., Rosen, D. M., McPherson, J. M., Conti, A., Siegel, N. R., Galluppi, G. R. & Piez, K. A. (1986) Cartilage-inducing factor-A. Apparent identity to transforming growth factor-beta. *Journal of Biological Chemistry* **261**, 5693–5695.

Simonet, W. S., Lacey, D. L., Dunstan, C. R., Kelley, M., Chang, M. S., Luthy, R., Nguyen, H. Q., Wooden, S., Bennett, L., Boone, T., Shimamoto, G., DeRose, M., Elliott, R., Colombero, A., Tan, H. L., Trail, G., Sullivan, J., Davy, E., Bucay, N., Renshaw-Gegg, L., Hughes, T. M., Hill, D., Pattison, W., Campbell, P., Sander, S., Van, G., Tarpley, J., Derby, P., Lee, R. & Boyle, W. J. (1997) Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* **89**, 309–319.

Somerman, M. J., Archer, S. Y., Imm, G. R. & Foster, R. A. (1988) A comparative study of human periodontal ligament cells and gingival fibroblasts in vitro. *Journal of Dental Research* **67**, 66–70.

Suda, T., Takahashi, N. & Martin, T. J. (1992) Modulation of osteoclast differentiation. *Endocrine Reviews* **13**, 66–80.

Suga, K., Saitoh, M., Fukushima, S., Takahashi, K., Nara, H., Yasuda, S. & Miyata, K. (2001) Interleukin-11 induces osteoblast differentiation and acts synergistically with bone morphogenetic protein-2 in C3H10T1/2 cells. *Journal of Interferon Cytokine Research* **21**, 695–707.

Suga, K., Saitoh, M., Kokubo, S., Fukushima, S., Kaku, S., Yasuda, S. & Miyata, K. (2003) Interleukin-11 acts synergistically with bone morphogenetic protein-2 to accelerate bone formation in a rat ectopic model.

Journal of Interferon Cytokine Research **23**, 203–207.

Suga, K., Saitoh, M., Kokubo, S., Nozaki, K., Fukushima, S., Yasuda, S., Sasamata, M. & Miyata, K. (2004) Synergism between interleukin-11 and bone morphogenetic protein-2 in the healing of segmental bone defects in a rabbit model. *Journal of Interferon Cytokine Research* **24**, 343–349.

Tabata, Y., Hong, L., Miyamoto, S., Miyao, M., Hashimoto, N. & Ikada, Y. (2000) Bone formation at a rabbit skull defect by autologous bone marrow cells combined with gelatin microspheres containing TGF-beta1. *Journal of Biomaterials Science, Polymer Edition* **11**, 891–901.

Takeuchi, Y., Watanabe, S., Ishii, G., Takeda, S., Nakayama, K., Fukumoto, S., Kaneta, Y., Inoue, D., Matsumoto, T., Harigaya, K. & Fujita, T. (2002) Interleukin-11 as a stimulatory factor for bone formation prevents bone loss with advancing age in mice. **277**, 49011–49018.

Trepicchio, W. L., Bozza, M., Pedneault, G. & Dorner, A. J. (1996) Recombinant human IL-11 attenuates the inflammatory response through down-regulation of proinflammatory cytokine release and nitric oxide production. *Journal of Immunology* **157**, 3627–3634.

Wahl, S. M., Costa, G. L., Mizel, D. E., Allen, J. B., Skaleric, U. & Mangan, D. F. (1993) Role of transforming growth factor beta in the pathophysiology of chronic inflammation. *Journal of Periodontology* **64**, 450–455.

Yamashita, H., Ten Dijke, P., Heldin, C. H. & Miyazono, K. (1996) Bone morphogenetic protein receptors. *Bone* **19**, 569–574.

Yasuda, H., Shima, N., Nakagawa, N., Mochizuki, S. I., Yano, K., Fujise, N., Sato, Y., Goto, M., Yamaguchi, K., Kuriyama, M., Kanno, T., Murakami, A., Tsuda, E., Morinaga, T. & Higashio, K. (1998a) Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): a mechanism by which OPG/OCIF inhibits osteoclastogenesis in vitro. *Endocrinology* **139**, 1329–1337.

Yasuda, H., Shima, N., Nakagawa, N., Yamaguchi, K., Kinosaki, M., Mochizuki, S., Tomoyasu, A., Yano, K., Goto, M., Murakami, A., Tsuda, E., Morinaga, T., Higashio, K., Udagawa, N., Takahashi, N. & Suda, T. (1998b) Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proceedings of National Academy of Science USA* **95**, 3597–3602.

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Clinical Relevance

Scientific rationale: Periodontal ligament is involved in bone and cementum formation in periodontal tissue. IL-11 has anti-inflammatory properties and augments bone formation collaborating with BMP-2. TGF-β suppresses the osteoblast-supported osteoclast differentiation. However, little is known about the effects of TGF-β on IL-11 production in periodontal ligament cells, and this needs to be investigated.

Principal findings: IL-11 mRNA expression and IL-11 production were augmented by TGF-β in both PDL and HGF, with higher values in PDL.

Practical implications: TGF-β might stimulate periodontal ligament fibroblasts to ameliorate inflammation and regenerate alveolar bone through the production of IL-11.

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RESEARCH ARTICLE

IL-11 Attenuates Liver Ischemia/Reperfusion Injury (IRI) through STAT3 Signaling Pathway in Mice

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Abstract

Background

The protective role of IL-11, an IL-6 family cytokine, has been implicated in ischemia/reperfusion injury (IRI) in the heart and kidney, but its role has not been elucidated in liver IRI. This study was designed to evaluate the effects of IL-11 and its mechanism of action on liver IRI in a mouse model.

Methods

A partial (70%) warm liver IRI was induced by interrupting the artery/portal vein blood supply to the left/middle liver lobes. IL-11 mRNA expression of ischemic liver after reperfusion was analyzed. Signal transducer and activator of transcription 3 (STAT3) was analyzed following IL-11 treatment in vivo and in vitro. Next, IL-11 was injected intraperitoneally (ip) 1 hour before ischemia. Liver injury was assessed based on serum alanine aminotransferase levels and histopathology. Apoptosis and inflammation were also determined in the ischemic liver. To analyze the role of STAT3 in IL-11 treatment, STAT3 siRNA or non-specific (NS) siRNA was used in vitro and in vivo.

Results

IL-11 mRNA expression was significantly increased after reperfusion in the ischemic liver. STAT3, as a target of IL-11, was activated in hepatocytes after IL-11 treatment in vivo and in vitro. Next, effects of IL-11/STAT3 signaling pathway were assessed in liver IRI, which showed IL-11 treatment significantly attenuated liver IRI, as evidenced by reduced hepatocellular function and hepatocellular necrosis/apoptosis. In addition, IL-11 treatment significantly inhibited the gene expressions of pro-inflammatory cytokines (TNF-α and IL-10) and chemokines (IP-10 and MCP-1). To determine the role of STAT3 in the hepatoprotective

effects of IL-11, STAT3 siRNA or NS siRNA was used prior to IL-11 treatment. The results showed STAT3 knockdown abrogated the protective effects of IL-11 in vitro and in vivo.

Conclusions

This work provides first-time evidence for the protective effect of IL-11 treatment on hepatocyte in liver IRI, through the activation of the STAT3 pathway.

Introduction

Ischemia-reperfusion injury (IRI) is a key contributing factor in liver dysfunction and failure after hepatic trauma, resection, liver transplantation, and circulatory shock [1–4]. An effective method for preventing or minimizing liver IRI is urgently needed in liver surgery. The factors/pathways have been involved in the hepatic IRI process include anaerobic metabolism, mitochondria damage, oxidative stress, endoplasmic reticulum stress, intracellular calcium overload, Kupffer cell (KC) activation, neutrophil infiltrations, and production of cytokines and chemokines [1–3]. The adverse factors mentioned above finally lead to cell death/apoptosis, which indicates that cell death/apoptosis is a significant and perhaps principal contributor to liver IRI [5–7]. Thus, understanding the sequence of events central to the cell death/apoptosis mechanism may potentially lead to treatments for liver IRI.

IL-11 is a hematopoietic IL-6 family cytokine with multifunctional effects. Indeed, IL-11 has thrombopoietic activity, and recombinant human IL-11 has been used for thrombocytopenia in clinical settings [8]. Different from other IL-6 family cytokines, IL-11 holds anti-inflammatory function against chronic inflammatory diseases, lipopolysaccharide-induced sepsis, etc [9–11]. Kimura's group reported that IL-11 played a cardioprotective role, and conferred resistance to heart IRI in a mouse model by enabling significant anti-necrotic/apoptotic effects [12]. In addition, IL-11 pretreatment also reduces IR-induced cell death/apoptosis by up-regulating Bcl-2 [13]. More importantly, IL-11 shares some similar effects to other IL-6 family members. It has been reported that IL-11 binding with gp130 receptor induces activation of STAT3, which is involved in many physiological and pathological processes [14]. Inhibitors of STAT3 phosphorylation or dominant-negative STAT3 mutants facilitate the expression of pro-apoptosis factors, suggesting that STAT3 plays a critical role in regulating cell proliferation and anti-apoptosis [15]. Furthermore, STAT3 knockout mice exhibit complete embryonic lethality [16]. Conditional ablation of STAT3 in myocardial cells leads to higher susceptibility to drug-induced heart failure [17]. To the best of our knowledge, there has been no report on IL-11 preconditioning before liver IRI. In the present study, we tested the hypothesis that exogenous IL-11 attenuates liver IRI by STAT3-mediated anti-necrotic/apoptotic effects.

Materials and Methods

Animals

Male C57BL/6 mice were purchased from the Laboratory Animal Resources Center of Nanjing Medical University (NMU). The animals were fed a laboratory diet with water and food and kept under constant environmental conditions with 12h light–dark cycles. Procedures were carried out in accordance with the Guidelines for Laboratory Animal Care. The animal protocol had been approved by the Institutional Animal Care & Use Committee (IACUC) of Nanjing Medical University (Protocol Number NMU08-092).

Surgical procedure and IL-11 treatment

The present study used a well-established mouse model of partial (70%) warm hepatic IRI [18]. Anesthesia was induced by 10% chloral hydrate (0.3g/kg, intraperitoneally). Mice were injected with heparin (100U/kg), and an atraumatic clip was used to interrupt the artery/portal vein blood supply to the left/middle liver lobes. After 90 minutes of ischemia, the clip was removed, and the mice were sacrificed (cervical dislocation) at required times after reperfusion. Some mice received a single injection of recombinant human IL-IL-11 (500μg/kg, ip) (PeproTech, Rocky Hill, NJ) or medium (PBS) 1 hour prior to ischemia. PBS injection was used as a control. Sham-operated controls underwent the same procedure but without vascular occlusion. To access effects of STAT3 on IL-11 treatment, STAT3 siRNA or NS siRNA (2mg/kg) was given intravenously 4 hours prior to ischemia [19]. Reports have previously documented the efficacy of this siRNA approach in the liver, with>40% of intravenously infused siRNA accumulating in the ischemic mouse livers [20].

Serum biochemical examination

Blood samples collected 6h after reperfusion was centrifuged to obtain serum. The serum level of alanine aminotransferase (sALT) or supernatant level of lactate dehydrogenase (LDH) was measured to assess the extent of hepatocyte damage using an automated chemical analyzer (Olympus Automated Chemistry Analyzer AU5400, Tokyo, Japan).

Histopathologic study

Liver specimens were fixed with 10% neutral formaldehyde and then embedded in paraffin. The specimens were sectioned at 4μm and stained with hematoxylin and eosin. The sections were used in histopathologic analysis by light microscopy. Sections were scored from 0 to 4 for sinusoidal congestion, vacuolization of hepatocyte cytoplasm, and parenchymal, as described by Suzuki et al [21].

Caspase-3 activity assay

Caspase-3 activity was assayed in liver tissues 6h after reperfusion. Frozen samples of ischemic tissues were homogenized with a Polytron homogenizer and centrifuged at 16,000g for 20 minutes. Activity was measured with an assay kit (Calbiochem) according to the manufacturer's instructions.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

Paraffin sections (4μm in thickness) were deparaffinized in toluene and then dehydrated in a graded series of ethanol solutions. Sections were stained by TUNEL using a commercially available kit (in situ cell death detection kit, Roche-Boehringer Mannheim, Germany).

Western blot analysis

Proteins were extracted from liver tissues subjected to ischemia or from cell lysates, and their concentrations were determined by the Bradford assay (Bio-Rad, CA). About 30 μg protein was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Sunshine Biotechnology, China). These membranes were blocked in skim milk powder (5% wt/vol) with phosphate buffered saline containing 0.1% Tween 20 (PBS-T) at 4°C overnight. Membranes were then incubated with primary antibodies for cleaved Caspase-3, P-STAT3, Bcl-2, Bax, β-actin (Cell Signaling Technology, Danvers, MA), and

STAT3 (Santa Cruz Biotechnology, Santa Cruz, CA). Following three washes with PBS-T, the membranes were incubated for 1h at room temperature with peroxidase-conjugated secondary antibody (Cell Signaling Technology, Danvers, MA). The final results were obtained by exposure to autoradiographic film (Kodak XAR film), and then visualized via a chemiluminescent detection system (ECL Substrate Western blot detection system, Pierce, IL).

Quantitative real-time PCR

Quantitative real-time PCR was performed using the DNA Engine with a Chromo 4 Detector (MJ Research, Waltham, MA). In a final reaction volume of 25μl, the following were added: 1×SuperMix (Platinum SYBR Green qPCR Kit; Invitrogen), cDNA, and 2.5μM of each primer. The amplification conditions were as follows: 50°C (2 min), 95°C (5 min), followed by 50 cycles at 95°C (15 sec) and 60°C (30 sec). The expression of the target genes (IL11, TNF-α, IL-6, IP-10 and MCP-1) (Invitrogen, Shanghai, China) was calculated based on the ratio of the gene of interest to the housekeeping gene HPRT. Primer sets (sense sequence and antisense sequence, respectively) for the following genes were: HPRT forward, 5'- TCA ACG GGG GAC ATA AAA GT-3', reverse, 5'- TGC ATT GTT TTA CCA GTG TCA A'; IL-11 forward: 5'- CTG CCC ACC TTG GCC ATG AG-3'; IL-11 reverse: 5'- CCA GGC GAG ACA TCA AGA AAG A-3'; TNF-α forward, 5'- GCC TCT TCT CAT TCC TGC TTG T-3', reverse, 5'- TTG AGA TCC ATG CCG TTG-3'; IL-6 forward, 5'- GCT ACC AAA CTG GAT ATA ATC AGG A-3', reverse, 5'- CCA GGT AGC TAT GGT ACT CCA GAA-3'; IP-10 forward. 5'-GCT GCC GTC ATT TTC TGC-3', reverse, 5'-TCT CAC TGG CCC GTC ATC-3'; MCP-1 forward, GGT GAT AAC CGC CCT AGC-3', reverse, 5'-TGT GTC GGC TGG ATA GGC-3'.

Cell culture and treatment

Mouse hepatocytes were isolated using a two-step in situ collagenase perfusion procedure [18]. Livers from the C57BL/6 mice were perfused in situ through the portal vein with ethylene glycol tetraacetic acid (EGTA) buffer (0.5mM EGTA, 137mM NaCl, 4.7mM KCl, 1.2mM KH2PO4, 0.65mM MgSO4, and 10.07mM HEPES at pH 7.4) at a flow rate of 5ml/min for 10 min, followed by collagenase buffer (67mM NaCl, 6.7mM KCl, 4.76mM CaCl2, 0.035% collagenase type II, and 10.07mM HEPES at pH 7.6) at a flow rate of 5ml/min for 15 min. After centrifugation, the hepatocytes were collected and seeded in DMEM containing 10% FBS, 100units/ml penicillin, and 100μg/ml streptomycin. Cells were preincubated with IL-11 (1μg/ml for 1h), then H_2O_2 (200μm for 24h) to induce cell death.

Knockdown of STAT3 expression using STAT3 siRNA transfection

Hepatocytes were grown and transiently transfected with STAT3 siRNA or NS siRNA using Transfection Reagent LipofectamineTM RNAiMAX (Invitrogen, CA, USA) according to the manufacturer's instructions. In brief, cells were seeded at 1 x 10^6 per well in 1.5ml of OPTI-medium (Invitrogen, CA, USA) in a 6-well plate. After 20h, the cells were transfected with 20nmol/ml STAT3 siRNA or NS siRNA. About 6h after transfection, the medium was changed to a regular medium, and the cells were treated as described above after 24h.

Statistical analysis

The data are presented as the mean ± SEM from at least three independent experiments. One-way analysis of variance test [—] was used in comparisons of three groups. Student's t-test [Π] was used for comparison of two groups. All P values were two-sided, and $P<0.05$ was considered to be statistically significant.

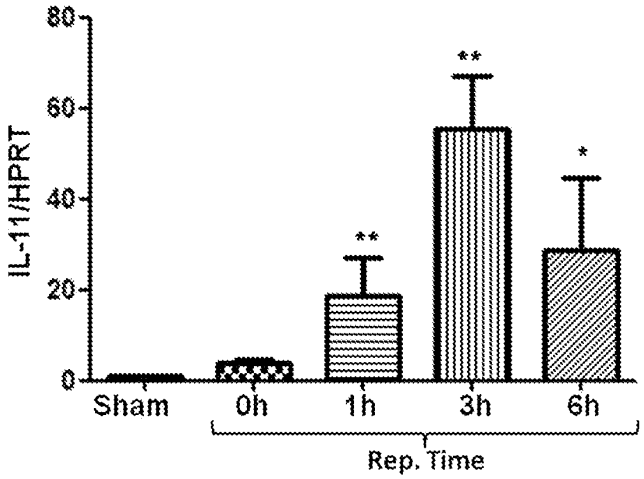

Fig 1. IL-11 expression was increased after IR. Mice were subjected to 90min of partial liver ischemia, followed by 0h, 1h, 3h and 6h reperfusion. Kinetics of IL-11 gene expression was analyzed in ischemic liver by RT-PCR. Expression of IL-11 was normalized with that of HPRT. Data are expressed as mean±SD (n = 6/group). *P<0.05, **P<0.001 vs sham group.

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Results

IL-11 is elevated in IR-stressed liver

To determine the effects of IL-11 on liver IRI, we first analyzed the gene expression of IL-11 in ischemic livers after various reperfusion time points. As shown in Fig 1, the expression of IL-11 was increased at 0h post reperfusion and reached its peak 3h post reperfusion. The data indicate that IL-11 was present in the liver 3h after reperfusion.

IL-11 activates STAT3 in liver and hepatocytes

IL-11, an IL-6 family cytokine, was supposed to activate STAT3. Here we determined whether administration of IL-11 stimulates STAT3 in liver by western blotting with antibody (Fig 2A). Phosphorylation of STAT3 was rapidly induced and recovered nearly to baseline after 2 hours. To further ascertain that phosphorylation of STAT3 occurred in hepatocytes, we analyzed the effects of IL-11 on STAT3 activity of hepatocytes in vitro. Fig 2B shows phosphorylation of STAT3 was also significantly elevated after IL-11 treatment, indicating that IL-11 administration may activate STAT3 within parenchymal cells in the liver.

IL-11 attenuates liver IRI

Next, we analyzed effects of IL-11 administration in liver IRI. Mouse livers were subjected to 90 min of warm ischemia 6h after reperfusion. sALT levels in each group were analyzed (Fig 3A). sALT levels were markedly increased in the IR group compared with that of the sham group (33.33±5.49 and 10610.00±1393.00, respectively; $P<0.01$). By contrast, when mice were pretreated with IL-11, sALT levels (3832.00±834.90, $P<0.01$) were significantly decreased compared with those in the IR control. Liver serum enzyme data were in line with liver pathological analysis (Fig 3B and 3C). The histological parameters in the sham (0.25±0.25), IR (3.20±0.37), and IL-11 administration (1.8±0.37) groups were observed according to Suzuki et al [26]. These data indicate that IL-11 treatment significantly attenuates IR-induced liver injury.

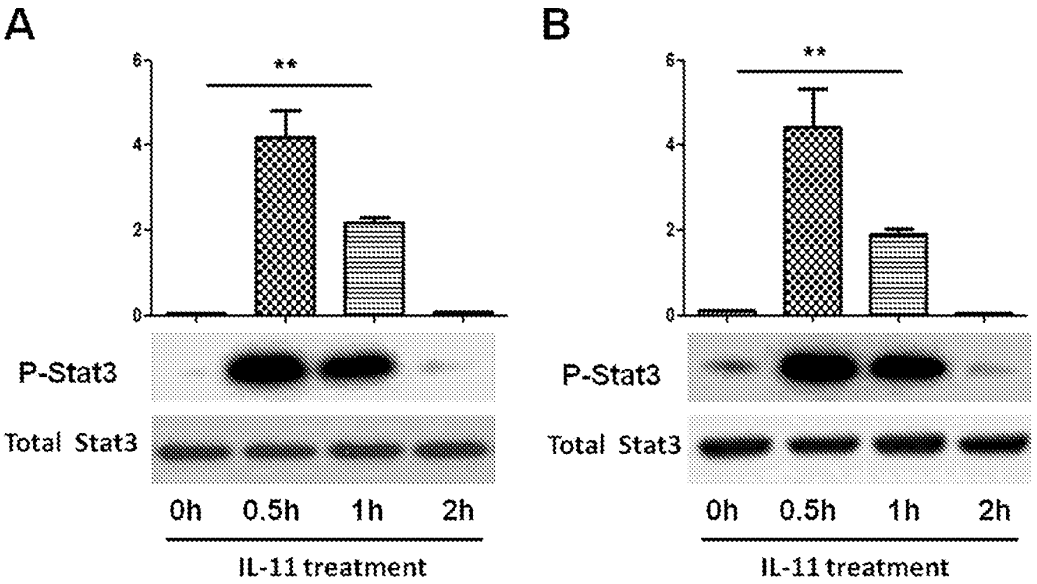

Fig 2. IL-11 activated STAT3 in hepatocytes in vivo and in vitro. (A) IL-11 was administered in mice for the indicated time. The lysates from liver were immunoblotted with anti-p-STAT3 and anti-STAT3 antibody. Representative data are shown (lower). Quantative analyses of p-STAT3 are shown (upper). Data are exprresed as mean±SD (n = 6/group). **P<0.001. (B) Hepatocytes were treated by IL-11 for the indicated time. The lysates from hepatocytes were immunoblotted with anti-p-STAT3 and anti-STAT3 antibody. Representative data are shown (lower). Quantative analyses of p-STAT3 are shown (upper). Data are expressed as mean±SD (n = 6/group), **P<0.001.

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IL-11 exhibits anti-apoptotic functions and reduces apoptosis

Apoptosis/necrosis is a central mechanism of cell death in liver IRI. In this study, hepatocellular apoptosis was analyzed in ischemic livers by TUNEL staining 6 hours after reperfusion. Our results showed TUNEL-positive cells were significantly lower in liver sections of the IL-11 treatment group compared with those in IR control group (Fig 4A and 4B). TUNEL-positive cells in the total hepatocytes of the three groups were (0.60±0.25)%, (10.20±1.28)%, and (4.00±0.63)%, respectively, indicating that hepatocellular apoptosis was significantly reduced by IL-11 administration. In addition, anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax were analyzed in ischemic liver, which showed that IL-11 treatment effectively upregulated Bcl-2 and inhibited Bax expression (Fig 4C). Caspase-cascade system, especially the component Caspase-3, plays a critical role during liver IRI. Caspase-3 affects apoptosis by component cleaved Caspase-3. Next, we assessed the expression of cleaved Caspase-3 by immune analysis, suggesting that IL-11 administration effectively inhibited expression of cleaved Caspase-3 during liver IRI (Fig 4C). Apoptotic active caspase-3 directly caused hepatocellular apoptosis and reflected the progress of apoptosis in the ischemic liver. Along with the TUNEL assay and expression of cleaved Caspases-3, Fig 4D shows that the activity of caspase-3 was significantly repressed after IL-11 preconditioning in ischemic liver tissue compared with the IR group (1.38 ±0.08 and 4.44±0.289, respectively; $P<0.001$).

IL-11 regulates inflammatory program and MPO activity in IR-stressed liver

A complex inflammatory program involving cytokines and chemokines is engaged in liver IRI. Inflammatory cytokines (TNF-α and IL-6) and chemokines (IP-10 and MCP-1) displayed

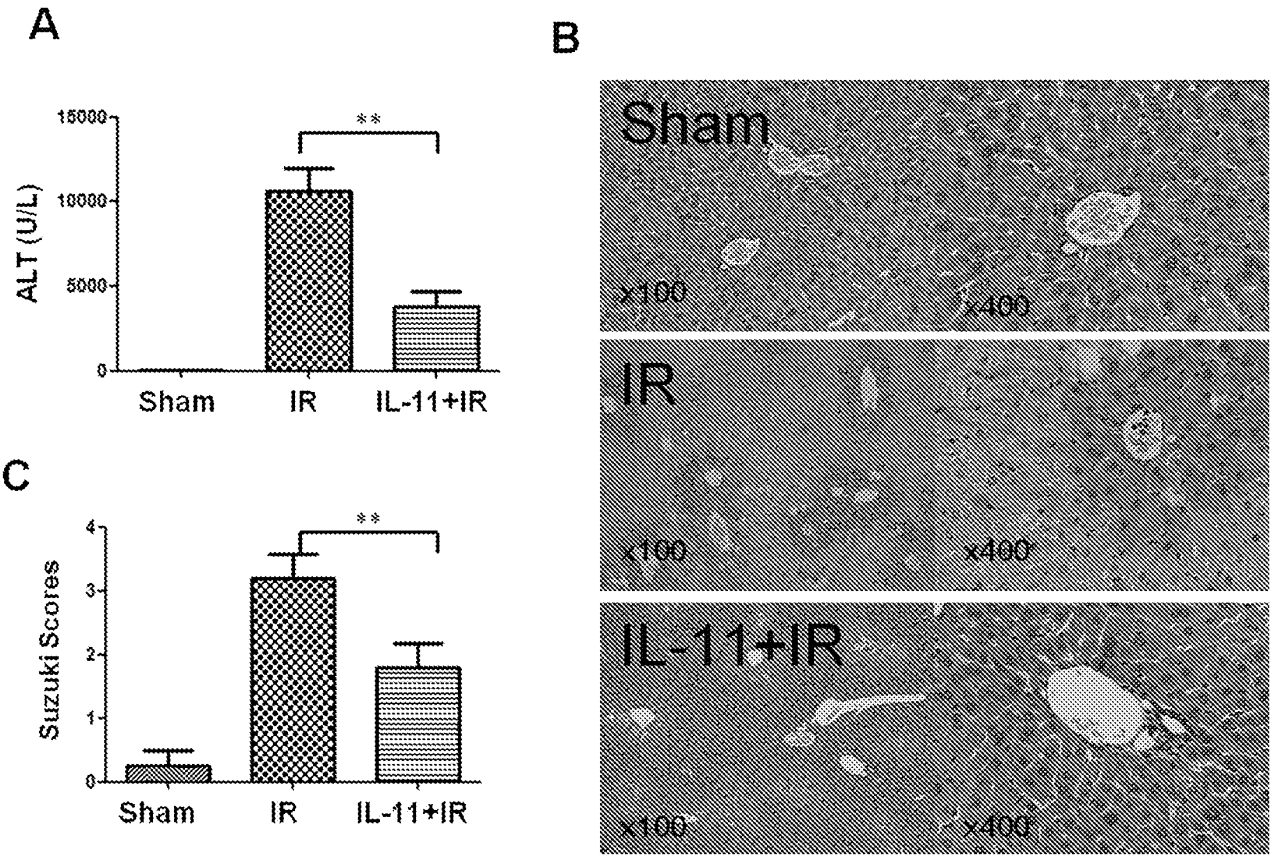

Fig 3. IL-11 treatment attenuated liver injury induced by IR. Mice were administered with recombinant human IL-IL-11 (500μg/kg, ip) or medium (PBS) 1 hour prior to ischemia, followed by 6h reperfusion. (A) sALT. (B) Histopathalogic analysis of livers harvested 6 hours after reperfusion. Sham group: Normal hepatic architecture; IR group: severe hepatic lobule distortion, sinusoidal congestion, apparent edema, vacuolization and massive necrosis; IL-11+IR group: mild vacuolization, punctate necrosis and edeman. (C) The severity of liver IRI by Suzuki's histological grading. Data are expressed as mean±SD (n = 6/group), **P<0.001.

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proinflammatory and proapoptotic roles in ischemic liver post-reperfusion. To further assess the hepatoprotective effects of IL-11 treatment, mRNA expressions of TNF-α, IL-6, IP-10 and MCP-1 were determined in ischemic liver after 6h of reperfusion by qRT-PCR. Fig 5A shows a significantly lower level of TNF-α (1.13.±0.13 and 0.31±0.04, respectively; $P<0.001$), IL-6 (6.73±1.12 and 2.43±0.33, respectively; $P<0.001$), IP-10 (9.69±1.63 and 2.93±0.39, respectively; $P<0.001$) and MCP-1 (4.08±0.70 and 1.22±0.27, respectively; $P<0.001$) in the IL-11 administration group compared with that in the IR group. These data indicated that IL-11 effectively inhibited the expression of inflammatory cytokines in ischemic liver after reperfusion. In addition, serum TNF-α was further analyzed by ELISA, which showed IL-11 treatment significantly decreased TNF-α secretion after liver IRI (Fig 5B). The protein level was consistent with gene expression. The MPO activity, reflecting neutrophil activity and infiltration, was reduced after reperfusion in IL-11 treated liver compared with controls (5.40±0.81U/g and 2.40±0.25U/g, respectively; $P<0.001$) (Fig 5C).

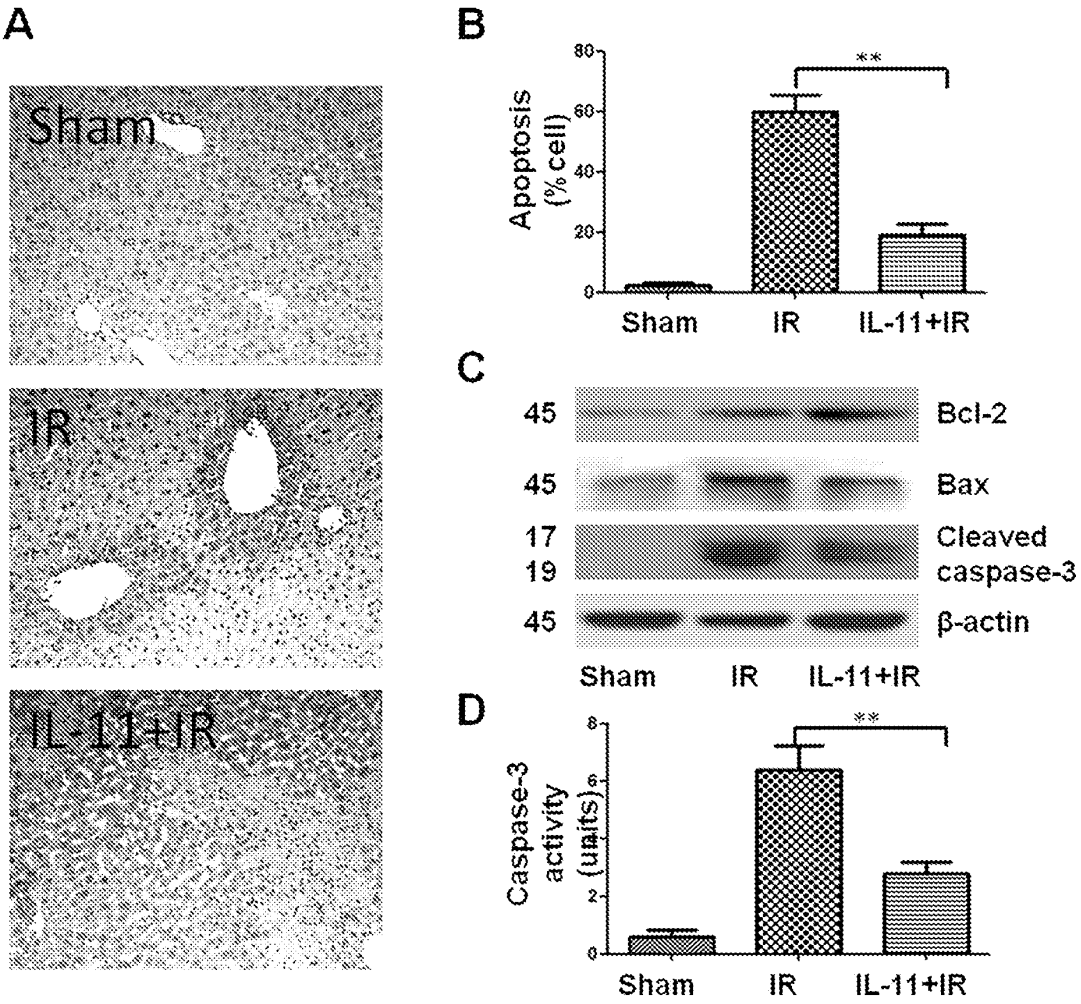

Fig 4. IL-11 treatment decreased hepatocellular apoptosis induced by IR. (A)Liver apoptosis was examined by TUNEL staining: Sham group, IR group and IL-11+IR group. (B) Apoptotic cells were quantified in six high-power fields (400x), and expressed as percentages of apoptotic cells among total cells. (C) Western blot-assisted detection of Bcl-2, Bax, Caspase-3 and β-actin. (D) Caspase-3 activity. Data are expressed as mean±SD (n = 6/group), **P<0.001.

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Activation of STAT3 is essential for IL-11-mediated protective role

To evaluate the effect of STAT3 activation in an IL-11-mediated protective role, primary hepatocytes were transiently transfected with STAT3 siRNA or NS siRNA. Next, STAT3 gene expression was assessed after IL-11 treatment by qRT-PCR, which showed STAT3 gene expression was significantly repressed compared with the NS siRNA control (Fig 6A). These data indicated that STAT3 expression was successfully knocked down in hepatocytes. Then, these transfected cells were treated with H_2O_2 to induce cell death. The released LDH level was checked in the supernatant after H_2O_2 treatment for 24h. Fig 6B shows that IL-11 treatment remarkably inhibited LDH release of hepatocytes after H_2O_2 treatment (14.00±4.32 and 14.80±1.99, respectively; $P<0.001$). However, STAT3 knockdown almost restored the decreased LDH release after IL-11 treatment (14.80±1.99 and 45.80±7.14, respectively; $P<0.001$). These data indicated STAT3 activation is necessary for IL-11-mediated protective role.

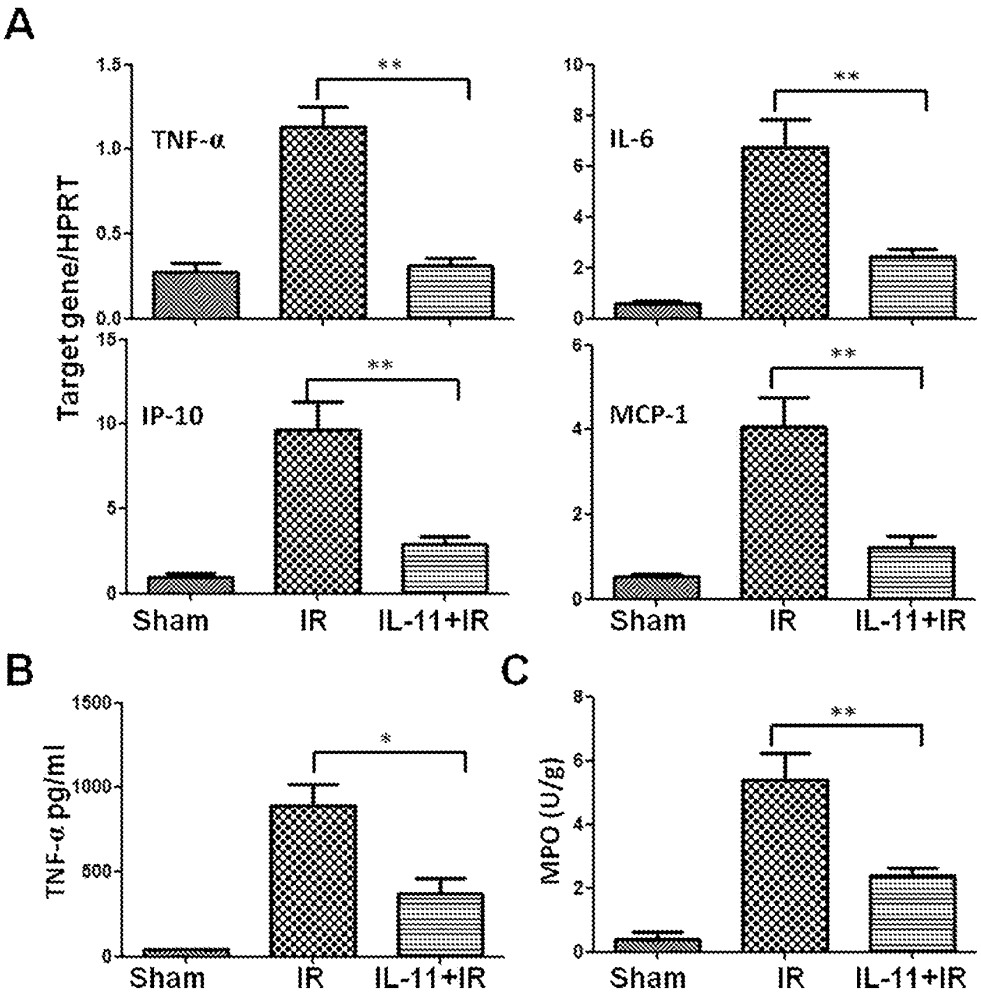

Fig 5. IL-11 treatment inhibited pro-inflammatory responeses induced by IR. (A) Cytokine gene (TNF-a, IL-6, IP-10 and MCP-1) expressions were analyzed in ischemic livers by RT-PCR analysis. Expressions of cytokine gene were normalized with that of HPRT. (B) TNF-α secretion was examined in serum by ELISA. (C) MPO activity in ischemic liver. Data are expressed as mean±SD (n = 6/group), *P<0.05, **P<0.001.

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Knockdown of STAT3 restores liver IRI in IL-11-treated mice

To further assess the effect of STAT3 activation in IL-11-mediated hepoto-protective role during liver IRI, mice were injected intravenously with STAT3 siRNA or NS siRNA prior to ischemia. As shown in Fig 7A, IL-11 treatment significantly increased expression of P-STAT3, which was reversed in STAT3 siRNA group. Fig 7B shows STAT3 siRNA treatment effectively neutralized the reduced sALT levels after IL-11treatment ($P<0.001$) (3795±879U/L versus 11460±1941U/L, respectively). These data correlated with Suzuki's histological grading of liver IRI (Fig 7C and 7D). Indeed, IL-11 resulted in minimal liver sinusoidal congestion, vacuolization without edema, or necrosis [Fig 7C (c); score = 1.80±0.37]. In contrast, livers in untreated or STAT3 siRNA-treated mice displayed moderate to severe edema and extensive hepatocellular necrosis [Fig 7C (b and d), Fig 7D (panels b and d); score = 3.60±0.25 and 3.40±0.40, respectively]. In addition, cleaved Caspase-3 was further analyzed in ischemic liver, which

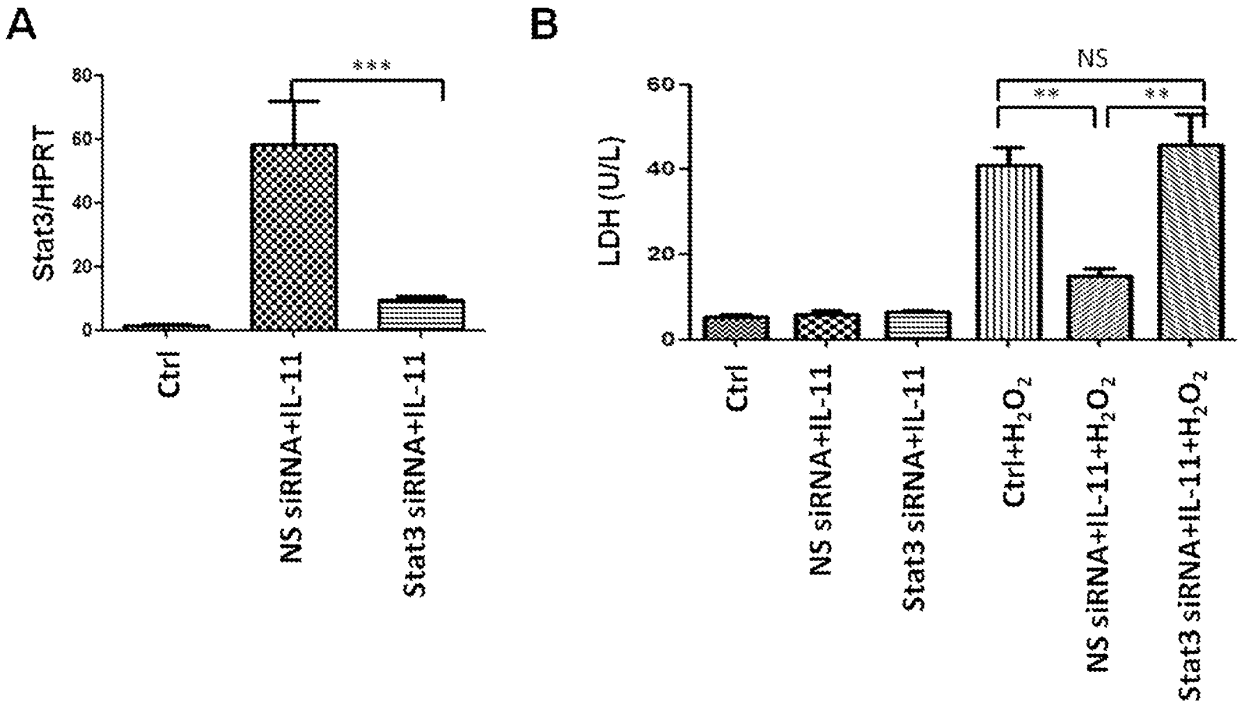

Fig 6. STAT3 knockdown abolished IL-11 protective effects in vitro. (A) STAT3 gene expression was analyzed in transfected hepatocytes by RT-PCR analysis. Expressions of gene were normalized with that of HPRT. (B) The released LDH level of hepatocytes after H_2O_2 treatment. Data are expressed as mean±SD (n = 4-6/group), **P<0.001, ***P<0.0001.

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showed IL-11 treatment partially inhibited IR-triggered cleaved Caspase-3 upregulataion (Fig 7A). These data indicated knockdown of STAT3 recreates liver IRI in IL-11-treated mice.

Discussion

IL-11 has displayed protective roles during ischemia-reperfusion injury (IRI) in the heart, kidney and intestine, but a similar role has not been elucidated in liver IRI [12, 13, 22, 23]. In this study, we have for the first time demonstrated that IL-11 pretreatment is a promising method for preventing or minimizing liver IRI. Indeed, IL-11 treatment improved liver function, attenuated histology damage, impaired proinflammatory cytokine/chemokine programs, and reduced hepatocellular death/apoptosis in IR-stressed livers. However, the IL-11 mediated protective role was partially impaired by STAT3 siRNA in vivo and in vitro. These findings demonstrate that IL-11 treatment attenuates liver IRI through activating the STAT3 signaling pathway.

IL-11 is a hematopoietic IL-6 family cytokine first identified from marrow-derived stromal cells. It is a key regulator of hematopoiesis and promotes megakaryocyte maturation [24]. IL-11 as well as its receptors is expressed in many tissues and cell types, including macrophages, hepatocytes, etc. in liver tissues [25]. Du's group for the first time reported the protective effects of IL-11 in models of cytoablative chemoradiotherapy in 1994. Then, some researchers have demonstrated that IL-11 play protective roles in various pathophysiologic states [25]. Different from other IL-6 family members, IL-11 administration reduces inflammatory responses in chronic inflammatory diseases, lipopolysaccharide-induced sepsis, macrophages inflammation,

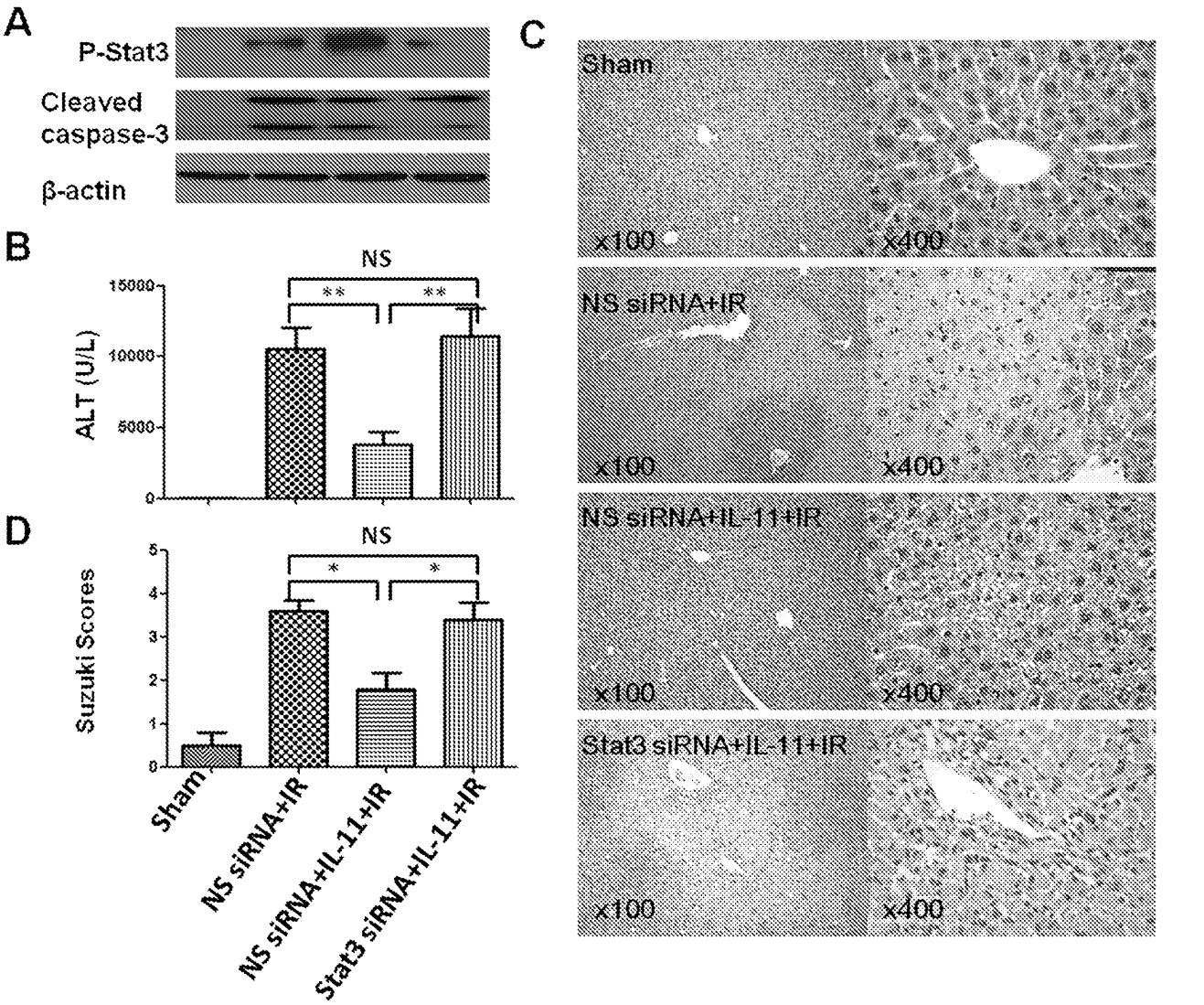

Fig 7. STAT3 knockdown abolished IL-11 protective effects in vivo. Mice were injected with STAT3 siRNA or NS siRNA 4h prior to ischemia, and administered with IL-11 1h prior to ischemia, followed by 6h reperfusion. (A) Western blot-assisted detection of P- STAT3, Cleaved Caspase-3 and β-actin. (B) sALT. (C) Histopathalogic analysis of livers harvested 6h after reperfusion. Sham group: Normal hepatic architecture; NS siRNA+IR group: severe hepatic lobule distortion, sinusoidal congestion, apparent edema, vacuolization and massive necrosis; NS siRNA+IL-11+IR group: mild vacuolization, punctate necrosis and edeman; STAT3 siRNA+IL-11+IR group: severe hepatic lobule distortion, sinusoidal congestion, apparent edema, vacuolization and massive necrosis. (D) The severity of liver IRI by Suzuki's histological grading. Data are expressed as mean±SD (n = 6/group), *P<0.05.

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nephrotoxic nephritis and T-cell mediated liver injury [9–11]. The present study also demonstrated that IL-11 treatment effectively inhibited inflammatory responses of ischemic liver, as evidenced by reducing pro-inflammatory cytokines (TNF-α and IL-6) and chemokines (IP-10 and MCP-1), and repressing MPO activtity. In addition to its anti-inflammatory properties, IL-11 treatment has been shown to attenuate necrotic and apoptotic cell death in many organs including the heart, intestines and endothelial cells [25]. In fact, apoptosis/necrosis is a key mechanism of cell death in liver IRI, which directly indicates the extent of liver damage. In our

study, hepatocellular death/necrosis as well as apoptosis was also observed in ischemic liver. Our data showed that IL-11 attenuated hepatocellular death/necrosis (ALT, Suzuki's score) as well as apoptosis (TUNEL staining, cleaved-Caspase 3 fragmentation) in ischemic liver after IRI. In addition, we also analyzed effects of IL-11 on hepatocellular death/necrosis by LDH release level in vitro, which was consistent with in vivo results. Thus, we conclude that IL-11 administration provides liver protection against IRI by reducing inflammation and necrosis/apoptosis.

The molecular mechanisms of IL-11 mediated signaling pathway for an anti-inflammatory and anti-necrosis/apoptosis might involve the activation of multiple intercellular pathways. After IL-11 binds to the IL-11 receptor, the ligand-receptor complex interacts with a common receptor subunit, glycoprotein 130 (gp130), leading to gp130-associated kinase-mediated tyrosine phosphorylation [26]. In vascular endothelial and intestinal epithelial cells, IL-11 protects against oxidant induced necrosis and apoptosis via mechanisms involving ERK, MAPK, AKT and/or induction of HSP25 [22, 27, 28]. In renal IRI, IL-11 performs renal protection by direct induction of sphingosine kinase-1 (SK-1) via nuclear translacation of HIF-1α. In cardiac myocytes, IL-11 treatment attenuates injury and fibrosis via Janus Kinase-Signal Transducer and Activation of Transducer 3 (JAK-STAT3) pathway activation [12, 23, 29]. Kawakami T et al have demonstrated rhIL-11 confers significant protection against CCl4-induced hepatic injury by virtue of its liver-specific HO-1 induction [30]. In addition, a previous studydemonstrated STAT3 activation after Ad-HO-1 treatment improved the hepatocellular function in a mouse model of segmental liver warm IRI [31]. In the present study, IL-11 treatment rapidly activated STAT3 in hepatocytes in vivo and in vitro, and reduced liver injury after reperfusion. However, IL-11 administration shows weak hepatocellularprotective effects in STAT3siRNA transfected hepatocytes or STAT3siRNA transfected mice. Therefore, hepatocyte is an important target in the action of IL-11, and IL-11-mediated protection of liver IRI is partially dependent on STAT3 activation of hepatocytes. Whether HO-1 is involved in this protective procedure will be further investigated in the future.

Evidence exists that the STAT3 signaling pathway transduces stress-activating extracellular chemical signals into cellular responses for a number of pathophysiological processes, such as immunity, inflammation and apoptosis, and is involved in liver IRI. The function of activated STAT3 is controversial; some studies have associated it with survival [31–33], while others have related it to cell death [34]. Previous studies have confirmed that STAT3 alterations affect Bcl-2 and Bax protein expression and induce inflammation and apoptosis in many types of tumor cells [35–37]. In mycosis fungoides tumor cells, some apoptosis-related genes, such as Bcl-2 and Bax, have been identified as STAT3 target genes [38]. In our study, STAT3 activation reduced hepatocellular necrosis/apoptosis and liver injury induced by IR after IL-11 treatment, but STAT3 knockdown restored the hepatocellular necrosis/apoptosis and liver injury in IL-11-treated mice. These data indicate that IL-11 treatment reduces hepatocellular necrosis/apoptosis by STAT3 activation. Lee et al demonstrated that co-activated NF-κB and STAT3 modulate Bax/Bcl-xL expression and promote cell survival in head and neck squamous cell carcinoma [38]. In primary cortical neurons and murine models of stroke, the activation of STAT3 pathway by secretoneurin has been found to exert neuroprotective effects and induce neuronal plasticity after hypoxia and ischemic insult [39]. Using a mouse model of myocardial infarction, Obana M et al demonstrated that IL-11 exerted protective effects against myocardial ischemic injury through IL-11R-mediated STAT3 activation, antiapoptotic signaling and proangiogenic activity [23]. In addition, STAT3 has been demonstrated to have an anti-inflammatory function in many pathophysiological processes [23]. Inflammatory response plays a pathogenic role in liver I/R injury, especially innate immune responses involved in cytokines and chemokines, including TNF-α, IL-6, IP-10, MCP-1 and so on [40–43]. Ke B et al

demonstrated that STAT3 activation repressed TLR4-drived inflammation by activating PI3K/Akt signaling during liver IRI [23]. Consistent with the above data, our results also showed that IL-11-induced STAT3 signaling inhibited pro-inflammatory cytokines and chemokines.

In conclusion, our findings demonstrate for the first time IL-11-mediated STAT3 attenuates IR-triggered liver injury. IL-11-mediated STAT3 signaling not only reduces hepatocellular apoptosis, but also inhibits inflammatory responses. Thus, our study provides a rationale for novel therapeutic approaches to the management of liver injury triggered by IR.

Author Contributions

Conceived and designed the experiments: MZ BL QC FL. Performed the experiments: MZ BL QC ZW ZX WL. Analyzed the data: MZ BL XY. Contributed reagents/materials/analysis tools: MZ BL QC XY. Wrote the paper: MZ BL QC FL. Primary responsibility for final content: FL.

References

1. Jaeschke H. Molecular mechanisms of hepatic ischemia-reperfusion injury and preconditioning. Am J Physiol Gastrointest Liver Physiol. 2002; 284: G15–G26. PMID: 12488232
2. Lentsch AB, Kato A, Yoshidome H, McMasters KM, Edwards MJ. Inflammatory mechanisms and therapeutic strategies for warm hepatic ischemia/reperfusion injury. Hepatology. 2000; 32: 69–173. PMID: 10975759
3. Serracino-Inglott F, Habib NA, Mathie RT. Hepatic ischemia-reperfusion injury. Am J Surg. 2001; 181: 160–166. PMID: 11425059
4. Que X, Debonera F, Xie J, Furth EE, Aldeguer X, Gelman AE, et al. Pattern of ischemia reperfusion injury in a mouse orthotopic liver transplant model. J Surg Res. 2004; 116: 262–268. PMID: 15013365
5. Shah KA, Shurey S, Green CJ. Characterization of apoptosis in intestinal ischaemia–reperfusion injury—A light and electron microscopic study. Int J Exp Pathol. 1997; 78: 355–63. PMID: 9462233
6. Noda T, Iwakiri R, Fujimoto K, Matsuo S, Aw TY. Programmed cell death induced by ischemia–reperfusion in rat intestinal mucosa. Am J Physiol. 1998; 274: G270–G276. PMID: 9486179
7. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. Science. 1995; 267: 1456–1462. PMID: 7878464
8. Gordon MS, McCaskill-Stevens WJ, Battiato LA, Loewy J, Loesch D, Breeden E, et al. A phase I trial of recombinant human interleukin-11 (neumega rhIL-11 growth factor) in women with breast cancer receiving chemotherapy. Blood. 1995; 87: 3615–3624.
9. Sands BE, Winston BD, Salzberg B, Safdi M, Barish C, Wruble L, et al. Randomized, controlled trial of recombinant human interleukin-11 in patients with active Crohn's disease. Aliment Pharmacol Ther. 2002; 16:399–406. PMID: 11876692
10. Trepicchio WL, Bozza M, Pedneault G, Dorner AJ. Recombinant human IL-11 attenuates the inflammatory response through down-regulation of proinflammatory cytokine release and nitric oxide production. J Immunol. 1996; 157: 3627–3634. PMID: 8871663
11. Trepicchio WL, Dorner AJ. The therapeutic utility of Interleukin-11 in the treatment of inflammatory disease. Expert Opin Investig Drugs. 1998; 7: 1501–1504. PMID: 15992047
12. Kimura R, Maeda M, Arita A, Oshima Y, Obana M, Ito T, et al. Identification of cardiac myocytes as the target of interleukin 11, a cardioprotective cytokine. Cytokine. 2007; 38: 107–115. PMID: 17629706
13. Du X, Liu Q, Yang Z, Orazi A, Rescorla FJ, Grosfeld JL, et al. Protective effects of interleukin-11 in a murine model of ischemic bowel necrosis. Am J Physiol. 1997; 272: G545–G552. PMID: 9124575
14. Yin T, Yasukawa K, Taga T, Kishimoto T, Yang YC. Identification of a 130-kilodalton tyrosine-phosphorylated protein induced by interleukin-11 as JAK2 tyrosine kinase, which associates with gp130 signal transducer. Exp Hematol. 1994; 22: 467–472. PMID: 8174677
15. Timofeeva OA, Tarasova NI, Zhang X, Chasovskikh S, Cheema AK, Wang H, et al. STAT3 suppresses transcription of proapoptotic genes in cancer cells with the involvement of its N-terminal domain. Proc Natl Acad Sci USA. 2013; 110: 1267–1272. doi: 10.1073/pnas.1211805110 PMID: 23288901
16. Schindler CW. Series introduction. JAK-STAT signaling in human disease. J Clin Invest. 2002; 109:1133–137. PMID: 11994400
17. Jacoby JJ, Kalinowski A, Liu MG, Zhang SS, Gao Q, Chai GX, et al. Cardiomyocyte-restricted knockout of STAT3 results in higher sensitivity to inflammation, cardiac fibrosis, and heart failure with advanced age. Proc Natl Acad Sci USA. 2003; 100: 12929–12934. PMID: 14566054

18. Rao J, Qin J, Qian X, Lu L, Wang P, Wu Z, et al. Lipopolysaccharide preconditioning protects hepatocytes from ischemia/reperfusion injury (IRI) through inhibiting ATF4-CHOP pathway in mice. PLoS One. 2013; 8: e65568. doi: 10.1371/journal.pone.0065568 PMID: 23750267

19. Rao J, Yue S, Fu Y, Zhu J, Wang X, Busuttil RW, et al. ATF6 mediates a pro-inflammatory synergy between ER stress and TLR activation in the pathogenesis of liver ischemia-reperfusion injury. Am J Transplant. 2014; 14: 1552–1561. doi: 10.1111/ajt.12711 PMID: 24909306

20. Soutschek J, Akinc A, Bramlage B, Charisse K, Constien R, Donoghue M, et al. Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. Nature. 2004; 432: 173–178. PMID: 15538359

21. Suzuki S, Nakamura S, Koizumi T, Sakaguchi S, Baba S, Muro H, et al. The beneficial effect of a prostaglandin I2 analog on ischemic rat liver. Transplantation. 1991: 52: 979–983. PMID: 1750084

22. Ropeleski MJ, Tang J, Walsh-Reitz MM, Musch MW, Chang EB. Interleukin-11-induced heat shock protein 25 confers intestinal epithelial-specific cytoprotection from oxidant stress. Gastroenterology. 2003; 124: 1358–1368. PMID: 12730876

23. Obana M, Maeda M, Takeda K, Hayama A, Mohri T, Yamashita T, et al. Therapeutic activation of signal transducer and activator of transcription 3 by interleukin-11 ameliorates cardiac fibrosis after myocardial infarction. Circulation. 2010; 121: 684–691. doi: 10.1161/CIRCULATIONAHA.109.883677 PMID: 20100971

24. Reynolds CH. Clinical efficacy of rhIL-11. Oncology (Williston Park). 2000; 14: 32–40.

25. Du X, Williams DA. Interleukin-11: review of molecular, cell biology, and clinical use. Blood. 1997; 189: 3897–3908.

26. Taga T, Kishimoto T. Gp130 and the interleukin-6 family of cytokines. Annu Rev Immunol. 1997; 15: 797–819. PMID: 9143707

27. Naugler KM, Baer KA, Ropeleski MJ. Interleukin-11 antagonizes Fas ligand-mediated apoptosis in IEC-18 intestinal epithelial crypt cells: role of MEK and Akt-dependent signaling. Am J Physiol Gastrointest Liver Physiol. 2008; 294: G728–G737. doi: 10.1152/ajpgi.00002.2007 PMID: 18202115

28. Waxman AB, Mahboubi K, Knickelbein RG, Mantell LL, Manzo N, Pober JS, et al. Interleukin-11 and interleukin-6 protect cultured human endothelial cells from H2O2-induced cell death. Am J Respir Cell Mol Biol. 2003; 29: 513–522. PMID: 12736073

29. Fujio Y, Maeda M, Mohri T, Obana M, Iwakura T, Hayama A, et al. (2011) Glycoprotein 130 Cytokine Signal as a Therapeutic Target Against Cardiovascular Diseases. J Pharmacol Sci. 2011; 117: 213–222. PMID: 22056652

30. Kawakami T, Takahashi T, Shimizu H, Nakahira K, Takeuchi M, Katayama H, et al. Highly liver-specific heme oxygenase-1 induction by interleukin-11 prevents carbon tetrachloride-induced hepatotoxicity. Int J Mol Med. 2006; 18: 537–546. PMID: 16964402

31. Ke B, Shen XD, Ji H, Kamo N, Gao F, Freitas MC, et al. HO-1-STAT3 axis in mouse liver ischemia/reperfusion injury: regulation of TLR4 innate responses through PI3K/PTEN signaling. J Hepatol. 2012; 56: 359–366. doi: 10.1016/j.jhep.2011.05.023 PMID: 21756853

32. Yue S, Rao J, Zhu J, Busuttil RW, Kupiec-Weglinski JW, Lu L, et al. Myeloid PTEN deficiency protects livers from ischemia reperfusion injury by facilitating M2 macrophage differentiation. J Immunol. 2014; 192: 5343–5353. doi: 10.4049/jimmunol.1400280 PMID: 24771857

33. Lou LX, Uemura T, Mani H, Yang C, Li W, Kadry Z, et al. Endogenous signal transducer and activator of transcription 3 is required for the protection of hepatocytes against warm ischemia/reperfusion injury. Liver Transpl. 2013; 119: 1078–1087.

34. Wen TC, Peng H, Hata R, Desaki J, Sakanaka M. Induction of phosphorylated-Stat3 following focal cerebral ischemia in mice. Neurosci Lett. 2001; 303: 153–156. PMID: 11323108

35. Nielsen M, Kaestel CG, Eriksen KW, Woetmann A, Stokkedal T, Kaltoft K, et al. Inhibition of constitutively activated Stat3 correlates with altered Bcl-2/Bax expression and induction of apoptosis in mycosis fungoides tumor cells. Leukemia. 1999; 13: 735–738. PMID: 10374878

36. Dinasarapu AR, Gupta S, Ram Maurya M, Fahy E, Min J, Sud M, et al. A combined omics study on activated macrophages-enhanced role of STATs in apoptosis, immunity and lipid metabolism. Bioinformatics. 2013; 29: 2735–2743. doi: 10.1093/bioinformatics/btt469 PMID: 23981351

37. Spehlmann ME, Manthey CF, Dann SM, Hanson E, Sandhu SS, Liu LY, et al. Trp53 deficiency protects against acute intestinal inflammation. J Immunol. 2013; 191: 837–847. doi: 10.4049/jimmunol.1201716 PMID: 23772033

38. Lee TL, Yeh J, Friedman J, Yan B, Yang X, Yeh NT, et al. A signal network involving coactivated NF-kappaB and STAT3 and altered p53 modulates BAX/BCL-XL expression and promotes cell survival of head and neck squamous cell carcinomas. Int J Cancer. 2008; 122:1987–1998. doi: 10.1002/ijc.23324 PMID: 18172861

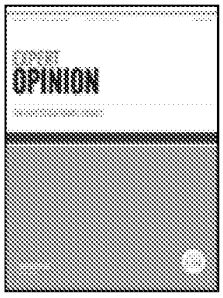
39. Shyu WC, Lin SZ, Chiang MF, Chen DC, Su CY, Wang HJ, et al. Secretoneurin promotes neuroprotection and neuronal plasticity via the Jak2/Stat3 pathway in murine models of stroke. J Clin Invest. 2008; 118:133–148. PMID: 18079966

40. Lentsch AB, Kato A, Yoshidome H, McMasters KM, Edwards MJ. Inflammatory mechanisms and therapeutic strategies for warm hepatic ischemia/reperfusion injury. Hepatology. 2000; 32: 169–173. PMID: 10915720

41. Liu J, Ren F, Cheng Q, Bai L, Shen X, Gao F, et al. Endoplasmic reticulum stress modulates liver inflammatory immune response in the pathogenesis of liver ischemia and reperfusion injury. Transplantation. 2012; 94: 211–217. doi: 10.1097/TP.0b013e318259d38e PMID: 22790388

42. Ren F, Duan Z, Cheng Q, Shen X, Gao F, Bai L, et al. Inhibition of glycogen synthase kinase 3 beta ameliorates liver ischemia reperfusion injury by way of an interleukin-10-mediated immune regulatory mechanism. Hepatology. 2011; 54: 687–696. doi: 10.1002/hep.24419 PMID: 21567437

43. Sano T, Izuishi K, Hossain MA, Inoue T, Kakinoki K, Hagiike M, et al. Hepatic preconditioning using lipopolysaccharide: association with specific negative regulators of the Toll-like receptor 4 signaling pathway. Transplantation. 2011, 91: 1082–1089. doi: 10.1097/TP.0b013e31821457cb PMID: 21403587



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The therapeutic utility of Interleukin-11 in the treatment of inflammatory disease

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The therapeutic utility of Interleukin-11 in the treatment of inflammatory disease

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Interleukin-11 (IL-11) is a pleiotropic cytokine that exhibits anti-inflammatory and mucosal protective effects in a variety of animal models of acute and chronic inflammation, such as mucositis, inflammatory bowel disease and autoimmune joint disease. This reduction in inflammation and epithelial damage is mediated in part through effects of recombinant human (rh) IL-11 on macrophage effector function and epithelial cell growth. *In vitro* studies indicate that rhIL-11 inhibits tumour necrosis factor (TNF)-α, IL-1β, IL-12, IL-6, and nitric oxide production from activated macrophages. Analysis of the effects of rhIL-11 on transcription factors that activate pro-inflammatory cytokines demonstrate that the level of induced nuclear factor kappa B (NF-κB) binding activity in the nucleus of rhIL-11-treated peritoneal macrophages is significantly reduced. Studies of normal intestinal epithelial cells indicate that rhIL-11 reduces the rate of cellular proliferation. Analysis of cell-cycle progression demonstrates that growth inhibition of epithelial cells by rhIL-11 correlates with delayed entry into S phase and suppression of pRB phosphorylation. IL-11 also protects intestinal crypt stem cells from radiation- or chemotherapy-induced insults. Such immunomodulatory and epithelial activities may contribute to the protective effects of this cytokine and support the clinical utility of rhIL-11 in the treatment of mucositis, as well as a variety of chronic inflammatory diseases, such as Crohn's disease and rheumatoid arthritis.

Keywords: *interleukin-11 (IL-11), immunomodulation, inflammatory disease*

Exp. Opin. Invest. Drugs (1998) 7(9):1501-1504

1. Introduction

Pro-inflammatory mediators have been directly implicated in the pathology of inflammatory diseases, such as inflammatory bowel disease (IBD), rheumatoid arthritis (RA) and mucositis [1-4]. For example, tumour necrosis factor (TNF)-α production is elevated in inflamed intestinal tissue of patients with Crohn's disease [5-7] and in serum of children with IBD [8]. Interleukin (IL)-1β production is elevated in macrophages isolated from colonic mucosa of patients with active IBD [9,10]. Furthermore, in synergy with IFN-λ, TNF-α and IL-1β have direct effects on intestinal epithelial cells to induce cell death and disrupt epithelial barrier function [11,12]. IL-1β, nitric oxide and TNF-α are also linked to the pathogenesis of RA [13-15].

Recent attempts to reduce the pathological consequences of an inflammatory response in the clinic have focused on blocking the production of pro-inflammatory mediators. Key targets have been TNF-α, IL-1β, and nitric oxide [16-19]. For example, administration of anti-TNF-α-neutralising

antibodies or inhibition of IL-1 or nitric oxide activity reduced the clinical symptoms and histological lesions of collagen-induced or streptococcal cell wall-induced arthritis in mice, rats and human [20,21]. Whilst these agents can block damage caused by pro-inflammatory molecules they do not directly promote tissue repair. The most highly effective therapeutics will do both.

IL-11 is a pleiotropic cytokine that has potent anti-inflammatory, as well as mucosal protective activities [22-26]. Therefore, IL-11 is a novel therapeutic that bridges the gap between pro-inflammatory cytokine blockade and tissue repair. The efficacy of this anti-inflammatory cytokine as a potential therapy for the treatment of IBD, RA and mucositis is under investigation.

2. IL-11: bioactivity on macrophages and mucosal epithelium

Interleukin-11 displays diverse effects on multiple cell types, including those of the myeloid, erythroid and megakaryocytic lineages (reviewed in [27-29]). More recently, rhIL-11 has demonstrated immunomodulatory as well as epithelial protective effects on macrophages and epithelial cells. rhIL-11 can down-regulate macrophage effector function through the inhibition of TNF-α, IL-1β, IL-12, and nitric oxide [23,30]. The anti-inflammatory activity of rhIL-11 on activated macrophages can be explained in part by the direct inhibition of pro-inflammatory cytokine gene expression [24,31]. Furthermore, this inhibition correlates with the reduction of NF-κB nuclear translocation and an increase in the levels of the NF-κB inhibitory proteins, IκB-α and IκB-β [24]. The biological activity of rhIL-11 on macrophages is direct and independent of other anti-inflammatory molecules, such as IL-10 and transforming growth factor (TGF)-β1. In an *in vivo* murine model of endotoxaemia, rhIL-11 also down-regulates serum levels of lipopolysaccharide (LPS)-induced pro-inflammatory cytokines [23]. Of particular note, rhIL-11 significantly reduces serum levels of interferon (IFN)-γ, indicating modulatory activity on T-cells or NK cells [23]. Thus, similar to, but distinct from IL-10 and TGF-β1, rhIL-11 acts as an anti-inflammatory cytokine primarily through modulation of immune effector cell function.

Recombinant human IL-11 also directly interacts with normal and damaged intestinal epithelium to affect proliferation and promote mucosal healing [22,26,32-34]. When normal intestinal epithelial cells (IEC-6 and IEC-18) were treated *in vitro* with rhIL-11, growth inhibition was observed [22,32]. Growth inhibition correlated with delayed entry into the S phase of the cell cycle and was associated with suppression of retinoblastoma protein (pRb) phosphorylation [22]. In a mouse model of bowel ischaemia, increased mitotic activity and suppression of apoptosis was observed in intestinal crypt stem cells following prophylactic administration of rhIL-11 [26]. Together, these studies indicate an ability of rhIL-11 to promote mucosal recovery following insult.

3. IL-11 as a novel therapeutic for the treatment of inflammatory disease

The ability of rhIL-11 to attenuate pro-inflammatory cytokine production and promote mucosal repair correlates with its ability to reduce inflammation and tissue damage in a variety of experimental animal models of chronic IBD and systemic inflammation. Administration of rhIL-11 reduces symptoms of IBD in transgenic rats expressing human HLA-B27 and β2-microglobulin genes [35]. The inflammatory cell infiltrates within these colonic lesions are composed primarily of activated macrophages and lymphocytes, and treatment with rhIL-11 significantly reduced clinical signs and histological lesions of colitis [35]. A protective effect of rhIL-11 on the colonic mucosa is also observed subsequent to intracolonic administration of trinitrobenzene sulfonic acid (TNBS) in Sprague-Dawley rats [36]. TNBS is a contact-sensitising agent that stimulates a CD4+ T-cell mediated delayed-type hypersensitivity (DTH) response to TNP hapten modified self-antigens. rhIL-11-treated rats showed a dose-related reduction in the severity of TNBS-induced colitis, enhanced mucus production and decreased myeloperoxidase activity, suggesting modulation of the inflammatory immune response and promotion of mucosal integrity [36].

Other preclinical animal studies have also demonstrated protective effects of rhIL-11 on intestinal mucosa. Administration of rhIL-11 enhanced survival of mice subjected to severe cytoablative therapy with fluorouracil and subiethal irradiation or following bowel ischaemia injury [26,37]. rhIL-11 administration also decreased the frequency, severity and duration of fluorouracil-induced oral mucositis in

the Syrian golden hamster [4,38]. Due to its immunomodulatory activities and ability to maintain the integrity, or promote the healing of the gastrointestinal mucosa, rhIL-11 has entered clinical trials for the treatment of Crohn's disease, RA and chemotherapy-induced mucositis.

4. Expert opinion

Down-regulation of the immune response with general immunomodulators such as steroids is the current standard therapy for the treatment of IBD and arthritis but is associated with limited efficacy and adverse effects. Currently, there is no effective therapy for the treatment of chemotherapy-induced mucositis. In severe mucositis cases, patients require dose de-escalation or interruption of therapy.

Inhibition of TNF-α function through the administration of anti-TNF-α monoclonal antibodies is being investigated for the treatment of Crohn's disease [39,40] and arthritis [20,21,41]. New therapies under investigation include the use of specific cytokine antagonists including receptor antagonists and anti-inflammatory cytokines [21]. The ability of IL-11 to modulate the production of several pro-inflammatory mediators and enhance epithelial protection distinguishes it from single target therapies described above. Thus, rhIL-11 is a novel therapeutic with potential to treat a variety of inflammatory disorders, such as Crohn's Disease, rheumatoid arthritis and chemotherapy- or radiation-induced mucositis.

Bibliography

1. ZHANG X, MORRISON DC: **Lipopolysaccharide-induced selective priming effects on tumour necrosis factor alpha and nitric oxide production in mouse peritoneal macrophages.** *J. Exp. Med.* (1993) **177**:511-516.
2. ALEXANDER HR, DOHERTY GM, BURESH CM, VENZON DJ, NORTON JA: **A recombinent human receptor antagonist to interleukin 1 improves survival after lethal endotoxemia in mice.** *J. Exp. Med.* (1991) **173**:1029-1032.
3. DING AH, NATHAN CF, STUEHR DJ: **Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production.** *J. Immunol.* (1988) **141**:2407-2412.
4. SONIS ST, VAN VUGT AG, MCDONALD J *et al.*: **Mitigating effects of interleukin 11 on consecutive courses of 5-fluorouracil-induced ulcerative mucositis in hamsters.** *Cytokine* (1997) **9**:605-612.
5. BREESE EJ, MICHIE CA, NICHOLLS SW *et al.*: **Tumour necrosis factor alpha producing cells in the intestinal mucosa of children with inflammatory bowel disease.** *Gastroenterology* (1994) **106**:1455-1466.
6. MACDONALD TT, HUTCHINGS P, CHOY M-Y, MURCH S, COOKE A: **Tumour necrosis factor alpha and interferon gamma production measured at the single cell level in normal and inflamed human intestine.** *Clin. Exp. Immunol.* (1990) **81**:301-305.
7. PLEVY SE, TARGAN SR, ANDUS T, TOYOD AH: **TNF-α mRNA levels differentiate mucosal inflammation in Crohn's disease from ulcerative colitis.** *J. Immunol.* (1993) **150**.10A.
8. MURCH SH, LAMKIN VA, SAVAGE MO, WALKER-SMITH JA, MACDONALD TT: **Serum levels of tumour necrosis factor alpha in childhood chronic inflammatory bowel disease.** *Gut* (1991) **32**:913-917.
9. MAHIDA YR, WU K, JEWELL DP: **Enhanced production of interleukin 1-β by mononuclear cells isolated from mucosa with active ulcerative colitis of Crohn's disease.** *Gut* (1989) **30**.835-838.
10. LICUMSKY M, SIMON PL, KARMELI F, RACHMILEWITZ D: **Role of IL-1 in inflammatory bowel disease. Enhanced production during active disease.** *Gut* (1990) **31**:686-689.
11. ABREU-MARTIN MT, VIDRICH AD, LYNCH H, TARGAN SR: **Divergent induction of apoptosis and IL-8 secretion in HT-29 cells in response to TNF-α and ligation of Fas antigen.** *J. Immunol.* (1995) **155**:4147-4154.
12. RODRIGUEZ P, HEYMAN M, CANDALH C, BLATON MA, BOUCHOUD C: **Tumour necrosis factor a induces morphological and functional alterations of intestinal HT29 cl.19A cell monolayers.** *Cytokine* (1995) **7**:441-448.
13. MCCARTNEY-FRANCIS N, ALLEN J, MIZEL D *et al.*: **Suppression of arthritis by an inhibitor of nitric oxide synthase.** *J. Exp. Med.* (1993) **178**:749-754.
14. GEIGER T, TOWBIN H, COSENTI-VARGAS A *et al.*: **Neutralization of IL-1β activity *in vivo* with a monoclonal antibody alleviates collagen-induced arthritis in DBA/1 mice and prevents the associated acute phase response.** *Clin. Exp. Rheumatol.* (1993) **19**:348-356.
15. WILLIAMS RO, FELDMANN M, MAINI RN: . **Antitumour necrosis factor ameliorates joint disease in murine collage-induced arthritis.** *Proc. Natl. Acad. Sci. USA* (1992)**89**:9784-9788.
16. DERKX B, TAMINIAU J, RADEMA S *et al.*: **Tumour necrosis factor antibody treatment in Crohn's disease.** *Lancet* (1993) **342**.173-174.
17. NATHAN C, XIE Q-W. **Nitric oxide synthases: roles, tolls, and controls.** *Cell* (1994) **78**:915-918.
18. BOGDAN C, VODOVOTZ Y, NATHAN CF: In. *Mononuclear Phagocytes: Activation. Role and Cytokinetics.* R. van Furth, (Ed.), Kluwer Academic Publishers, Dordrecht, The Netherlands (1991).

19. EIGLER A, SINHA B, HARTMANN G, ENDRES sincerely: **Taming TNF-α: strategies to restrain this proinflammatory cytokine.** *Immunol. Today* (1997)**18**:487-492.

20. FELDMANN M, ELLIOTT MJ, WOODY JN, MAINI RN: **Anti-tumour necrosis factor-alpha therapy of rheumatoid arthritis.** *Adv. Immunol.* (1997) **64**:283-350.

21. KOOPMAN WJ, MORELAND LW. **Rheumatoid arthritis: anticytokine therapies on the horizon [Editorial].** *Ann. Intern. Med.* (1998)**128**:231-233.

22. PETERSON RL, BOZZA MM, DORNER AJ: **Interleukin-11 induces intestinal epithelial cell growth arrest through effects on retinoblastoma protein phosphorylation.** *Am. J. Pathol.* (1996) **149**:895-902.

23. TREPICCHIO WL, BOZZA M, PEDNEAULT G, DORNER AJ: **Recombinant human IL-11 attenuates the inflammatory response through down-regulation of proinflammatory cytokine release and nitric oxide production.** *J. Immunol.* (1996) **157**:3627-3634.

24. TREPICCHIO WL, WANG L, BOZZA M, DORNER AJ: **IL-11 regulates macrophage effector function through the inhibition of nuclear factor-kappaB.** *J. Immunol.* (1997) **159**:5661-5670

25. DU XX, WILLIAMS DA: **Interleukin-11: a multifunctional growth factor derived from the hematopoietic microenvironment.** *Blood* (1994) **83**:2023-2030.

26. DU X, LIU Q, YANG Z, ORAZI A: **Protective effects of interleukin-11 in a murine model of ischemic bowel necrosis.** *Am. J. Physiol.* (1997) **272**:G545-552.

27. DORNER AJ, GOLDMAN SJ, KEITH JCJ: **Interleukin-11: biological activity and clinical studies.** *BioDrugs* (1997) **6**:418-429.

28. GOLDMAN SJ: **Preclinical biology of interleukin 11: a multifunctional hematopoietic cytokine with potent thrombopoietic activity.** *Stem. Cells (Dayt)* (1995) **13**:462-471.

29. DU X, WILLIAMS DA: **Interleukin-11: review of molecular, cell biology, and clinical use.** *Blood* (1997) **89**:3897-3908.

30. REDLICH CA, GAO X, ROCKWELL S, KELLEY M, ELIAS JA: **IL-11 enhances survival and decreases TNF-α production after radiation-induced thoracic injury.** *J. Immunol.* (1996) **157**:1705-1710.

31. LENG SX, ELIAS JA: **Interleukin-11 inhibits macrophage interleukin-12 production.** *J. Immunol.* (1997) **159**:2161-2168.

32. BOOTH C, POTTEN CS: **Effects of IL-11 on the growth of intestinal epithelial cells *in vitro*.** *Cell Prolif.* (1995) **28**:581-94.

33. ORAZI A, DU X, YANG Z, KASHAR M, WILLIA DA: **Lab Inv Interleukin-11 prevents apoptosis and accelerates recovery** of small intestinal mucosa in mice treated with combined chemotherapy and radiation. *Lab. Inv.* (1996) **75**:33-42

34 POTTEN CS. **Interleukin-11 protects the clonogenic stem cells in murine small-intestinal crypts from impairment of their reproductive capacity by radiation.** *Int. J. Cancer* (1995)**62**:356-361.

35 KEITH JC, ALBERT JRL, SONIS ST, PFEIFFER CJ, SCHAUB RG: **IL-11, a pleiotropic cytokine: exciting new effects of IL-11 on gastrointestinal mucosal biology.** *Stem Cells* (1994) **1**:79-89.

36 QIU BS, PFEIFFER CJ, KEITH JC Jr: **Protection by recombinant human interleukin-11 against experimental TNB-induced colitis in rats.** *Dig Dis. Sci.* (1996) **41**:1625-1630.

37. DU XX, DOERSCHUK CM, ORAZI A, WILLIAMS DA: **A bone marrow stromal-derived growth factor, interleukin-11, stimulates recovery of small intestinal mucosal cells after cytoablative therapy.** *Blood* (1994) **83**:33-37.

38. SONIS S, MUSKA A, O'BRIEN J *et al.*: **Alteration in the frequency, severity and duration of chemotherapy-induced mucositis in hamsters by interleukin-11.** *Eur. J. Cancer B. Oral Oricol.* (1995) **31B**:261-266.

39. VAN DULLEMEN HM, VAN DEVENTER SJ, HOMMES DW *et al.*: **Treatment of Crohn's disease with antitumour necrosis factor chimeric monoclonal antibody.** *Gastroenterology* (1995) **109**:129-135.

40. TARGAN SR, HANAUER SB, VAN DEVENTER SJ *et al.*: **A short-term study of chimeric monoclonal antibody cA2 to tumour necrosis factor alpha for Crohn's disease. Crohn's Disease cA2 Study Group.** *New Engl. J. Med.* (1997) **337**:1029-35

41 ELLIOTT MJ, MAINI RN, FELDMANN M *et al.*: **Repeated therapy with monoclonal antibody to tumour necrosis factor alpha (cA2) in patients with rheumatoid arthritis.** *Lancet* (1994) **344**:1125-1127.

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Cellular and molecular mechanisms of fibrosis

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Abstract

Fibrosis is defined by the overgrowth, hardening, and/or scarring of various tissues and is attributed to excess deposition of extracellular matrix components including collagen. Fibrosis is the end result of chronic inflammatory reactions induced by a variety of stimuli including persistent infections, autoimmune reactions, allergic responses, chemical insults, radiation, and tissue injury. Although current treatments for fibrotic diseases such as idiopathic pulmonary fibrosis, liver cirrhosis, systemic sclerosis, progressive kidney disease, and cardiovascular fibrosis typically target the inflammatory response, there is accumulating evidence that the mechanisms driving fibrogenesis are distinct from those regulating inflammation. In fact, some studies have suggested that ongoing inflammation is needed to reverse established and progressive fibrosis. The key cellular mediator of fibrosis is the myofibroblast, which when activated serves as the primary collagen-producing cell. Myofibroblasts are generated from a variety of sources including resident mesenchymal cells, epithelial and endothelial cells in processes termed epithelial/endothelial-mesenchymal (EMT/EndMT) transition, as well as from circulating fibroblast-like cells called fibrocytes that are derived from bone-marrow stem cells. Myofibroblasts are activated by a variety of mechanisms, including paracrine signals derived from lymphocytes and macrophages, autocrine factors secreted by myofibroblasts, and pathogen-associated molecular patterns (PAMPS) produced by pathogenic organisms that interact with pattern recognition receptors (i.e. TLRs) on fibroblasts. Cytokines (IL-13, IL-21, TGF-β1), chemokines (MCP-1, MIP-1β), angiogenic factors (VEGF), growth factors (PDGF), peroxisome proliferator-activated receptors (PPARs), acute phase proteins (SAP), caspases, and components of the renin–angiotensin–aldosterone system (ANG II) have been identified as important regulators of fibrosis and are being investigated as potential targets of antifibrotic drugs. This review explores our current understanding of the cellular and molecular mechanisms of fibrogenesis.

Keywords

fibrosis; myofibroblast; collagen; wound healing; liver; lung

Introduction

In contrast to acute inflammatory reactions, which are characterized by rapidly resolving vascular changes, oedema and neutrophilic inflammation, fibrosis typically results from chronic inflammation — defined as an immune response that persists for several months and in which inflammation, tissue remodelling and repair processes occur simultaneously. Despite having distinct aetiological and clinical manifestations, most chronic fibrotic disorders have

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in common a persistent irritant that sustains the production of growth factors, proteolytic enzymes, angiogenic factors and fibrogenic cytokines, which stimulate the deposition of connective tissue elements that progressively remodel and destroy normal tissue architecture [1–3].

Damage to tissues can result from various stimuli, including infections, autoimmune reactions, toxins, radiation and mechanical injury. The repair process typically involves two distinct phases: a regenerative phase, in which injured cells are replaced by cells of the same type, leaving no lasting evidence of damage; and a phase known as fibroplasia or fibrosis, in which connective tissues replaces normal parenchymal tissue. Although initially beneficial, the repair process becomes pathogenic when it is not controlled appropriately, resulting in substantial deposition of ECM components in which normal tissue is replaced with permanent scar tissue [4]. In some diseases, such as idiopathic pulmonary fibrosis, liver cirrhosis, cardiovascular fibrosis, systemic sclerosis and nephritis, extensive tissue remodelling and fibrosis can ultimately lead to organ failure and death (Table 1).

Wound healing versus fibrosis

When epithelial and/or endothelial cells are damaged, they release inflammatory mediators that initiate an anti-fibrinolytic coagulation cascade [5], which triggers blood-clot formation and formation of a provisional ECM. Platelets are exposed to ECM components, triggering aggregation, clot formation and haemostasis. Platelet degranulation also promotes vasodilation and increased blood vessel permeability, while myofibroblasts (activated collagen secreting, α-SMA$^+$ fibroblasts) and epithelial and/or endothelial cells produce MMPs, which disrupt the basement membrane, allowing inflammatory cells to be easily recruited to the site of injury. Growth factors, cytokines and chemokines are also produced, which stimulates the proliferation and recruitment of leukocytes across the provisional ECM. Some of the early responders include macrophages and neutrophils, which eliminate tissue debris, dead cells and any invading organisms. They also produce cytokines and chemokines, which are mitogenic and chemotactic for endothelial cells, which begin to surround the injured site. They also help form new blood vessels as epithelial/endothelial cells migrate towards the centre of the wound. During this period, lymphocytes and other cells become activated and begin secreting profibrotic cytokines and growth factors, such as TGFβ, IL-13 and PDGF [6–8], which further activate the macrophages and fibroblasts. Activated fibroblasts transform into α-SMA-expressing myofibroblasts as they migrate along the fibrin lattice into the wound. Following activation, the myofibroblasts promote wound contraction, the process in which the edges of the wound migrate towards the centre. Finally, epithelial and/or endothelial cells divide and migrate over the basal layers to regenerate the damaged tissue, which completes the wound-healing process. However, chronic inflammation and repair can trigger an excessive accumulation of ECM components, which leads to the formation of a permanent fibrotic scar. Collagen turnover and ECM remodelling is regulated by various MMPs and their inhibitors, which include the tissue inhibitors of metalloproteinases (TIMPs). Shifts in synthesis versus catabolism of the ECM regulate the net increase or decrease of collagen within the wound [9]. Fibrosis occurs when the synthesis of new collagen by myofibroblasts exceeds the rate at which it is degraded, such that the total amount of collagen increases over time.

The cellular origins of myofibroblasts

Local tissue myofibroblasts were originally believed to be the primary producers of ECM components following injury [5]; however, it is now thought that fibroblasts can be derived from multiple sources [10]. In addition to resident mesenchymal cells, myofibroblasts are derived from epithelial cells in a process termed epithelial–mesenchymal transition (EMT) [10–12]. More recently, it was suggested that a similar process occurs with endothelial cells,

termed endothelial–mesenchymal transition (EndMT) [13]. Bucala and colleagues also identified a unique circulating fibroblast-like cell derived from bone marrow stem cells [14]. These blood-borne mesenchymal stem cell progenitors have a fibroblast/myofibroblast-like phenotype (they express CD34, CD45 and type I collagen) and are now commonly called fibrocytes [15–18]. Finally, in some tissues, resident fibroblasts are not the only source of myofibroblasts. For example, in liver fibrosis the resident hepatic stellate cell (HSC) appears to be the primary source of myofibroblasts, although bone-marrow-derived cells can also contribute [18,19]. Because it is now thought that fibrocytes and EMT-derived myofibroblasts participate with resident mesenchymal cells in the reparative process, there has been growing interest in dissecting the role of the various myofibroblast subpopulations in fibroproliferative disease [20]. Because bone marrow-derived fibrocytes must find their way to sites of tissue injury to participate in wound healing and fibrosis, there has been a great deal of interest in understanding the role of chemokines and acute phase proteins, such as serum amyloid P (SAP), in the development and recruitment of myofibroblasts [20–22]. Because fibrocytes and EMT-derived myofibroblasts produce a variety of factors that are involved in the fibrotic process [10], interrupting their development, recruitment and/or activation could provide a unique therapeutic approach to treat a variety of fibrotic diseases.

Innate and adaptive immune mechanisms regulate myofibroblast activity

Many fibrotic disorders are thought to have an infectious aetiology, with bacteria, viruses, fungi and multicellular parasites driving chronic inflammation and the development of fibrosis. It was recently suggested that conserved pathogen-associated molecular patterns (PAMPs) found on these organisms help maintain myofibroblasts at a heightened state of activation [23]. Bacteria living in the gut can also contribute to the activation of myofibroblasts [24]. PAMPs are pathogen byproducts, such as lipoproteins, bacterial DNA and double-stranded RNA, which are recognized by pattern recognition receptors (PRRs) found on a wide variety of cells, including fibroblasts [25]. The interaction between PAMPs and PRRs serves as a first line of defence during infection and activates numerous proinflammatory cytokine and chemokine responses. In addition, because fibroblasts express a variety of PRRs, including Toll-like receptors (TLRs), Toll ligands can directly activate fibroblasts and promote their differentiation into collagen-producing myofibroblasts [23,24,26]. Thus, inhibiting TLR signalling might represent a novel approach to treat fibrotic disease.

Nevertheless, pathogenic organisms are not responsible for all fibrotic disorders. Therefore, additional mechanisms must also participate in the activation of myofibroblasts. For example, in the case of systemic sclerosis (SSc), fibroblasts obtained from lesional skin or fibrotic lungs have a constitutively activated myofibroblast-like phenotype, characterized by enhanced ECM synthesis, constitutive secretion of cytokines and chemokines and increased expression of cell surface receptors [27–29]. Because most of the characteristics of fibroblasts from patients with SSc are reproduced in normal human fibroblasts following stimulation with TGFβ, it is thought that the SSc fibroblast phenotype is maintained by an autocrine TGFβ signal. However, TGFβ/SMAD3-independent mechanisms have also been proposed [30,31], including a role for viruses such as CMV, which stimulate the production of auto-antibodies and connective tissue growth factor (CTGF), both of which are known to participate in the activation of myofibroblasts [28,32]. Epigenetic changes may also contribute to the persistent activation of myofibroblasts [33]. B cells have also been implicated, either by producing autoanti-bodies or by secreting IL-6, a well-known fibroblast growth factor [34]. Still other studies have argued that Th2-type cytokines derived from a variety of cellular sources are critically involved in the mechanism of fibrosis [35–38]. Therefore, paracrine signals derived from activated lymphocytes, autocrine factors produced by fibroblasts, as well as molecules derived from pathogenic organisms can cooperate to initiate and maintain myofibroblast activation.

Chemokines regulate fibrogenesis by controlling myofibroblast recruitment

Chemokines are leukocyte chemoattractants that cooperate with profibrotic cytokines in the development of fibrosis by recruiting myofibroblasts, macrophages and other key effector cells to sites of tissue injury. Although a large number of chemokine signalling pathways are involved in the mechanism of fibrogenesis, the CC- and CXC-chemokine receptor families have consistently exhibited important regulatory roles. Specifically, CCL3 (macrophage inflammatory protein 1α) and CC-chemokines such as CCL2 (monocyte chemoattractant protein-1), which are chemotactic for mononuclear phagocytes, were identified as profibrotic mediators. Macrophages and epithelial cells are believed to be the key sources of CCL3, and studies in the bleomycin model of pulmonary fibrosis showed that anti-CCL3 antibodies could significantly reduce the development of fibrosis [39,40]. Similar results were obtained when CCL2 was neutralized, suggesting that a variety of CC-chemokines are involved [41,42]. Subsequent studies with CC-chemokine receptor 1 (CCR1)- and CCR2-deficient mice produced similar results, confirming critical roles for CCL3/CCL2-mediated signalling pathways in fibrogenesis [43–47]. Interestingly, in several of these blocking studies, the absence of fibrosis was associated with decreased IL-4/IL-13 expression [44,48], suggesting a direct link between CC-chemokine activity and the production of profibrotic cytokines such as IL-13. IL-13 is a potent inducer of several CC-chemokines, including CCL3, CCL4 (MIP-1β), CCL20 (MIP-3α), CCL2, CCL11, CCL22 (macrophage-derived chemokine) and CCL6 (C10), among others, suggesting that a positive feedback mechanism exists between IL-13 and the CC-chemokine family [49,50]. As seen with anti-CCL3 and anti-CCL2 antibody treatment, antibodies to CCL6 significantly attenuated lung remodelling responses in IL-13-transgenic mice [50] as well as in mice challenged with bleomycin [49], indicating non-redundant roles for a variety of CC-chemokines in the pathogenesis of fibrosis. In mice, CXC chemokine receptor 4 (CXCR4), CC chemokine receptor 7 (CCR7) and CCR2 have also been shown to regulate the recruitment of fibrocytes to the lung [20,21]. Thus, interrupting specific chemokine signalling pathways could have a significant impact on the treatment of a variety of fibroproliferative diseases.

Th1 and Th2 cells differentially regulate organ fibrosis

Chronic inflammatory reactions are typically characterized by a large infiltrate of mononuclear cells, including macrophages, lymphocytes, eosinophils and plasma cells. Lymphocytes are mobilized to sites of injury and become activated following contact with various antigens, which stimulate the production of lymphokines that further activate macrophages and other local inflammatory cells. Thus, there is significant activation of the adaptive immune response in many chronic inflammatory diseases. Although inflammation typically precedes the development of fibrosis, results from a variety of experimental models suggest that fibrosis is not always characterized by persistent inflammation, implying that the mechanisms regulating fibrosis are to a certain extend distinct from those controlling inflammation. Findings from our own studies of schistosomiasis-induced liver fibrosis support this theory [35]. In this model, fibrosis develops progressively in response to schistosome eggs that are deposited in the liver, which induce a chronic granulomatous response. As in many other experimental models of fibrosis, $CD4^+$ T cells play a prominent role in the progression of the disease. Studies conducted with multiple cytokine-deficient mice have demonstrated that liver fibrosis is strongly linked with the development of a $CD4^+$ Th2 cell response (involving IL-4, IL-5, IL-13 and IL-21) [51–55].

Several experimental models of fibrosis in addition to our own have also documented potent antifibrotic activities for the Th1-associated cytokines IFNγ and IL-12. In schistosomiasis, while treatment with IFNγ or IL-12 has no effect on the establishment of infection, collagen deposition associated with chronic granuloma formation is substantially decreased [51].

Similar results have been obtained in models of pulmonary, liver and kidney fibrosis [56–59]. These findings suggest that it might be possible to develop an antifibrosis vaccine based on immune deviation [51,60], in which the profibrotic effects of the Th2 response are switched off in favour of an antifibrotic Th1 response. Indeed, similar approaches have been proposed for individuals suffering from allergic airway inflammation [61], which is also driven by Th2-type responses. Studies investigating the gene expression patterns of fibrotic tissues found that markedly different gene expression profiles are induced during Th1 and Th2 polarized responses [62,63]. As might be expected, a large number of IFNγ-induced genes are upregulated in the tissues of mice exhibiting Th1-polarized responses, with no evidence of significant activation of the fibrosis-associated genes in this setting [62–64]. Instead, two major groups of genes were identified in Th1-polarized mice: those associated with the acute-phase reaction and apoptosis (cell death), findings which may explain the extensive tissue damage that is commonly observed when Th1 responses continue unchecked [65]. By contrast, several genes known to be involved in the mechanisms of wound healing and fibrosis were upregulated in animals exhibiting Th2-polarized inflammation [62,63]. The regulation and function of a few of the genes, including procollagens I, III and VI, arginase-1 [66], lysyl oxidase [67,68], matrix metalloproteinase-2 (MMP-2) [69,70], MMP-9 [71,72] and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) [73,74], have been investigated in some detail. Several additional Th2-linked genes [62,63], including haem oxygenase, procollagen III, secreted phosphoprotein 1, procollagen V, reticulocalbin and fibrillin 1 have also been reported in the fibrotic lungs of bleomycin-treated mice [75] and in CCl_4-stimulated rat hepatic stellate cells (collagen-producing cells in the liver) [76], providing further evidence that fibrosis is often associated with the development of Th2-type responses.

Unique roles for the Th2 cytokines IL-4, IL-5, IL-13 and IL-21 in fibrosis

The Th2 cytokines IL-4, IL-5, IL-13 and IL-21 each have distinct roles in the regulation of tissue remodelling and fibrosis. IL-4 is found at increased levels in the bronchoalveolar lavage fluids of patients with idiopathic pulmonary fibrosis (IPF) [77], in the pulmonary interstitium of individuals with cryptogenic fibrosing alveolitis [78] and in peripheral blood mononuclear cells (PBMCs) of those suffering from periportal fibrosis [79]. Development of post-irradiation fibrosis is also associated with increased production of IL-4 [80]. Although the extent to which IL-4 participates in fibrosis varies in different diseases, it has long been considered a potent profibrotic mediator. In fact, studies have suggested that IL-4 is nearly twice as effective as TGFβ [81], another potent profibrotic cytokine that has been extensively studied [82]. Receptors for IL-4 are found on many mouse [83] and human fibroblast subtypes [84] and *in vitro* studies showed the synthesis of the extracellular matrix proteins, types I and III collagen and fibronectin, following IL-4 stimulation. One of the first *in vivo* reports to investigate the contribution of IL-4 was a study of schistosomiasis in mice, in which neutralizing antibodies to IL-4 were shown to significantly reduce the development of hepatic fibrosis [52]. Inhibitors of IL-4 were also found to reduce dermal fibrosis in a chronic skin graft rejection model and in a mouse model of scleroderma [85,86].

IL-13 shares many functional activities with IL-4 because both cytokines exploit the same IL-4Rα/Stat6 signalling pathways [87]. However, with the development of IL-13 transgenic and knockout mice [88,89], as well as IL-13 antagonists [53,90], unique and non-redundant roles for IL-13 and IL-4 have been revealed in numerous models. When IL-4 and IL-13 were inhibited independently, IL-13 was identified as the dominant effector cytokine of fibrosis in several experimental models of fibrosis [38,53,91–94]. In schistosomiasis, although the egg-induced inflammatory response was unaffected by IL-13 blockade, collagen deposition decreased by more than 85% [53,95], despite continued and undiminished production of IL-4 [53,96]. Related studies have also shown a dominant role for IL-13 in the pathogenesis of pulmonary fibrosis. Over-expression of IL-13 in the lung triggered significant subepithelial

airway fibrosis in mice in the absence of any additional inflammatory stimulus [89], while treatment with anti-IL-13 antibody markedly reduced collagen deposition in the lungs of animals challenged with *A. fumigatus* conidia [91] or bleomycin [49]. In contrast, transgenic mice that over-expressed IL-4 displayed little evidence of subepithelial airway fibrosis, despite developing an intense inflammatory response in the lung [97]. Interestingly, two recent studies suggested that IL-13-regulated responses [98], including lung fibrosis [99], could develop in the absence of IL-4Rα or Stat6-mediated signalling, suggesting that IL-13 can exploit an additional signalling mechanism that is distinct from the IL-4Rα/Stat6-signalling pathway. Indeed, a recent report suggested that TGFβ1-driven pulmonary fibrosis might in some cases be dependent on IL-13-mediated signalling through the IL-13Rα2 chain [100], which was originally thought to operate exclusively as a decoy receptor for IL-13 and as an inhibitor of fibrosis [53,101].

IL-5 and eosinophils have also been shown to regulate tissue fibrogenesis. The differentiation, activation and recruitment of eosinophils is highly dependent on IL-5, and eosinophils are an important source of fibrogenic cytokines, including TGFβ1 and IL-13. IL-5 and tissue eosinophils have been observed in a variety of diseases, including skin allograft rejection and pulmonary fibrosis [86,102,103]. However, studies with neutralizing anti-IL-5 antibodies and IL-5 knockout mice have often yielded conflicting results [104]. Early experiments with neutralizing anti-IL-5 monoclonal antibodies showed no reduction in liver fibrosis following *S. mansoni* infection, even though tissue eosinophil responses were markedly reduced [105]. Although negative findings were also reported in some of the skin and lung fibrosis models [105,106], other studies observed significant reductions in fibrosis when IL-5 activity was neutralized [86,107–110]. A recent study demonstrated that although excessive amounts of IL-5 can exacerbate bleomycin-induced fibrosis, IL-5$^{-/-}$ mice showed no impairment in fibrosis [111], suggesting that IL-5 and/or eosinophils act as amplifiers rather than as direct mediators of fibrosis. In mice deficient in IL-5 and/or CCL11 (eotaxin), tissue eosinophilia was abolished and the ability of CD4$^+$ Th2 cells to produce the profibrotic cytokine IL-13 was significantly impaired [112]. Eosinophils were also found to be an important source of IL-13 in the schistosomiasis-induced model of liver fibrosis [55]. IL-5 and eosinophils can also regulate the TGFβ response in the lungs of mice [109,113]. Thus, one of the key roles of IL-5 and eosinophils may be to facilitate production of important profibrotic cytokines like IL-13 and/or TGFβ, which function as the key mediators of fibrosis.

Finally, similar to IL-5 [55], IL-21/IL-21R signalling was recently shown to promote fibrosis by facilitating the development of the CD4$^+$ Th2 response [54]. IL-21R-signalling was also critical for Th2-cell survival and for the migration Th2 cells to the peripheral tissues [114]. In addition to supporting the development of Th2 responses, IL-21 also increased IL-4 and IL-13 receptor expression on macrophages [54], which enhances the development of alternatively activated macrophages that are believed to be important regulators of fibrosis [66,115].

Distinct and overlapping roles for TGFβ and Th2-type cytokines in fibrosis

TGFβ has been the most intensively studied regulator of the ECM and has been linked with the development of fibrosis in a number of diseases [116–119]. There are three isotypes of TGFβ in mammals, TGFβ1, -2 and -3, all exhibiting similar biological activity [120]. Although a variety of cell types produce and respond to TGFβ [82], tissue fibrosis is primarily attributed to the TGFβ1 isoform, with circulating monocytes and tissue macrophages being the predominant cellular sources. In macrophages, the primary level of control is not in the regulation of TGFβ1 mRNA expression, but in the regulation of both the secretion and activation of latent TGFβ1. TGFβ1 is stored inside the cell as a disulphide-bonded homodimer, non-covalently bound to a latency-associated protein (LAP), which keeps TGFβ inactive. Binding of the cytokine to its receptors requires dissociation of the LAP, a process that is

catalysed by several agents, including cathepsins, plasmin, calpain, thrombospondin, integrin-$\alpha v\beta 6$ and matrix metalloproteinases [82,120,121], many of which have become potential targets of antifibrotic drugs. Once activated, TGFβ signals through transmembrane receptors that trigger signalling intermediates known as Smad proteins, which modulate transcription of important target genes, including procollagen I and III [122]. Dermal fibrosis following irradiation [123] and renal interstitial fibrosis induced by unilateral ureteral obstruction [116] are both reduced in Smad3-deficient mice, confirming an important role for the TGFβ signalling pathway. Macrophage-derived TGFβ1 is thought to promote fibrosis by directly activating resident mesenchymal cells including epithelial cells, which differentiate into collagen-producing myofibroblasts via EMT. Interestingly, a recent paper showed that the loss of TGFβ signalling in fibroblasts triggers intraepithelial neoplasia, suggesting that TGFβ1 signalling critically regulates the activity of fibroblasts as well as the oncogenic potential of neighbouring epithelial cells [124]. In the bleomycin model of fibrosis, alveolar macrophages are thought to produce nearly all of the active TGFβ that promotes pulmonary fibrosis [125]. Nevertheless, Smad3/TGFβ1-independent mechanisms of fibrosis have also been demonstrated in the lung and other tissues [30,126,127], suggesting that profibrotic mediators such as IL-4, IL-5, IL-13 and IL-21 can act separately from the TGFβ/Smad-signalling pathway to stimulate collagen deposition.

There is also evidence that Th2 cytokines cooperate with TGFβ to induce fibrosis. IL-13 induces the production of latent TGFβ1 in macrophages and can also serve as an indirect activator of TGFβ by upregulating expression of proteins that cleave the LAP [128,129]. Indeed, IL-13 is a potent stimulator of both MMP and cathepsin-based proteolytic pathways that activate TGFβ [74,129]. Thus, the significant tissue remodelling associated with polarized Th2 responses may involve a pathway wherein IL-13-expressing $CD4^+$ Th2 cells trigger macrophage production of TGFβ1, which then serves as the major stimulus for fibroblast activation and collagen deposition [100,128,130]. In support of this hypothesis, when TGFβ1 activity was neutralized in the lungs of IL-13-transgenic mice, development of subepithelial fibrosis was significantly reduced [128]. However, related studies observed enhanced pulmonary pathology when the TGFβ/Smad signalling pathway was blocked [131,132], suggesting that TGFβ suppresses rather than induces tissue remodelling in some settings. The source of TGFβ1 appears to be critical, since macrophage-derived TGFβ1 is often profibrotic [128], while T cell-derived TGFβ1 appears to play a suppressive role [133]. Some studies investigating the mechanisms of IL-13-driven fibrosis also reported no reduction in fibrosis in MMP-9-, Smad3- and TGFβ1-deficient mice, suggesting that IL-13 can operate independently from TGFβ1 [30]. This may explain the unexpected failure of Smad/TGFβ inhibitors in some blocking studies [126,127]. Thus, it remains unclear to what extent IL-13 must act through TGFβ1 to trigger fibrosis. Given that numerous antifibrotic therapies are focused on inhibiting the TGFβ1 signalling pathway [82,134], it will be important to determine whether the collagen-inducing activity of IL-13 is dependent on TGFβ1 or whether IL-13 and other profibrotic mediators [135] can also operate independently, as has been suggested in some studies [30, 53,135].

Vascular changes often accompany the development of fibrosis

In addition to fibroproliferation and deposition of ECM components, the pathogenesis of IPF, systemic sclerosis (SSc), liver fibrosis and many other fibrotic diseases, including many fibrotic diseases of the eye, are characterized by substantial vascular remodelling, which often occurs prior to the development of fibrosis. In the case of systemic sclerosis, vascular changes are a prominent and early manifestation of the disease, with impaired angiogenesis leading to the progressive disappearance of blood vessels [28,29]. It has been suggested that reduced numbers of circulating bone marrow-derived $CD34^+$ endothelial progenitor cells, as well as their impaired differentiation into mature endothelial cells, might be contributing to the early

vascular defects in SSc [136]. In contrast to SSc, where fibrosis is associated with the loss of blood vessels, fibrosis and traction retinal detachments associated with advanced diabetic retinopathy (DR) are characterized by uncontrolled vascular proliferation [137]. Indeed, the common pathway for many fibrotic eye diseases, including age-related macular degeneration (ARMD) [138], is injury to the cornea and/or retina, which results in inflammatory changes, tissue oedema, hypoxia-driven neovascularization and ultimately fibrosis. Once new blood vessels begin to grow in the eye, they are prone to haemorrhage, leading to further activation of the wound-healing response, and ultimately development of severe fibrosis [139]. Therefore, prevention of the primary vascular abnormality has been the most promising therapeutic strategy for many diseases of the eye. Because various members of the CXC-chemokine family exhibit potent angiogenic or angiostatic activity [140], targeting the CXC-chemokine family might offer a unique approach to regulate angiogenesis and fibrosis.

Angiotensin II plays a critical role in fibrosis

Although all major components of the renin–angiotensin–aldosterone system exhibit profibrotic activity, ANG II appears to be the dominant hormone responsible for cardiac fibrosis in hypertensive heart disease [141]. ANG II also plays an important role in the development of renal and hepatic fibrosis [142]. ANG II, produced locally by activated macrophages and fibroblasts, is thought to exert its effects by directly inducing NADPH oxidase activity, stimulating TGFβ1 production and triggering fibroblast proliferation and differentiation into collagen-secreting myofibroblasts [143,144]. In addition to its effects on TGFβ1 secretion and activation, ANG II also enhances TGFβ1 signalling by increasing SMAD2 levels and by augmenting the nuclear translocation of phosphorylated SMAD3. TGFβ1, in turn, augments the production of interstitial collagens, fibronectin and proteoglycans by cardiac myofibroblasts [2]. It also stimulates its own production in myofibroblasts, thereby establishing an autocrine cycle of myofibroblast differentiation and activation. Studies have shown that overexpression of TGFβ1 in transgenic mice can lead to cardiac hypertrophy, characterized by both interstitial fibrosis and hypertrophic growth of cardiac myocytes [145]. Patients suffering from idiopathic hypertrophic cardiomyopathy and dilated cardiomyopathy also have increased levels of TGFβ1 in the left ventricular myocardium [146]. Therefore, therapies that target the renin–angiotensin–aldosterone system or TGFβ1 pathways might provide effective strategies to slow the progression of fibrosis in hypertensive heart disease, progressive renal disease and hepatic fibrosis [144,147,148].

Endogenous mechanisms that slow the progression of fibrosis

Regulatory T cells (Tregs) and IL-10

IL-10 functions as a general immunosuppressive cytokine, which down-regulates chronic inflammatory responses through many mechanisms [149]. Consistent with its role as a suppressive cytokine, IL-10 has been shown to inhibit fibrosis in numerous models. Mice treated with IL-10 develop significantly less liver, lung and pancreatic fibrosis when challenged with carbon tetrachloride (CCl4), bleomcyin and cerulein, respectively [150–153]. In contrast, IL-10-deficient mice are much more susceptible to these fibrosis-inducing compounds. IL-10 has also been shown to significantly suppress the synthesis of type I collagens in human scar tissue-derived fibroblasts [154], indicating that it can directly inhibit fibrosis [155]. The severity of liver fibrosis in a subset of patients chronically infected with hepatitis C virus was also reduced by IL-10 treatment [156]. However, despite its success in some clinical studies, the mechanism by which IL-10 confers protection from fibrosis remains unclear. In the schistosomiasis model, IL-10 deficiency alone has little effect on the progression of hepatic fibrosis [157]. However, when IL-10$^{-/-}$ mice were crossed with IFN$\gamma^{-/-}$, IL-12$^{-/-}$ or IL-13Rα2$^{-/-}$ animals, liver fibrosis developed at a highly accelerated rate, suggesting that IL-10 cooperates with Th1 cytokines and the IL-13 decoy receptor to suppress collagen

deposition [74,158,159]. In support of these findings, a study of human *S. mansoni* infection found that most cases of severe periportal fibrosis are associated with low IL-10 and IFNγ production [79].

The IL-13 decoy receptor (IL-13Rα2)

Soluble IL-13Rα2-Fc is a highly effective inhibitor of IL-13 [90], which can ameliorate the progression of established fibrotic disease [53,95,160]. IL-13Rα2 inhibits IL-13 by blocking its interaction with the signalling type II IL-4R complex [90,98,161]. Consistent with its proposed activity as a decoy receptor [162], mice with targeted deletion of IL-13Rα2 displayed enhanced IL-13 activity [101]. When the IL-13Rα2-deficient mice were infected with *S. mansoni*, the development of IL-13-dependent liver fibrosis increased significantly [163]. Fibrosis increased despite the fact that there was no change in the inflammatory response. These findings suggested that IL-13Rα2 directly inhibits the ECM-remodelling activity of IL-13. However, the decoy receptor did play a significant role in the down-regulation of the inflammatory response in chronically infected animals [164]. In fact, the chronically infected IL-13R$\alpha 2^{-/-}$ mice showed a marked exacerbation in granulomatous inflammation. They also developed severe liver fibrosis and portal hypertension, which led to their rapid death following infection. Thus, the IL-13 decoy receptor was identified as a critical life-sustaining inhibitor of Th2-driven inflammation and fibrosis.

Can progressive fibrosis be reversed and normal tissue architecture restored?

Although the ability to repair damaged tissues without scarring would be ideal, in most chronic inflammatory diseases repair cannot be accomplished solely by the regeneration of parenchymal cells, even in tissues where significant regeneration is possible, such as the liver. Repair of damaged tissues must then occur by replacing non-regenerated parenchymal cells with connective tissues, which in time leads to significant fibrosis and scarring. Thus, development of therapeutic strategies that limit the progression of fibrosis without adversely affecting the overall repair process would represent an important technological advance.

It is controversial whether advanced fibrosis can be reversed to the extent that normal tissue architecture is restored completely. Indeed, there is substantial evidence that, if fibrosis is sufficiently advanced, reversal is no longer possible. Because advanced fibrosis is often hypocellular, it has been suggested that incomplete ECM degradation (irreversible fibrosis) develops when the appropriate cellular mediators (the source of MMPs) are no longer present [165]. Thus, ongoing inflammation might be required for the successful resolution of fibrotic disease [166]. Not surprisingly, the source and identity of key MMPs that mediate the resolution of fibrosis are being intensively investigated. Recent studies demonstrated that macrophage depletion at the onset of fibrosis resolution could retard ECM degradation and the loss of activated HSCs [115]. This suggests that macrophages are essential for initiating ECM degradation, perhaps by producing MMPs. Therefore, it might be possible to reverse what was once thought to be irreversible fibrosis [167]. Successful elimination of HBV and HCV in chronically infected individuals is often associated with marked regression of disease, providing evidence that human hepatic fibrosis is at least partially reversible [167]. Similar observations have also been reported in schistosomiasis patients following treatment with praziquantel, a drug that eliminates the causative pathogen [168]. Current approaches aimed at treating fibrosis are primarily directed at inhibiting cytokines (TGFβ1, IL-13), chemokines, specific MMPs, adhesion molecules (integrins) and inducers of angiogenesis, such as VEGF [138]. Although many of these treatments could prove highly successful, ideally, the best therapy would lead to the complete restoration of the damaged tissue, or minimally, restore homeostasis to the areas that drive the fibrotic response [169]. One way to restore homeostasis

would be to eliminate the collagen-producing cell. Indeed, apoptosis of hepatic stellate cells (HSCs) have been observed during the resolution of liver fibrosis [170]. Thus, methods that inhibit fibroblast proliferation and activation or actively induce myofibroblast apoptosis could help slow the progression of fibrosis [8,171,172]. Cell-based therapies using adult bone marrow-derived progenitor/stem cell technologies might also prove highly successful for the treatment of fibrosis. Stem cell therapies have already proved successful at restoring cardiac function in injured hearts [173], therefore they might prove successful for a wide variety of fibroproliferative disorders.

Moving experimental antifibrotic strategies into the clinic

As discussed in this review, there is a growing list of novel mediators and pathways that could be exploited in the development of antifibrotic drugs. These include cytokine, chemokine and TLR antagonists, angiogenesis inhibitors, anti-hypertensive drugs, TGFβ signalling modifiers, B cell-depleting antibodies and stem/progenitor cell transplantation strategies, to name just a few. As there are many potential targets and strategies, what we need now is a well thought-out plan for translating the available experimental information into clinically effective drugs. However, there are challenging roadblocks ahead that must be overcome before any treatment can reach the clinic. The most difficult obstacle will be to design effective clinical trials with well-defined clinical endpoints. Non-invasive techniques, such as serum markers, improved imaging techniques or other clinical features that can quickly quantify changes in the natural history of the disease (rate of disease progression, etc.) are desperately needed. Host genetic factors, such as single nucleotide polymorphisms (SNPs), may also be exploited to determine the relative risk of developing fibrosis. Recently, a predictive seven-gene signature was identified in chronic hepatitis C patients at high risk of developing cirrhosis [174]. In future studies, it will be important to explore what impact these or other SNPs have on fibrosis in other organ systems. Nearly 45% of all deaths in the developed world are attributed to some type of chronic fibroproliferative disease. Therefore, the demand for antifibrotic drugs that are both safe and effective is great and will likely continue to increase in the coming years.

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References

1. Wynn TA. Common and unique mechanisms regulate fibrosis in various fibroproliferative diseases. J Clin Invest 2007;117(3):524–529. [PubMed: 17332879]
2. Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA. Myofibroblasts and mechano-regulation of connective tissue remodelling. Nat Rev Mol Cell Biol 2002;3(5):349–363. [PubMed: 11988769]
3. Friedman SL. Mechanisms of disease: mechanisms of hepatic fibrosis and therapeutic implications. Nat Clin Pract Gastroenterol Hepatol 2004;1(2):98–105. [PubMed: 16265071]
4. Schuppan D, Ruehl M, Somasundaram R, Hahn EG. Matrix as a modulator of hepatic fibrogenesis. Semin Liver Dis 2001;21(3):351–372. [PubMed: 11586465]
5. Kumar, V.; Abbas, AK.; Fausto, N. Tissue renewal and repair: regeneration, healing, and fibrosis. In: Kumar, V.; Abbas, AK.; Fausto, N., editors. Pathologic Basis of Disease. Elsevier Saunders; Philadelphia, PA: 2005. p. 87-118.
6. Li MO, Wan YY, Sanjabi S, Robertson AK, Flavell RA. Transforming growth factor-beta regulation of immune responses. Annu Rev Immunol 2006;24:99–146. [PubMed: 16551245]
7. Wynn TA. IL-13 effector functions. Annu Rev Immunol 2003;21:425–456. [PubMed: 12615888]
8. Parsons CJ, Takashima M, Rippe RA. Molecular mechanisms of hepatic fibrogenesis. J Gastroenterol Hepatol 2007;22(suppl 1):S79–84. [PubMed: 17567474]

9. Pardo A, Selman M. Matrix metalloproteases in aberrant fibrotic tissue remodelling. Proc Am Thorac Soc 2006;3(4):383–388. [PubMed: 16738205]
10. Quan TE, Cowper SE, Bucala R. The role of circulating fibrocytes in fibrosis. Curr Rheumatol Rep 2006;8(2):145–150. [PubMed: 16569374]
11. Zeisberg EM, Tarnavski O, Zeisberg M, Dorfman AL, McMullen JR, Gustafsson E, et al. Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. Nat Med 2007;13(8):952–961. [PubMed: 17660828]Epub: 29 July 2007
12. Willis BC, du Bois RM, Borok Z. Epithelial origin of myofibroblasts during fibrosis in the lung. Proc Am Thorac Soc 2006;3(4):377–382. [PubMed: 16738204]
13. Kalluri R, Neilson EG. Epithelial–mesenchymal transition and its implications for fibrosis. J Clin Invest 2003;112(12):1776–1784. [PubMed: 14679171]
14. Bucala R, Spiegel LA, Chesney J, Hogan M, Cerami A. Circulating fibrocytes define a new leukocyte subpopulation that mediates tissue repair. Mol Med 1994;1(1):71–81. [PubMed: 8790603]
15. Ebihara Y, Masuya M, Larue AC, Fleming PA, Visconti RP, Minamiguchi H, et al. Hematopoietic origins of fibroblasts: II. *In vitro* studies of fibroblasts, CFU-F, and fibrocytes. Exp Hematol 2006;34 (2):219–229. [PubMed: 16459190]
16. Brittan M, Hunt T, Jeffery R, Poulsom R, Forbes SJ, Hodivala-Dilke K, et al. Bone marrow derivation of pericryptal myofibroblasts in the mouse and human small intestine and colon. Gut 2002;50(6): 752–757. [PubMed: 12010874]
17. Direkze NC, Forbes SJ, Brittan M, Hunt T, Jeffery R, Preston SL, et al. Multiple organ engraftment by bone-marrow-derived myofibroblasts and fibroblasts in bone-marrow-transplanted mice. Stem Cells 2003;21(5):514–520. [PubMed: 12968105]
18. Forbes SJ, Russo FP, Rey V, Burra P, Rugge M, Wright NA, et al. A significant proportion of myofibroblasts are of bone marrow origin in human liver fibrosis. Gastroenterology 2004;126(4): 955–963. [PubMed: 15057733]
19. Russo FP, Alison MR, Bigger BW, Amofah E, Florou A, Amin F, et al. The bone marrow functionally contributes to liver fibrosis. Gastroenterology 2006;130(6):1807–1821. [PubMed: 16697743]
20. Phillips RJ, Burdick MD, Hong K, Lutz MA, Murray LA, Xue YY, et al. Circulating fibrocytes traffic to the lungs in response to CXCL12 and mediate fibrosis. J Clin Invest 2004;114(3):438–446. [PubMed: 15286810]
21. Moore BB, Kolodsick JE, Thannickal VJ, Cooke K, Moore TA, Hogaboam C, et al. CCR2-mediated recruitment of fibrocytes to the alveolar space after fibrotic injury. Am J Pathol 2005;166(3):675–684. [PubMed: 15743780]
22. Haudek SB, Xia Y, Huebener P, Lee JM, Carlson S, Craw-ford JR, et al. Bone marrow-derived fibroblast precursors mediate ischemic cardiomyopathy in mice. Proc Natl Acad Sci USA 2006;103 (48):18284–18289. [PubMed: 17114286]Epub: 17 November 2006
23. Meneghin MD, Hogaboam C. Infectious disease, the innate immune response, and fibrosis. J Clin Invest 2007;117(3):530–538. [PubMed: 17332880]
24. Otte JM, Rosenberg IM, Podolsky DK. Intestinal myofibroblasts in innate immune responses of the intestine. Gastroenterology 2003;124(7):1866–1878. [PubMed: 12806620]
25. Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol 2004;4(7):499–511. [PubMed: 15229469]
26. Coelho AL, Hogaboam CM, Kunkel SL. Chemokines provide the sustained inflammatory bridge between innate and acquired immunity. Cytokine Growth Factor Rev 2005;16(6):553–560. [PubMed: 15967703]Epub: 20 June 2005
27. Strehlow D, Korn JH. Biology of the scleroderma fibroblast. Curr Opin Rheumatol 1998;10(6):572–578. [PubMed: 9812218]
28. Abraham DJ, Varga J. Scleroderma: from cell and molecular mechanisms to disease models. Trends Immunol 2005;26(11):587–595. [PubMed: 16168711]Epub: 15 September 2005
29. Varga J, Abraham D. Systemic sclerosis: a prototypic multisystem fibrotic disorder. J Clin Invest 2007;117(3):557–567. [PubMed: 17332883]
30. Kaviratne M, Hesse M, Leusink M, Cheever AW, Davies SJ, McKerrow JH, et al. IL-13 activates a mechanism of tissue fibrosis that is completely TGFβ-independent. J Immunol 2004;173(6):4020–4029. [PubMed: 15356151]

31. Moustakas A, Heldin CH. Non-Smad TGFβ signals. J Cell Sci 2005;118(16):3573–3584. [PubMed: 16105881]
32. Markiewicz M, Smith EA, Rubinchik S, Dong JY, Trojanowska M, LeRoy EC. The 72-kDa IE-1 protein of human cytomegalovirus (HCMV) is a potent inducer of connective tissue growth factor (CTGF) in human dermal fibroblasts. Clin Exp Rheumatol 2004;22(3 suppl 33):S31–34. [PubMed: 15344595]
33. Wang Y, Fan PS, Kahaleh B. Association between enhanced type I collagen expression and epigenetic repression of the *FLI1* gene in scleroderma fibroblasts. Arthritis Rheum 2006;54(7):2271–2279. [PubMed: 16802366]
34. Hasegawa M, Fujimoto M, Takehara K, Sato S. Pathogenesis of systemic sclerosis: altered B cell function is the key linking systemic autoimmunity and tissue fibrosis. J Dermatol Sci 2005;39(1):1–7. [PubMed: 15885984]
35. Wynn TA. Fibrotic disease and the T(H)1/T(H)2 paradigm. Nat Rev Immunol 2004;4(8):583–594. [PubMed: 15286725]
36. Ong CJ, Ip S, Teh SJ, Wong C, Jirik FR, Grusby MJ, et al. A role for T helper 2 cells in mediating skin fibrosis in tight-skin mice. Cell Immunol 1999;196(1):60–68. [PubMed: 10486156]
37. Lakos G, Melichian D, Wu M, Varga J. Increased bleomycin-induced skin fibrosis in mice lacking the Th1-specific transcription factor T-bet. Pathobiology 2006;73(5):224–237. [PubMed: 17314493]
38. Aliprantis AO, Wang J, Fathman JW, Lemaire R, Dorfman DM, Lafyatis R, et al. Transcription factor T-bet regulates skin sclerosis through its function in innate immunity and via IL-13. Proc Natl Acad Sci USA 2007;104(8):2827–2830. [PubMed: 17307869]Epub: 16 February 2007
39. Smith RE, Strieter RM, Phan SH, Lukacs NW, Huffnagle GB, Wilke CA, et al. Production and function of murine macrophage inflammatory protein-1α in bleomycin-induced lung injury. J Immunol 1994;153(10):4704–4712. [PubMed: 7525712]
40. Smith RE, Strieter RM, Zhang K, Phan SH, Standiford TJ, Lukacs NW, et al. A role for C–C chemokines in fibrotic lung disease. J Leukoc Biol 1995;57(5):782–787. [PubMed: 7539030]
41. Lloyd CM, Minto AW, Dorf ME, Proudfoot A, Wells TN, Salant DJ, et al. RANTES and monocyte chemoattractant protein-1 (MCP-1) play an important role in the inflammatory phase of crescentic nephritis, but only MCP-1 is involved in crescent formation and interstitial fibrosis. J Exp Med 1997;185(7):1371–1380. [PubMed: 9104823]
42. Belperio JA, Keane MP, Burdick MD, Lynch JP III, Xue YY, Berlin A, et al. Critical role for the chemokine MCP-1/CCR2 in the pathogenesis of bronchiolitis obliterans syndrome. J Clin Invest 2001;108(4):547–556. [PubMed: 11518728]
43. Tokuda A, Itakura M, Onai N, Kimura H, Kuriyama T, Matsushima K. Pivotal role of CCR1-positive leukocytes in bleomycin-induced lung fibrosis in mice. J Immunol 2000;164(5):2745–2751. [PubMed: 10679116]
44. Blease K, Mehrad B, Standiford TJ, Lukacs NW, Kunkel SL, Chensue SW, et al. Airway remodelling is absent in CCR1$^{-/-}$ mice during chronic fungal allergic airway disease. J Immunol 2000;165(3):1564–1572. [PubMed: 10903765]
45. Anders HJ, Vielhauer V, Frink M, Linde Y, Cohen CD, Blattner SM, et al. A chemokine receptor CCR-1 antagonist reduces renal fibrosis after unilateral ureter ligation. J Clin Invest 2002;109(2):251–259. [PubMed: 11805137]
46. Moore BB, Paine R III, Christensen PJ, Moore TA, Sitterding S, Ngan R, et al. Protection from pulmonary fibrosis in the absence of CCR2 signalling. J Immunol 2001;167(8):4368–4377. [PubMed: 11591761]
47. Zhu Z, Ma B, Zheng T, Homer RJ, Lee CG, Charo IF, et al. IL-13-induced chemokine responses in the lung: role of CCR2 in the pathogenesis of IL-13-induced inflammation and remodelling. J Immunol 2002;168(6):2953–2962. [PubMed: 11884467]
48. Gao JL, Wynn TA, Chang Y, Lee EJ, Broxmeyer HE, Cooper S, et al. Impaired host defence, hematopoiesis, granulomatous inflammation and type 1-type 2 cytokine balance in mice lacking CC chemokine receptor 1. J Exp Med 1997;185(11):1959–1968. [PubMed: 9166425]
49. Belperio JA, Dy M, Burdick MD, Xue YY, Li K, Elias JA, et al. Interaction of IL-13 and C10 in the pathogenesis of bleomycin-induced pulmonary fibrosis. Am J Respir Cell Mol Biol 2002;27(4):419–427. [PubMed: 12356575]

50. Ma B, Zhu Z, Homer RJ, Gerard C, Strieter R, Elias JA. The C10/CCL6 chemokine and CCR1 play critical roles in the pathogenesis of IL-13-induced inflammation and remodelling. J Immunol 2004;172(3):1872–1881. [PubMed: 14734772]
51. Wynn TA, Cheever AW, Jankovic D. Poindexter RW, Caspar P, Lewis FA, et al. An IL-12-based vaccination method for preventing fibrosis induced by schistosome infection. Nature 1995;376 (6541):594–596. [PubMed: 7637808]
52. Cheever AW, Williams ME, Wynn TA, Finkelman FD, Seder RA, Cox TM, et al. Anti-IL-4 treatment of *Schistosoma mansoni*-infected mice inhibits development of T cells and non-B, non-T cells expressing Th2 cytokines while decreasing egg-induced hepatic fibrosis. J Immunol 1994;153(2): 753–759. [PubMed: 8021510]
53. Chiaramonte MG, Donaldson DD, Cheever AW, Wynn TA. An IL-13 inhibitor blocks the development of hepatic fibrosis during a T-helper type 2-dominated inflammatory response. J Clin Invest 1999;104(6):777–785. [PubMed: 10491413]
54. Pesce J, Kaviratne M, Ramalingam TR, Thompson RW, Urban JF Jr, Cheever AW, et al. The IL-21 receptor augments Th2 effector function and alternative macrophage activation. J Clin Invest 2006;116(7):2044–2055. [PubMed: 16778988]Epub: 15 June 2006
55. Reiman RM, Thompson RW, Feng CG, Hari D, Knight R, Cheever AW, et al. Interleukin-5 (IL-5) augments the progression of liver fibrosis by regulating IL-13 activity. Infect Immun 2006;74(3): 1471–1479. [PubMed: 16495517]
56. Gurujeyalakshmi G, Giri SN. Molecular mechanisms of antifibrotic effect of interferon-γ in bleomycin-mouse model of lung fibrosis: downregulation of TGFβ and procollagen I and III gene expression. Exp Lung Res 1995;21(5):791–808. [PubMed: 8556994]
57. Keane MP, Belperio JA, Burdick MD, Strieter RM. IL-12 attenuates bleomycin-induced pulmonary fibrosis. Am J Physiol Lung Cell Mol Physiol 2001;281(1):L92–97. [PubMed: 11404251]
58. Oldroyd SD, Thomas GL, Gabbiani G, El Nahas AM. Interferon-γ inhibits experimental renal fibrosis. Kidney Int 1999;56(6):2116–2127. [PubMed: 10594787]
59. Poynard T, Yuen MF, Ratziu V, Lai CL. Viral hepatitis C. Lancet 2003;362(9401):2095–2100. [PubMed: 14697814]
60. Hesse M, Cheever AW, Jankovic D, Wynn TA. NOS-2 mediates the protective anti-inflammatory and antifibrotic effects of the Th1-inducing adjuvant, IL-12, in a Th2 model of granulomatous disease. Am J Pathol 2000;157(3):945–955. [PubMed: 10980133]
61. Hessel EM, Chu M, Lizcano JO, Chang B, Herman N, Kell SA, et al. Immunostimulatory oligonucleotides block allergic airway inflammation by inhibiting Th2 cell activation and IgE-mediated cytokine induction. J Exp Med 2005;202(11):1563–1573. [PubMed: 16314434]Epub: 28 November 2005
62. Hoffmann KF, McCarty TC, Segal DH, Chiaramonte M, Hesse M, Davis EM, et al. Disease fingerprinting with cDNA microarrays reveals distinct gene expression profiles in lethal type 1 and type 2 cytokine-mediated inflammatory reactions. FASEB J 2001;15(13):2545–2547. [PubMed: 11641263]
63. Sandler NG, Mentink-Kane MM, Cheever AW, Wynn TA. Global gene expression profiles during acute pathogen-induced pulmonary inflammation reveal divergent roles for Th1 and Th2 responses in tissue repair. J Immunol 2003;171(7):3655–3667. [PubMed: 14500663]
64. Zeeberg BR, Qin H, Narasimhan S, Sunshine M, Cao H, Kane DW, et al. High-throughput GoMiner, an 'industrial-strength' integrative gene ontology tool for interpretation of multiple-microarray experiments, with application to studies of common variable immune deficiency (CVID). BMC Bioinformat 2005;6:168.
65. Walker LS, Abbas AK. The enemy within: keeping self-reactive T cells at bay in the periphery. Nat Rev Immunol 2002;2(1):11–19. [PubMed: 11908514]
66. Hesse M, Modolell M, La Flamme AC, Schito M, Fuentes JM, Cheever AW, et al. Differential regulation of nitric oxide synthase-2 and arginase-1 by type 1/type 2 cytokines *in vivo*: granulomatous pathology is shaped by the pattern of L-arginine metabolism. J Immunol 2001;167(11):6533–6544. [PubMed: 11714822]

67. Decitre M, Gleyzal C, Raccurt M, Peyrol S, Aubert-Foucher E, Csiszar K, et al. Lysyl oxidase-like protein localizes to sites of *de novo* fibrinogenesis in fibrosis and in the early stromal reaction of ductal breast carcinomas. Lab Invest 1998;78(2):143–151. [PubMed: 9484712]
68. Akiri G, Sabo E, Dafni H, Vadasz Z, Kartvelishvily Y, Gan N, et al. Lysyl oxidase-related protein-1 promotes tumor fibrosis and tumor progression *in vivo*. Cancer Res 2003;63(7):1657–1666. [PubMed: 12670920]
69. Wang S, Hirschberg R. BMP7 antagonizes TGFβ-dependent fibrogenesis in mesangial cells. Am J Physiol Renal Physiol 2003;284(5):F1006–1013. [PubMed: 12676736]
70. Cheng S, Lovett DH. Gelatinase A (MMP-2) is necessary and sufficient for renal tubular cell epithelial–mesenchymal transformation. Am J Pathol 2003;162(6):1937–1949. [PubMed: 12759250]
71. Underwood DC, Osborn RR, Bochnowicz S, Webb EF, Rieman DJ, Lee JC, et al. SB 239063, a p38 MAPK inhibitor, reduces neutrophilia, inflammatory cytokines, MMP-9, and fibrosis in lung. Am J Physiol Lung Cell Mol Physiol 2000;279(5):L895–902. [PubMed: 11053025]
72. Heymans S, Lupu F, Terclavers S, Vanwetswinkel B, Herbert JM, Baker A, et al. Loss or inhibition of uPA or MMP-9 attenuates LV remodelling and dysfunction after acute pressure overload in mice. Am J Pathol 2005;166(1):15–25. [PubMed: 15631996]
73. Kim H, Oda T, Lopez-Guisa J, Wing D, Edwards DR, Soloway PD, et al. TIMP-1 deficiency does not attenuate interstitial fibrosis in obstructive nephropathy. J Am Soc Nephrol 2001;12(4):736–748. [PubMed: 11274235]
74. Vaillant B, Chiaramonte MG, Cheever AW, Soloway PD, Wynn TA. Regulation of hepatic fibrosis and extracellular matrix genes by the Th response: New insight into the role of tissue inhibitors of matrix metalloproteinases. J Immunol 2001;167(12):7017–7026. [PubMed: 11739522]
75. Kaminski N, Allard JD, Pittet JF, Zuo F, Griffiths MJ, Morris D, et al. Global analysis of gene expression in pulmonary fibrosis reveals distinct programs regulating lung inflammation and fibrosis. Proc Natl Acad Sci USA 2000;97(4):1778–1783. [PubMed: 10677534]
76. Kristensen DB, Kawada N, Imamura K, Miyamoto Y, Tateno C, Seki S, et al. Proteome analysis of rat hepatic stellate cells. Hepatology 2000;32(2):268–277. [PubMed: 10915733]
77. Emura M, Nagai S, Takeuchi M, Kitaichi M, Izumi T, Nishimura K, et al. *In vitro* production of B cell growth factor and B cell differentiation factor by peripheral blood mononuclear cells and bronchoalveolar lavage T lymphocytes from patients with idiopathic pulmonary fibrosis. Clin Exp Immunol 1990;82(1):133–139. [PubMed: 2208788]
78. Wallace WA, Ramage EA, Lamb D, Howie SE. A type 2 (Th2-like) pattern of immune response predominates in the pulmonary interstitium of patients with cryptogenic fibrosing alveolitis (CFA). Clin Exp Immunol 1995;101(3):436–441. [PubMed: 7664490]
79. Booth M, Mwatha JK, Joseph S, Jones FM, Kadzo H, Ireri E, et al. Periportal fibrosis in human *Schistosoma mansoni* infection is associated with low IL-10, low IFNγ, high TNFα, or low RANTES, depending on age and gender. J Immunol 2004;172(2):1295–1303. [PubMed: 14707108]
80. Buttner C, Skupin A, Reimann T, Rieber EP, Unteregger G, Geyer P, et al. Local production of interleukin-4 during radiation-induced pneumonitis and pulmonary fibrosis in rats: macrophages as a prominent source of interleukin-4. Am J Respir Cell Mol Biol 1997;17(3):315–325. [PubMed: 9308918]
81. Fertin C, Nicolas JF, Gillery P, Kalis B, Banchereau J, Maquart FX. Interleukin-4 stimulates collagen synthesis by normal and scleroderma fibroblasts in dermal equivalents. Cell Mol Biol 1991;37(8): 823–829. [PubMed: 1807791]
82. Letterio JJ, Roberts AB. Regulation of immune responses by TGFβ. Annu Rev Immunol 1998;16:137–161. [PubMed: 9597127]
83. Sempowski GD, Beckmann MP, Derdak S, Phipps RP. Subsets of murine lung fibroblasts express membrane-bound and soluble IL-4 receptors. Role of IL-4 in enhancing fibroblast proliferation and collagen synthesis. J Immunol 1994;152(7):3606–3614. [PubMed: 7908305]
84. Doucet C, Brouty-Boye D, Pottin-Clemenceau C, Canonica GW, Jasmin C, Azzarone B. Interleukin (IL)-4 and IL-13 act on human lung fibroblasts. Implication in asthma. J Clin Invest 1998;101(10): 2129–2139. [PubMed: 9593769]

85. Ong C, Wong C, Roberts CR, Teh HS, Jirik FR. Anti-IL-4 treatment prevents dermal collagen deposition in the tight-skin mouse model of scleroderma. Eur J Immunol 1998;28(9):2619–2629. [PubMed: 9754550]

86. Le Moine A, Flamand V, Demoor FX, Noel JC, Surquin M, Kiss R, et al. Critical roles for IL-4, IL-5, and eosinophils in chronic skin allograft rejection. J Clin Invest 1999;103(12):1659–1667. [PubMed: 10377172]

87. Zurawski SM, Vega F Jr, Huyghe B, Zurawski G. Receptors for interleukin-13 and interleukin-4 are complex and share a novel component that functions in signal transduction. EMBO J 1993;12(7): 2663–2670. [PubMed: 8101483]

88. McKenzie GJ, Emson CL, Bell SE, Anderson S, Fallon P, Zurawski G, et al. Impaired development of Th2 cells in IL-13-deficient mice. Immunity 1998;9(3):423–432. [PubMed: 9768762]

89. Zhu Z, Homer RJ, Wang Z, Chen Q, Geba GP, Wang J, et al. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. J Clin Invest 1999;103(6):779–788. [PubMed: 10079098]

90. Donaldson DD, Whitters MJ, Fitz LJ, Neben TY, Finnerty H, Henderson SL, et al. The murine IL-13 receptor $\alpha 2$: molecular cloning, characterization, and comparison with murine IL-13 receptor $\alpha 1$. J Immunol 1998;161(5):2317–2324. [PubMed: 9725226]

91. Blease K, Jakubzick C, Westwick J, Lukacs N, Kunkel SL, Hogaboam CM. Therapeutic effect of IL-13 immunoneutralization during chronic experimental fungal asthma. J Immunol 2001;166(8): 5219–5224. [PubMed: 11290806]

92. Kumar RK, Herbert C, Yang M, Koskinen AM, McKenzie AN, Foster PS. Role of interleukin-13 in eosinophil accumulation and airway remodelling in a mouse model of chronic asthma. Clin Exp Allergy 2002;32(7):1104–1111. [PubMed: 12100061]

93. Keane MP, Gomperts BN, Weigt S, Xue YY, Burdick MD, Nakamura H, et al. IL-13 is pivotal in the fibro-obliterative process of bronchiolitis obliterans syndrome. J Immunol 2007;178(1):511–519. [PubMed: 17182591]

94. Kolodsick JE, Toews GB, Jakubzick C, Hogaboam C, Moore TA, McKenzie A, et al. Protection from fluorescein isothiocyanate-induced fibrosis in IL-13-deficient, but not IL-4-deficient, mice results from impaired collagen synthesis by fibroblasts. J Immunol 2004;172(7):4068–4076. [PubMed: 15034018]

95. Chiaramonte MG, Cheever AW, Malley JD, Donaldson DD, Wynn TA. Studies of murine schistosomiasis reveal interleukin-13 blockade as a treatment for established and progressive liver fibrosis. Hepatology 2001;34(2):273–282. [PubMed: 11481612]

96. Fallon PG, Richardson EJ, McKenzie GJ, McKenzie AN. Schistosome infection of transgenic mice defines distinct and contrasting pathogenic roles for IL-4 and IL-13: IL-13 is a profibrotic agent. J Immunol 2000;164(5):2585–2591. [PubMed: 10679097]

97. Rankin JA, Picarella DE, Geba GP, Temann UA, Prasad B, DiCosmo B, et al. Phenotypic and physiologic characterization of transgenic mice expressing interleukin 4 in the lung: lymphocytic and eosinophilic inflammation without airway hyperreactivity. Proc Natl Acad Sci USA 1996;93 (15):7821–7825. [PubMed: 8755560]

98. Webb DC, Mahalingam S, Cai Y, Matthaei KI, Donaldson DD, Foster PS. Antigen-specific production of interleukin (IL)-13 and IL-5 cooperate to mediate IL-4Rα-independent airway hyperreactivity. Eur J Immunol 2003;33(12):3377–3385. [PubMed: 14635046]

99. Blease K, Schuh JM, Jakubzick C, Lukacs NW, Kunkel SL, Joshi BH, et al. Stat6-deficient mice develop airway hyperresponsiveness and peribronchial fibrosis during chronic fungal asthma. Am J Pathol 2002;160(2):481–490. [PubMed: 11839568]

100. Fichtner-Feigl S, Strober W, Kawakami K, Puri RK, Kitani A. IL-13 signalling through the IL-13$\alpha 2$ receptor is involved in induction of TGF$\beta 1$ production and fibrosis. Nat Med 2006;12(1): 99–106. [PubMed: 16327802]Epub: 4 December 2005

101. Wood N, Whitters MJ, Jacobson BA, Witek J, Sypek JP, Kasaian M, et al. Enhanced interleukin (IL)-13 responses in mice lacking IL-13 receptor $\alpha 2$. J Exp Med 2003;197(6):703–709. [PubMed: 12642602]

102. Gharaee-Kermani M, Phan SH. Lung interleukin-5 expression in murine bleomycin-induced pulmonary fibrosis. Am J Respir Cell Mol Biol 1997;16(4):438–447. [PubMed: 9115755]

103. Ochkur SI, Jacobsen EA, Protheroe CA, Biechele TL, Pero RS, McGarry MP, et al. Coexpression of IL-5 and eotaxin-2 in mice creates an eosinophil-dependent model of respiratory inflammation with characteristics of severe asthma. J Immunol 2007;178(12):7879–7889. [PubMed: 17548626]
104. Williams TJ. The eosinophil enigma. J Clin Invest 2004;113(4):507–509. [PubMed: 14966558]
105. Sher A, Coffman RL, Heiny S, Scott P, Cheever AW. Interleukin 5 is required for the blood and tissue eosinophilia but not granuloma formation induced by infection with *Schistosoma mansoni*. Proc Natl Acad Sci USA 1990;87:61–64. [PubMed: 2104985]
106. Hao H, Cohen DA, Jennings CD, Bryson JS, Kaplan AM. Bleomycin-induced pulmonary fibrosis is independent of eosinophils. J Leukoc Biol 2000;68(4):515–521. [PubMed: 11037973]
107. Gharaee-Kermani M, McGarry B, Lukacs N, Huffnagle G, Egan RW, Phan SH. The role of IL-5 in bleomycin-induced pulmonary fibrosis. J Leukoc Biol 1998;64(5):657–666. [PubMed: 9823772]
108. Blyth DI, Wharton TF, Pedrick MS, Savage TJ, Sanjar S. Airway subepithelial fibrosis in a murine model of atopic asthma: suppression by dexamethasone or anti-interleukin-5 antibody. Am J Respir Cell Mol Biol 2000;23(2):241–246. [PubMed: 10919992]
109. Cho JY, Miller M, Baek KJ, Han JW, Nayar J, Lee SY, et al. Inhibition of airway remodelling in IL-5-deficient mice. J Clin Invest 2004;113(4):551–560. [PubMed: 14966564]
110. Trifilieff A, Fujitani Y, Coyle AJ, Kopf M, Bertrand C. IL-5 deficiency abolishes aspects of airway remodelling in a murine model of lung inflammation. Clin Exp Allergy 2001;31(6):934–942. [PubMed: 11422160]
111. Huaux F, Liu T, McGarry B, Ullenbruch M, Xing Z, Phan SH. Eosinophils and T lymphocytes possess distinct roles in bleomycin-induced lung injury and fibrosis. J Immunol 2003;171(10): 5470–5481. [PubMed: 14607953]
112. Mattes J, Yang M, Mahalingam S, Kuehr J, Webb DC, Simson L, et al. Intrinsic defect in T cell production of interleukin (IL)-13 in the absence of both IL-5 and eotaxin precludes the development of eosinophilia and airways hyperreactivity in experimental asthma. J Exp Med 2002;195(11): 1433–1444. [PubMed: 12045241]
113. Tanaka H, Komai M, Nagao K, Ishizaki M, Kajiwara D, Takatsu K, et al. Role of IL-5 and eosinophils in allergen-induced airway remodelling in mice. Am J Respir Cell Mol Biol 2004;19:19.
114. Frohlich A, Marsland BJ, Sonderegger I, Kurrer M, Hodge MR, Harris NL, et al. IL-21 receptor signalling is integral to the development of Th2 effector responses *in vivo*. Blood 2007;109(5): 2023–2031. [PubMed: 17077330]Epub: 31 October 2006
115. Duffield JS, Forbes SJ, Constandinou CM, Clay S, Partolina M, Vuthoori S, et al. Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. J Clin Invest 2005;115 (1):56–65. [PubMed: 15630444]
116. Sato M, Muragaki Y, Saika S, Roberts AB, Ooshima A. Targeted disruption of TGFβ1/Smad3 signalling protects against renal tubulointerstitial fibrosis induced by unilateral ureteral obstruction. J Clin Invest 2003;112(10):1486–1494. [PubMed: 14617750]
117. Border WA, Noble NA, Yamamoto T, Harper JR, Yamaguchi Y, Pierschbacher MD, et al. Natural inhibitor of transforming growth factor-β protects against scarring in experimental kidney disease. Nature 1992;360(6402):361–364. [PubMed: 1280332]
118. Clouthier DE, Comerford SA, Hammer RE. Hepatic fibrosis, glomerulosclerosis, and a lipodystrophy-like syndrome in PEPCK-TGFβ1 transgenic mice. J Clin Invest 1997;100(11):2697–2713. [PubMed: 9389733]
119. Sime PJ, Xing Z, Graham FL, Csaky KG, Gauldie J. Adeno-vector-mediated gene transfer of active transforming growth factor-β1 induces prolonged severe fibrosis in rat lung. J Clin Invest 1997;100 (4):768–776. [PubMed: 9259574]
120. Gorelik L, Flavell RA. Transforming growth factor-β in T-cell biology. Nat Rev Immunol 2002;2 (1):46–53. [PubMed: 11905837]
121. Munger JS, Huang X, Kawakatsu H, Griffiths MJ, Dalton SL, Wu J, et al. The integrin $\alpha v\beta 6$ binds and activates latent TGFβ1: a mechanism for regulating pulmonary inflammation and fibrosis. Cell 1999;96(3):319–328. [PubMed: 10025398]
122. Roberts AB, Russo A, Felici A, Flanders KC. Smad3: a key player in pathogenetic mechanisms dependent on TGFβ. Ann NY Acad Sci 2003;995:1–10. [PubMed: 12814934]

123. Flanders KC, Sullivan CD, Fujii M, Sowers A, Anzano MA, Arabshahi A, et al. Mice lacking Smad3 are protected against cutaneous injury induced by ionizing radiation. Am J Pathol 2002;160(3): 1057–1068. [PubMed: 11891202]

124. Bhowmick NA, Chytil A, Plieth D, Gorska AE, Dumont N, Shappell S, et al. TGFβ signalling in fibroblasts modulates the oncogenic potential of adjacent epithelia. Science 2004;303(5659):848–851. [PubMed: 14764882]

125. Khalil N, Corne S, Whitman C, Yacyshyn H. Plasmin regulates the activation of cell-associated latent TGFβ1 secreted by rat alveolar macrophages after *in vivo* bleomycin injury. Am J Respir Cell Mol Biol 1996;15(2):252–259. [PubMed: 8703482]

126. Ma LJ, Yang H, Gaspert A, Carlesso G, Barty MM, Davidson JM, et al. Transforming growth factor-β-dependent and-independent pathways of induction of tubulointerstitial fibrosis in $\beta 6^{-/-}$ mice. Am J Pathol 2003;163(4):1261–1273. [PubMed: 14507636]

127. Ashcroft GS, Yang X, Glick AB, Weinstein M, Letterio JL, Mizel DE, et al. Mice lacking Smad3 show accelerated wound healing and an impaired local inflammatory response. Nat Cell Biol 1999;1 (5):260–266. [PubMed: 10559937]

128. Lee CG, Homer RJ, Zhu Z, Lanone S, Wang X, Koteliansky V, et al. Interleukin-13 induces tissue fibrosis by selectively stimulating and activating transforming growth factor-β1. J Exp Med 2001;194(6):809–821. [PubMed: 11560996]

129. Lanone S, Zheng T, Zhu Z, Liu W, Lee CG, Ma B, et al. Overlapping and enzyme-specific contributions of matrix metalloproteinases-9 and-12 in IL-13-induced inflammation and remodelling. J Clin Invest 2002;110(4):463–474. [PubMed: 12189240]

130. Elias JA, Zhu Z, Chupp G, Homer RJ. Airway remodelling in asthma. J Clin Invest 1999;104(8): 1001–1006. [PubMed: 10525034]

131. Nakao A, Miike S, Hatano M, Okumura K, Tokuhisa T, Ra C, et al. Blockade of transforming growth factor beta/Smad signalling in T cells by overexpression of Smad7 enhances antigen-induced airway inflammation and airway reactivity. J Exp Med 2000;192(2):151–158. [PubMed: 10899902]

132. Hansen G, McIntire JJ, Yeung VP, Berry G, Thorbecke GJ, Chen L, et al. $CD4^+$ T helper cells engineered to produce latent TGFβ1 reverse allergen-induced airway hyperreactivity and inflammation. J Clin Invest 2000;105(1):61–70. [PubMed: 10619862]

133. Kitani A, Fuss I, Nakamura K, Kumaki F, Usui T, Strober W. Transforming growth factor (TGF)-β1-producing regulatory T cells induce Smad-mediated interleukin 10 secretion that facilitates coordinated immunoregulatory activity and amelioration of TGFβ1-mediated fibrosis. J Exp Med 2003;198(8):1179–1188. [PubMed: 14557415]Epub: 13 October 2003

134. Daniels CE, Wilkes MC, Edens M, Kottom TJ, Murphy SJ, Limper AH, et al. Imatinib mesylate inhibits the profibrogenic activity of TGFβ and prevents bleomycin-mediated lung fibrosis. J Clin Invest 2004;114(9):1308–1316. [PubMed: 15520863]

135. Oriente A, Fedarko NS, Pacocha SE, Huang SK, Lichtenstein LM, Essayan DM. Interleukin-13 modulates collagen homeostasis in human skin and keloid fibroblasts. J Pharmacol Exp Ther 2000;292(3):988–994. [PubMed: 10688614]

136. Del Papa N, Quirici N, Soligo D, Scavullo C, Cortiana M, Borsotti C, et al. Bone marrow endothelial progenitors are defective in systemic sclerosis. Arthritis Rheum 2006;54(8):2605–2615. [PubMed: 16868984]

137. Wilkinson-Berka JL. Angiotensin and diabetic retinopathy. Int J Biochem Cell Biol 2006;38(5–6): 752–765. [PubMed: 16165393]Epub: 1 September 2005

138. Rattner A, Nathans J. Macular degeneration: recent advances and therapeutic opportunities. Nat Rev Neurosci 2006;7(11):860–872. [PubMed: 17033682]Epub: 11 October 2006

139. Friedlander M. Fibrosis and diseases of the eye. J Clin Invest 2007;117(3):576–586. [PubMed: 17332885]

140. Strieter RM, Gomperts BN, Keane MP. The role of CXC chemokines in pulmonary fibrosis. J Clin Invest 2007;117(3):549–556. [PubMed: 17332882]

141. Watanabe T, Barker TA, Berk BC. Angiotensin II and the endothelium: diverse signals and effects. Hypertension 2005;45(2):163–169. [PubMed: 15630047]Epub: 3 January 2005

142. Mezzano SA, Ruiz-Ortega M, Egido J. Angiotensin II and renal fibrosis. Hypertension 2001;38(3 Pt 2):635–638. [PubMed: 11566946]

143. Rosenkranz S. TGFβ1 and angiotensin networking in cardiac remodelling. Cardiovasc Res 2004;63 (3):423–432. [PubMed: 15276467]

144. Bataller R, Schwabe RF, Choi YH, Yang L, Paik YH, Lindquist J, et al. NADPH oxidase signal transduces angiotensin II in hepatic stellate cells and is critical in hepatic fibrosis. J Clin Invest 2003;112(9):1383–1394. [PubMed: 14597764]

145. Rosenkranz S, Flesch M, Amann K, Haeuseler C, Kilter H, Seeland U, et al. Alterations of β-adrenergic signalling and cardiac hypertrophy in transgenic mice overexpressing TGFβ1. Am J Physiol Heart Circ Physiol 2002;283(3):H1253–1262. [PubMed: 12181157]

146. Li RK, Li G, Mickle DA, Weisel RD, Merante F, Luss H, et al. Overexpression of transforming growth factor-β1 and insulin-like growth factor-I in patients with idiopathic hypertrophic cardiomyopathy. Circulation 1997;96(3):874–881. [PubMed: 9264495]

147. Berk BC, Fujiwara K, Lehoux S. ECM remodelling in hypertensive heart disease. J Clin Invest 2007;117(3):568–575. [PubMed: 17332884]

148. Iwano M, Neilson EG. Mechanisms of tubulointerstitial fibrosis. Curr Opin Nephrol Hypertens 2004;13(3):279–284. [PubMed: 15073485]

149. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol 2001;19:683–765. [PubMed: 11244051]

150. Thompson K, Maltby J, Fallowfield J, McAulay M, Millward-Sadler H, Sheron N. Interleukin-10 expression and function in experimental murine liver inflammation and fibrosis. Hepatology 1998;28(6):1597–1606. [PubMed: 9828224]

151. Louis H, Van Laethem JL, Wu W, Quertinmont E, Degraef C, Van den Berg K, et al. Interleukin-10 controls neutrophilic infiltration, hepatocyte proliferation, and liver fibrosis induced by carbon tetrachloride in mice. Hepatology 1998;28(6):1607–1615. [PubMed: 9828225]

152. Arai T, Abe K, Matsuoka H, Yoshida M, Mori M, Goya S, et al. Introduction of the interleukin-10 gene into mice inhibited bleomycin-induced lung injury *in vivo*. Am J Physiol Lung Cell Mol Physiol 2000;278(5):L914–922. [PubMed: 10781421]

153. Demols A, Van Laethem JL, Quertinmont E, Degraef C, Delhaye M, Geerts A, et al. Endogenous interleukin-10 modulates fibrosis and regeneration in experimental chronic pancreatitis. Am J Physiol Gastrointest Liver Physiol 2002;282(6):G1105–1112. [PubMed: 12016137]

154. Wangoo A, Laban C, Cook HT, Glenville B, Shaw RJ. Interleukin-10- and corticosteroid-induced reduction in type I procollagen in a human *ex vivo* scar culture. Int J Exp Pathol 1997;78(1):33–41. [PubMed: 9166103]

155. Wang SC, Ohata M, Schrum L, Rippe RA, Tsukamoto H. Expression of interleukin-10 by *in vitro* and *in vivo* activated hepatic stellate cells. J Biol Chem 1998;273(1):302–308. [PubMed: 9417080]

156. Nelson DR, Tu Z, Soldevila-Pico C, Abdelmalek M, Zhu H, Xu YL, et al. Long-term interleukin 10 therapy in chronic hepatitis C patients has a proviral and anti-inflammatory effect. Hepatology 2003;38(4):859–868. [PubMed: 14512873]

157. Wynn TA, Cheever AW, Williams ME, Hieny S, Caspar P, Kühn R, et al. IL-10 regulates liver pathology in acute murine schistosomiasis *mansoni* but is not required for immune down-modulation of chronic disease. J Immunol 1998;160:5000–5008. [PubMed: 9590249]

158. Hoffmann KF, Cheever AW, Wynn TA. IL-10 and the dangers of immune polarization: excessive type 1 and type 2 cytokine responses induce distinct forms of lethal immunopathology in murine schistosomiasis. J Immunol 2000;164(12):6406–6416. [PubMed: 10843696]

159. Wilson MS, Elnekave E, Mentink-Kane M, Hodges MG, Pesce JT, Ramalingam TR, et al. IL-13Ra2 and IL-10 coordinately suppresss airway inflammation, airway-hyperreactivity, and fibrosis in mice. J Clin Invest 2007;117:2941–2951. [PubMed: 17885690]

160. Taube C, Duez C, Cui ZH, Takeda K, Rha YH, Park JW, et al. The role of IL-13 in established allergic airway disease. J Immunol 2002;169(11):6482–6489. [PubMed: 12444158]

161. Mattes J, Yang M, Siqueira A, Clark K, MacKenzie J, McKenzie AN, et al. IL-13 induces airways hyperreactivity independently of the IL-4R α chain in the allergic lung. J Immunol 2001;167(3): 1683–1692. [PubMed: 11466392]

162. Feng N, Lugli SM, Schnyder B, Gauchat JF, Graber P, Schlagenhauf E, et al. The interleukin-4/ interleukin-13 receptor of human synovial fibroblasts: overexpression of the nonsignalling interleukin-13 receptor α2. Lab Invest 1998;78(5):591–602. [PubMed: 9605184]

163. Chiaramonte MG, Mentink-Kane M, Jacobson BA, Cheever AW, Whitters MJ, Goad ME, et al. Regulation and function of the interleukin 13 receptor $\alpha 2$ during a T helper cell type 2-dominant immune response. J Exp Med 2003;197(6):687–701. [PubMed: 12642601]

164. Mentink-Kane MM, Cheever AW, Thompson RW, Hari DM, Kabatereine NB, Vennervald BJ, et al. IL-13 receptor $\alpha 2$ down-modulates granulomatous inflammation and prolongs host survival in schistosomiasis. Proc Natl Acad Sci USA 2004;101(2):90–586.Epub: 29 December 2003

165. Issa R, Zhou X, Constandinou CM, Fallowfield J, Millward-Sadler H, Gaca MD, et al. Spontaneous recovery from micronodular cirrhosis: evidence for incomplete resolution associated with matrix cross-linking. Gastroenterology 2004;126(7):808–1795.

166. Iredale JP. Models of liver fibrosis: exploring the dynamic nature of inflammation and repair in a solid organ. J Clin Invest 2007;117(3):539–548. [PubMed: 17332881]

167. Fallowfield JA, Kendall TJ, Iredale JP. Reversal of fibrosis: no longer a pipe dream? Clin Liver Dis 2006;10(3):481–497. [PubMed: 17162224]

168. Vennervald BJ, Dunne DW. Morbidity in schistosomiasis: an update. Curr Opin Infect Dis 2004;17 (5):439–447. [PubMed: 15353964]

169. Lund RD, Wang S, Klimanskaya I, Holmes T, Ramos-Kelsey R, Lu B, et al. Human embryonic stem cell-derived cells rescue visual function in dystrophic RCS rats. Cloning Stem Cells 2006;8 (3):189–199. [PubMed: 17009895]

170. Iredale JP, Benyon RC, Pickering J, McCullen M, Northrop M, Pawley S, et al. Mechanisms of spontaneous resolution of rat liver fibrosis. Hepatic stellate cell apoptosis and reduced hepatic expression of metalloproteinase inhibitors. J Clin Invest 1998;102(3):538–549. [PubMed: 9691091]

171. Thannickal VJ, Toews GB, White ES, Lynch JP 3rd, Martinez FJ. Mechanisms of pulmonary fibrosis. Annu Rev Med 2004;55:395–417. [PubMed: 14746528]

172. Wright MC, Issa R, Smart DE, Trim N, Murray GI, Primrose JN, et al. Gliotoxin stimulates the apoptosis of human and rat hepatic stellate cells and enhances the resolution of liver fibrosis in rats. Gastroenterology 2001;121(3):685–698. [PubMed: 11522753]

173. Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. J Cell Biochem 2006;98(5): 1076–1084. [PubMed: 16619257]

174. Huang H, Shiffman ML, Friedman S, Venkatesh R, Bzowej N, Abar OT, et al. A seven gene signature identifies the risk of developing cirrhosis in patients with chronic hepatitis C. Hepatology 2007;46 (2):297–306. [PubMed: 17461418]

Table 1

Major tissues affected by fibrosis and possible contributing factors

- *Liver*—Viral hepatitis, schistosomiasis, and alcoholism are leading causes of cirrhosis worldwide.
- *Lung*—The interstitial lung diseases (ILDs) include a diverse set of disorders in which pulmonary inflammation and fibrosis are the final common pathological manifestations. There are more than 150 different causes of ILDs, including sarcoidosis, silicosis, drug reactions and infections, as well as collagen vascular diseases, such as rheumatoid arthritis and systemic sclerosis (scleroderma). Idiopathic pulmonary fibrosis, the most common type of ILD, has no known cause
- *Kidney disease*—Diabetes damages and scars the kidneys, which can lead to a progressive loss of function. Untreated hypertension can contribute
- *Heart and vascular disease*—Following a heart attack, scar tissue can impair the ability of the heart to pump blood. Hypertension, atherosclerosis and restenosis also contribute
- *Eye*—Macular degeneration, retinal and vitreal retinopathy can lead to blindness
- *Skin*—Including keloids and hypertrophic scars. Systemic sclerosis and scleroderma, burns and genetic factors may also contribute
- *Pancreas*—Poorly understood but possible autoimmune/hereditary causes
- *Intestine*—Crohn's disease/inflammatory bowel disease. Pathogenic orgnanisms
- *Brain*—Alzheimer's disease, AIDS
- *Bone marrow*—Cancer and ageing
- *Multi-organ fibrosis*—(a) Due to surgical complications; scar tissue can form between internal organs, causing contracture, pain and, in some cases, infertility; (b) chemotherapeutic drug-induced fibrosis; (c) radiation-induced fibrosis as a result of cancer therapy/accidental exposure; (d) mechanical injuries

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IL-11 is a crucial determinant of cardiovascular fibrosis

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Abstract

Fibrosis is a common pathology in cardiovascular disease[1]. In the heart, fibrosis causes mechanical and electrical dysfunction[1,2] and in the kidney, it predicts the onset of renal failure[3]. Transforming growth factor β1 (TGFβ1) is the principal pro-fibrotic factor[4,5], but its inhibition is associated with side effects due to its pleiotropic roles[6,7]. We hypothesized that downstream effectors of TGFβ1 in fibroblasts could be attractive therapeutic targets and lack upstream toxicity. Here we show, using integrated imaging–genomics analyses of primary human fibroblasts, that upregulation of interleukin-11 (IL-11) is the dominant transcriptional response to TGFβ1 exposure and required for its pro-fibrotic effect. IL-11 and its receptor (IL11RA) are expressed specifically in fibroblasts, in which they drive non-canonical, ERK-dependent autocrine signalling that is required for fibrogenic protein synthesis. In mice, fibroblast-specific *Il11* transgene expression or Il-11 injection causes heart and kidney fibrosis and organ failure, whereas genetic deletion of *Il11ra1* protects against disease. Therefore, inhibition of IL-11 prevents fibroblast activation across organs and species in response to a range of important pro-fibrotic stimuli. These results reveal a central role of IL-11 in fibrosis and we propose that inhibition of IL-11 is a potential therapeutic strategy to treat fibrotic diseases.

Trans-differentiation of fibroblasts into activated myofibroblasts, which express α-smooth muscle actin (*ACTA2*) and secrete extracellular matrix (ECM) proteins, is a defining feature of fibrosis[8]. We automated the quantification of myofibroblasts and ECM production in primary human cardiac fibroblast cultures ($n = 84$; Extended Data Table 1 and Supplementary Table 1), and performed RNA sequencing (RNA-seq) on paired unstimulated and TGFβ1-stimulated samples (Extended Data Fig. 1). Genes were ranked on the basis of the magnitude and significance of their differential expression and their correlation with myofibroblasts. Typical fibrosis genes such as *COMP* and *NOX4*[9] were among the most-upregulated genes (Fig. 1a and Supplementary Table 2). Gene set enrichment analysis showed upregulation of genes important for protein secretion (Supplementary Table 3).

Notably, *IL11* expression showed the most positive correlation with myofibroblast numbers ($r = 0.47$, $P_{adjusted} = 6.44 \times 10^{-6}$; Spearman's correlation) (Fig. 1a) and upregulation of *IL11* expression (8.5-fold, $P_{adjusted} = 6 \times 10^{-218}$; DESeq2[10]) defined the dominant transcriptional response of cardiac fibroblasts to TGFβ1. *IL11* expression is highly specific to fibroblasts, especially when stimulated, but is undetectable in most healthy human tissues and cells[11,12] (Extended Data Fig. 2). To further explore the biological context of IL-11, we examined the expression of its receptor (*IL11RA*) compared to the receptor of IL-6 (*IL6R*), a close family member, across 512 cell lines[12]. *IL6R* was present at high levels in immune cells, whereas *IL11RA* was most highly expressed in fibroblasts (Fig. 1b).

To investigate *Il11* expression *in vivo*, we performed single-cell RNA-seq of hearts from a $Pln^{R9C/+}$ mouse, which has a cardiac fibrosis phenotype[13], and a wild-type control mouse (Fig. 1c and Extended Data Fig. 3). Both *Il11* ($P = 5.6 \times 10^{-8}$) and *Il1ra1* ($P = 2.2 \times 10^{-16}$) were enriched in fibroblasts and *Il11* was highly expressed in fibroblast subpopulations that had transcriptional features of TGFβ1 activation or ECM production (Fig. 1d). *Il11*-expressing cells were most common in fibrotic $Pln^{R9C/+}$ hearts and we confirmed Il-11 protein upregulation in this model (Extended Data Fig. 3).

IL11 has been linked to haematopoiesis[14] and tumorigenesis[15], among other roles. In contrast to a previous cardiac study[16], we found that recombinant human IL-11 (rhIL-11) is strongly pro-fibrotic in cardiac fibroblasts, increasing myofibroblasts and ECM production, motility, contraction and invasion (Fig. 1e and Extended Data Fig. 4). To better understand the apparent contradiction between our data and the previous work in which rhIL-11 was used in mouse models[16], we tested whether rhIL-11 could activate mouse cardiac fibroblasts. rhIL-11 was mostly ineffective in mouse cardiac fibroblasts, whereas recombinant mouse Il-11 (rmIl-11) activated mouse cardiac and renal fibroblasts (Extended Data Fig. 4). rhIL-11 strongly activated human renal fibroblasts.

Our findings implicate a pro-fibrotic role for IL-11 downstream of TGFβ1. In the presence of neutralizing anti-IL-11 antibodies, the pro-fibrotic effects of TGFβ1 were greatly diminished across a wide range of fibrosis assays (Fig. 1f–i and Extended Data Fig. 4). We made ligand traps by fusing IL11RA and gp130 (IL11RA:gp130) and these traps inhibited the pro-fibrotic effects of TGFβ1 in a dose- dependent manner. The specificity of IL-11 inhibition was further confirmed through inhibition of IL11RA using receptor-blocking antibodies or by short interfering RNA (siRNA), all of which attenuated the effects of TGFβ1. By contrast, anti-IL-6 antibodies had no effect on TGFβ1- induced cardiac fibroblast activation (Extended Data Fig. 4).

We next investigated the consequences of IL-11 signalling in cardiac fibroblasts using RNA-seq. Surprisingly, the effect of IL-11 on the transcriptome was negligible, whereas TGFβ1-driven transcriptional regulation in cardiac fibroblasts from the same patients remained profound (Fig. 2a, b). We repeated the experiment using cardiac fibroblasts from multiple patients and consistently observed very little effect of IL-11 on mRNA levels but, in the cell culture supernatants of identical samples, we reproducibly documented pro-fibrotic protein secretion. Therefore, the effects of IL-11 on cardiac fibroblasts are mainly at the protein

level and are unrelated to transcriptional changes (Extended Data Fig. 5). This differs from effects of IL-11 on cancer cells, in which JAK–STAT signalling is involved[17].

IL-11 can bind to free IL11RA, which is shed from cardiac fibroblast membranes (Extended Data Fig. 5) and signal *in trans* in cells that express *GP130* (also known as *IL6STP1*)[18]. We generated an IL11RA:IL-11 fusion protein (hyperIL-11), which mimics the *trans*signalling complex[19]. Concentrations of hyperIL-11 as low as 0.2 ng ml^{-1} activated cardiac fibroblasts (Extended Data Fig. 5), supporting a role for IL-11 *trans*-signalling in fibrosis.

We found that both *IL11* and *IL11RA* are expressed in fibroblasts, which implies that an autocrine signalling loop exists. Using hyperIL-11, which is not detected in an IL-11 enzyme-linked immunosorbent assay (ELISA), we confirmed the existence of autocrine IL-11 signalling in cardiac fibroblasts. This autocrine, feed-forward loop of hyperIL-11-induced IL-11 secretion is dependent on *de novo* IL-11 protein synthesis and secretion (Fig. 2c and Extended Data Fig. 5), but is independent of *IL11* RNA levels. In separate experiments, rhIL-11 (not detected by IL-11 ELISA) *cis*-signalling also strongly induced endogenous IL-11 secretion, and this also occurred in the absence of changes in *IL11* mRNA levels (Extended Data Fig. 5).

TGFβ1 activation of non-canonical ERK signalling in fibroblasts is important for fibrosis[4,5] and we observed that IL-11 also activated ERK in cardiac fibroblasts and that both TGFβ1 and IL-11 required ERK to induce pro-fibrotic phenotypes (Fig. 2d and Extended Data Fig. 5). Because the effects of IL-11 on pro-fibrotic gene expression are post-transcriptional, we suggest that this phenomenon may be driven, in part, by activation of ERK and its downstream substrates (Extended Data Fig. 5).

We therefore investigated the activation of STAT, SMAD, ERK and kinases that are important for protein synthesis in cardiac fibroblasts in response to stimulation by a range of pro-fibrotic factors. These factors included established stimuli for cardiac fibrosis (endothelin-1 (END1), angiotensin II (AngII) and PDGF) and other key pro-fibrotic cytokines (*OSM*, *bFGF*, *CTGF* and *IL13*)[1,5]. IL-11 activation of STAT in cardiac fibroblasts was negligible, consistent with its lack of transcriptional effects in this cell type. As expected, TGFβ1 activated SMAD, but the only pathway that was consistently activated by all stimuli was the ERK pathway (Fig. 2e). All pro-fibrotic stimuli that were tested induced changes in IL-11 protein levels but not mRNA; only TGFβ1 increased SMAD-dependent *IL11* transcription (Fig. 2f and Extended Data Fig. 5). Remarkably, as seen with TGFβ1, fibroblast activation in response to all pro-fibrotic stimuli that were tested was dependent on IL-11 signalling (Fig. 2g and Extended Data Fig. 6).

We studied the fibrotic response of cardiac fibroblasts from *Il11ra1*$^{-/-}$ mice[20]. TGFβ1-induced transcriptional regulation in *Il11ra1*$^{-/-}$ cardiac fibroblasts was maintained and similar to that of *Il11ra1*$^{+/+}$ (wild-type) cardiac fibroblasts ($R^2 = 0.94$, $P < 2.2 \times 10^{-16}$, Spearman's correlation; Extended Data Fig. 7). However, protein-based assays showed that cardiac fibroblasts from *Il11ra1*$^{-/-}$ mice did not increase synthesis of ECM proteins or become myofibroblasts upon stimulation (Fig. 2h and Extended Data Fig. 7), again demonstrating the effect of IL-11 at the protein level.

We investigated the role of Il-11 in a mouse model of myocardial infarction, but rather than injecting rhIL-11, as had been done in the previous study[16], we administered rmIl-11. We measured epicardial activation, a defining feature of active fibrosis in myocardial infarction[21] and found that rmIl-11 robustly stimulated fibroblasts in the epicardium (Fig. 3a–c) and caused ventricular impairment (Fig. 3d, e). We then tested the effects of rmIl-11 injection in healthy mice using regimens similar to those used for rhIL-11 in patients with cancer[22]. rmIL-11 injection in Col1a1–GFP reporter mice[23] resulted in specific activation of fibroblasts in the epicardium and renal interstitium (Fig. 3f). rmIl-11-treated mice had high circulating levels of TGFβ1, but not of other pro-inflammatory factors and features of cardiac and renal impairment along with cardiovascular fibrosis (Fig. 3g–i and Extended Data Fig. 7).

To investigate the effects of autocrine Il-11 signalling in fibroblasts *in vivo*, we generated rmIl-11-transgenic mice, which we crossed with inducible Col1a2–Cre mice (Il-11-Tg; see Methods). Il-11 was expressed after induction with tamoxifen and within two weeks, there was widespread activation of cardiac and renal fibroblasts and accumulation of collagen (Fig. 3j, k). This was accompanied by a reduction in cardiac function (Fig. 3l), increased serum TGFβ1 and an increase in serum urea and creatinine, which are biomarkers of renal failure (Extended Data Fig. 7).

Il-11 expression was found to be increased in three preclinical models of cardiovascular fibrosis (Fig. 4a, e, i). Therefore, using knockout and wild-type mice we determined whether inhibition of Il-11 could reduce fibrosis in these models. After either AngII infusion or transverse aortic constriction, less fibrosis occurred in the hearts of knockout mice compared to wild-type mice (Fig. 4b, c, f, g). This effect was independent of loading conditions (Extended Data Fig. 8). Similarly, after folateinduced kidney damage, knockout mice had reduced renal fibrosis (Fig. 4j, k). Deletion of Il11ra1 signalling resulted in reduced ERK signalling across all models tested, whereas p38 signalling was unaffected (Fig. 4d, h, l and Extended Data Fig. 9).

IL-11 was discovered owing to its ability to sustain an IL-6-dependent haematopoetic cell line when secreted from fibroblastic cells[24], but was later found to be redundant for haematopoiesis[20]. Here we show that *IL11* is a crucial fibrosis gene acting downstream of TGFβ1 and many other pro-fibrotic factors. We believe that the importance of IL-11 in fibroblasts may have gone unnoticed because its effects are apparent only at the post-transcriptional level (Extended Data Fig. 9). We highlight that *IL11RA* is not only expressed in fibroblasts but also in other cells and that IL-11 signalling may therefore be important in other cell types.

We note that rhIL-11 has been given to patients with myocardial infarction[25] and it is possible that the use of rhIL-11 in cancer patients[22] causes fibrosis-related side effects. We therefore suggest that the use of rhIL-11 in humans should be reviewed. *IL11* is highly upregulated in fibroblasts from patients with idiopathic pulmonary fibrosis or systemic sclerosis, by a 100-fold and 30-fold, respectively[26]; this suggests a role for IL-11 in fibrotic human disease beyond the cardiovascular system. IL-11 inhibitors may be particularly effective in treating fibrosis, because they would target a nodal point of pro-fibrotic

signalling. Because of these results, and target safety data from human[27] and mouse knockouts[20], we propose that IL-11 is a potential therapeutic target.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

METHODS

Data reporting

No statistical methods were used to predetermine sample size. The experiments were not randomized.

Patient cohort

Patients ($n = 84$, aged between ≥ 21 and ≤ 81) undergoing coronary artery bypass grafting (CABG) at the National Heart Centre Singapore were recruited to the study, which was approved by the SingHealth Centralised Institutional Review Board (CIRB; 2013/103/C). Patients with valvular heart disease or previous atrial intervention were excluded. Atrial biopsies (94.6 ± 59.5 mg) were collected from the right atrium and were used to grow primary atrial fibroblasts. A summary of patient data is provided in Extended Data Table 1 and detailed patient data are presented in Supplementary Table 1.

Recombinant proteins

Commercial recombinant proteins—Human CTGF (PHG0286, Biosource), human endothelin 1 (1160/100U, Tocris), human bFGF (233-FB-025, R&D Systems), human IL-11 (PHC0115, Life Technologies), human IL-13 (PHC0134, Biosource), human oncostatin M (PHC5015, Biosource), human PDGF (220-BB-010, R&D Systems), human angiotensin II (A9525, Sigma-Aldrich), and human TGFβ1 (PHP143B, Bio-Rad).

Custom recombinant proteins—Recombinant mouse IL-11 (UniProtKB: P47873) and human IL-11 (UniProtKB: P20809) were synthesized without the signal peptide. HyperIL-11 was constructed using a fragment of IL11RA (amino acid residues 1–317 consisting of domains 1–3; UniProtKB: Q14626) and IL-11 (amino acid residues 22–199, UniProtKB: P20809) with a 20-amino-acid-long linker: GPAGQSGGGGGSGGGSGGGSV. Decoy receptors were constructed using a fragment of gp130 (amino acid residues 1–326 consisting of domains 1–3, UniProtKB: P40189) and IL11RA (amino acid residues 109–308 consisting of domains 2–3, UniProtKB: Q14626) with either a 33-amino-acid-long linker: GGGGSTRGSAGSGGSATGSGSAAGSGDSVRRGS or a 50-amino-acid-long linker: GGGGSTRGQLHTQPEVEPQVDSPAPPRPSLQPHPRLLDHRDSVEQVAVG. All custom recombinant proteins were synthesized by GenScript using a mammalian expression system.

Primary fibroblast culture

Human cardiac fibroblasts were prepared as follows: right atrial biopsies were weighed, minced into 1–2 mm^3 pieces, and placed in 6-cm dishes. Human cardiac fibroblasts were grown and maintained in DMEM (11995- 065, Gibco) supplemented with 20% fetal bovine

serum (FBS, 10500, Hyclone) and 1% penicillin–streptomycin (15140-122, Gibco), in a humidified atmosphere at 37 °C and 5% CO_2. The medium was renewed every 2–3 days. At 80–90% confluence, cells were passaged using standard trypsinization techniques. This protocol was also used to isolate fibroblasts from mouse atria, ventricles and kidneys. Human primary kidney fibroblasts (H-6016; CellBiologics) were cultured in specific medium (M2267, CellBiologics). All experiments were carried out at low cell passage (< P4) and cells were cultured in serum-free media for 16 h before treatment.

Antibodies and inhibitors used in this study were as follows: IgG type 2a (MAB003, R&D Systems), anti-IL-11 antibody (MAB218, R&D Systems), anti- IL11RA antibody (MAB1977, R&D Systems), brefeldin A (B7651, Sigma-Aldrich), cycloheximide (C1988, Sigma-Aldrich), PD98059 (9900, Cell Signaling), and U0126 (9930, Cell Signaling).

Operetta platform and image analysis

Fibroblasts were seeded in 96-well black CellCarrier plates (6005550, Perkin-Elmer) at a density of 1×10^4 cells per well. Following experimental conditions, cells were rinsed in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde (PFA, 28908, Life Technologies). Cells were permeabilized with 0.1%Triton X-100 in PBS. EdU–AlexaFluor488 was incorporated using a Click-iT EdU labelling kit (C10350, Life Technologies) according to the manufacturer's protocol. Non-specific sites were blocked using blocking solution (0.5% BSA and 0.1% Tween-20 in PBS). Cells were incubated overnight at 4 °C with antibodies: α-smooth muscle actin (ACTA2, ab7817, Abcam), collagen I (ab292, Abcam), periostin (POSTN; ab14041, Abcam). All primary antibodies were diluted 1:500 in blocking solution. Following wash buffer (0.25% BSA and 0.1% Tween-20 in PBS) rinses, cells were incubated with the appropriate AlexaFluor488-conjugated secondary antibodies (1:1,000) for 1 h at room temperature in the dark. Cells were counter-stained with rhodamine–phalloidin (1:1,000, R415, Life Technologies) and DAPI (1 μg ml^{-1}, D1306, Life Technologies) in blocking solution. Plates were scanned and images were collected with an Operetta high-content imaging system 1483 (PerkinElmer). Each condition was assayed from at least three wells and a minimum of seven fields per well. The quantification of $ACTA2^+$ and EdU^+ cells was done using Harmony software version 3.5.2 (PerkinElmer). The measurement of collagen I and POSTN fluorescence intensity per area was performed with Columbus version 2.7.1 (PerkinElmer).

Scratch wound and migration assay

Human cardiac fibroblast migration was determined using *in vitro* scratch wound assays and Boyden chamber assays in duplicate per patient sample. Scratch wound assays were performed on confluent monolayers of fibroblasts. After synchronizing in low serum medium (DMEM containing 0.2% FBS) for 24 h, a linear scratch was created with a sterile pipette tip and cells were subjected to different treatments for 24 h. Changes in the wound area were imaged at 0 and 24 h and quantified using ImageJ software (version 1.49). Boyden chamber assays were performed using a Cell Migration Assay kit (Cell Biolabs) as per the manufacturer's protocol. Fibroblasts (5×10^4 cells per well) were seeded inside trans-well inserts, and could migrate towards the experimental conditions as in the wound assays for 24 h.

Collagen gel contraction assay

Collagen gel contraction assays were performed in duplicate per patient sample of cardiac fibroblasts (8×10^4 cells per well) using a Cell Contraction Assay kit (Cell Biolabs) as per the manufacturer's protocol. The cells were treated with stimuli similar to the scratch wound assays, except that cells were cultured in DMEM containing 1% FBS. The gels were imaged every 24 h for 72 h and gel area was quantified using ImageJ software (version 1.49).

siRNA transfection

Human cardiac fibroblasts were seeded in 96-well black CellCarrier (PerkinElmer) plates (1×10^4 cells per well) and transfected with 12.5 nM On-Targetplus siRNAs (Dharmacon) in serum-free Opti-MEM medium and DMEM containing 10% FBS (ratio 1:9) using Lipofectamine RNAiMax (13778-150, Life Technologies). The cells were transfected for 24 h and subsequently cultured in DMEM containing 1%FBS overnight before subjected to different treatment conditions.

RNA-seq library preparation

Total RNA was isolated from human cardiac fibroblasts using RNeasy columns (Qiagen). RNA was quantified using a Qubit RNA High-Sensitivity Assay kit (Life Technologies) and assessed for degradation on the basis of their RNA integrity number using the Bioanalyzer RNA 6000 Nano assay (Agilent Technologies). TruSeq Stranded mRNA Library Prep kit (Illumina) was used to assess transcript abundance following standard instructions from the manufacturer. The final libraries were quantified using KAPA library quantification kits (KAPA Biosystems) on a StepOnePlus Real-Time PCR system (Applied Biosystems) according to the manufacturer's protocol. The quality and average fragment size of the final libraries were determined using a LabChip GX DNA High Sensitivity Reagent Kit (Perkin Elmer). Libraries were pooled and sequenced on a HiSeq 2500 in High Output mode using 75-bp paired-end sequencing chemistry.

RNA-seq analysis

RNA-seq data pre-processing—Raw sequencing data (.bcl files) were demultiplexed into FastQ files with Illumina's bcl2fastq (version 1.8.4) based on unique index pairs. TopHat (version 2.0.12) was used for mapping the reads to the human genome (GRCh38.78), with the following parameters: number of threads, prefilter multihits, read mismatches, read edit distance, mate inner distance and read realign edit distance[29]. Gene level counts were computed using HTSeq[30] (version 0.6.1) with the same human genome reference used for mapping (Ensembl version 78). Ribosomal genes (Ensembl gene biotype 'rRNA') and genes located in chromosomes other than 1–22, X and Y were discarded.

Differential expression of stimulated versus non-stimulated fibroblasts—Differential expression between the stimulated and non-stimulated samples was computed at the gene level from gene counts using the DESeq2[10] R package (version 1.10.1). A pre-filtering step was used by considering only genes with counts above one across all samples (36,352 genes remained after this filter). To account for patient effects and technical artefacts, patient ID, sex and RNA concentration were added as covariates in the differential

expression model. The baseline comparison was set to non-stimulation. DESeq2 independentFiltering parameter was set to False. The rest of the parameters were left as default.

Functional enrichment of differential expression results

Gene Ontology (GO) functional enrichment of the differential expression results was performed with Gene Set Enrichment Analysis (GSEA) software (version 2.2.2). Human Ensembl genes IDs included in DESeq2[10] output were mapped gene symbols by retrieving 'hgnc_symbol' (using the biomaRt R package and Ensembl version 78). Genes without gene symbol were removed (the final number of genes was 21,412). Then all genes were ranked by the corresponding DESeq2 output Wald statistic ('stat' in DESeq2 output, defined as estimate of the $\log_2$ fold change divided by its standard error).

One functional GSEA run was performed for each GO category (BP, CC and MF) retrieved from the Molecular Signatures Database gene sets (version 5.2, gene sets queried using gene symbols)[31]. A total of three GSEA runs was carried out. GSEA was run in classic pre-rank mode with 10,000 permutations to assess the false discovery rate (FDR). In the GSEA runs, maximum cluster size was set to 5,000 and minimum cluster size was set to 10.

Computation of variation in gene expression

TPM were computed as follows. Gene read counts were divided by gene length in kilobases (gene length was computed using featureCounts software, version 1.5.1). This results in reads per kilobase (RPK). To obtain then TPM values, RPKs were divided by the sample 'per million' scaling factor (defined as the total number of gene counts in a sample divided by a million). The removeBatchEffect function from the limma R package (version 3.26.9) was used to remove technical batch effects. In removeBatchEffect, the expression matrix was set to the $\log_2$(TPM + 1) expression matrix, the batch1 variable was set to 'library preparation batch' and the covariates variable was set to 'RNA concentration'. The obtained matrix was split into non-stimulated and stimulated. Then the removeBatchEffect function was run a second time to remove patient sex and race effects separately. From this output data, the delta of each gene was computed.

Correlation between the delta of gene expression and delta ACTA2

$ACTA2^+$ cells were measured in samples from the 84 patients as described above (Operetta image analysis). Outlier measurements were removed from the $ACTA2^+$ cells measurements. To select the measurements to be removed, we plotted the distribution of the s.d. in the measurements of each patient ($n = 3$). We removed the most distant point in the top one percentile of patients with the highest s.d. This procedure was carried out in the TGFβ1-stimulated and non- stimulated measurement separately. For each patient sample, mean ACTA2 levels were computed in baseline and stimulated fibroblasts. The corresponding delta ACTA2 was derived (defined as the difference between the mean stimulated and the mean nonstimulated). Spearman's rank correlation coefficient and the corresponding Student's *t*-test *P* value were computed between the stimulated and non-stimulated delta of each gene and the delta ACTA2 by using the function corAndPvalue from the WGCNA R package (version 1.51)[32].

RNA-seq analysis in the test cohort (patients, *n* = 9; mouse, *n* = 18)

Raw sequencing data (.bcl files) were demultiplexed into individual FastQ read files with Illumina's bcl2fastq version 2.16.0.10 on the basis of unique index pairs. The adaptor sequences and low quality reads and/or bases were trimmed using Trimmomatic version 0.36[33] and the read quality was assessed using FastQC version 0.11.5[34]. High-quality reads were mapped to Ensembl human GRCh38 version 86 reference or mouse GRCm38 version 86 reference genomes using Spliced Transcripts Alignment to a Reference (STAR) version 2.5.2b[35].

STAR alignment options were selected on the basis of the parameters used in the ENCODE project. Strand-specific raw counts of uniquely mapped (paired-end) reads were summarized with featureCounts[36] to get gene-level quantification of genomic features: featureCounts -t exon -g gene_id -s 2 –p. Differential expression was performed with DESeq2[10] version 1.14.1 using raw read counts from featureCounts. We performed a minimal pre-filtering to remove genes that have no reads or only a single read across all samples to reduce the data size and speed up the analysis process.

Sample IDs were included as covariates in DESeq2[10] design formula to remove batch effect due to samples and increase the sensitivity for finding differences among the conditions. Basal condition was always used as the reference level for pair-wise comparisons. Shrinkage MA-plot was generated to show the $\log_2$ fold changes over the mean of normalized counts and points are coloured red if the adjusted *P* value was less than 0.1.

GTEx/Fantom analysis

Two independent analyses were carried to test the tissue specificity of the expression of IL-11. These analyses used tissue expression data from the Genotype–Tissue Expression (GTEx)[11] project and the primary cell expression data from FANTOM5[12]. The test was designed to find those genes which had an expression profile that most closely matched an 'idealized' expression profile in which a gene is only expressed in stimulated fibroblasts. To do this, we used the Jennson–Shannon divergence (JSD) index, which is a measure of similarity between two probability distributions. We compared each distribution from FANTOM or GTEx incorporating our own expression data on stimulated fibroblasts to the idealized distribution. As a result, all genes with a low distance (according to JSD) to this idealized distribution are genes that are specific to stimulated fibroblasts and as such represent good candidates for further investigation.

GTEx data processing

GTEx[11] project RNA-seq V6p gene read counts (file GTEx_Analysis_v6p_RNA-seq_RNA-SeQCv1.1.8_gene_reads.gct.gz), reference annotation file (gencode.v19.genes.v6p_model.patched_contigs.gtf.gz GTEx V6p) and sample attributes (file GTEx_Data_V6_Annotations_SampleAttributesDS.txt) were downloaded from https://www.gtexportal.org/home/. TPM were computed as stated above using the downloaded gene reference annotation file. All samples provided by GTEx were used (n = 8,555), classified by tissue type as included in the column 'SMTS' of the sample attributes file, 30 tissues in total. Our gene TPM dataset consisting of 84 samples with unstimulated and TGFβ1-

stimulated fibroblasts was added ($n = 168$) to this GTEx data and all samples were quantile-normalized.

FANTOM data processing

Expression levels of all genes in primary cell types with replicates were downloaded from the FANTOM5 web resource (119 cell types). Because the FANTOM5 data are at the level of transcription start site expression derived from CAGE sequencing, we calculated the gene level expression by summing all counts that were assigned to a given gene. These were then normalized by library size in order to calculate the TPM for each gene. We then incorporated the TPM values from the stimulated fibroblasts and quantile normalized the data to ensure that data from the different techniques were comparable. In order to compare the expression profiles of *IL11RA* and *IL6R*, we extracted the TPM for these two genes across 511 different primary cell samples that covered cell types from all lineages. In each case, for which the expression of either *IL11RA* or *IL6R* is above the level of noise, we highlight these cell types and categorize them as described in the FANTOM5 cell type ontology.

JSD computation

On the basis of the distribution of the TPM gene expression levels in the 168 fibroblasts samples before quantile normalization, genes with average $\log_2(\text{TPM} + 1)$ higher than 2 were selected (Extended Data Fig. 2). In addition, only protein-coding genes were considered. The final number of genes included in the GTEx + fibroblasts and FANTOM + fibroblast analysis was 10,736 and 9,888, respectively.

The JSD was computed between the probability distribution of each gene and an idealized distribution, representing the situation in which a gene is only expressed in stimulated fibroblasts. The probability distribution of each gene was computed as the median gene expression in each condition, that is, GTEx tissue, FANTOM cell type or unstimulated or stimulated fibroblast, divided by the sum of the median gene expression across all conditions. The idealized probability distribution was defined as probability of 1×10^{-20} in all conditions, except for stimulated fibroblasts (with probability ~ 1). JSD was computed using the R package jsd (version 0.1).

Single-cell RNA-seq

Single-cell suspensions of non-cardiomyocytes were derived from adult left ventricles as previously described[37] either from male, 18-week-old transgenic mice[13] overexpressing PLN^{R9C+} or from FVB littermates. Single cells were isolated, lysed and subsequently RNA was reverse-transcribed and converted into cDNA libraries for RNA-seq analysis using a Chromium Controller and a Chromium Single Cell 3′ v2 Reagent kit (Genomics 10×) following the manufacturer's protocol. Libraries derived from PLN^{R9C+} or FVB mice were pooled together for DNA sequencing on a NextSeq 500 (Illumina) using a high-output kit (150 cycles) to a mean depth of > 60,000 reads per cell. Alignment of reads to the genome and generation of gene counts per cell was performed by Cell Ranger 1.2 software (Genomics 10×). Cells of sufficient complexity were clustered using tSNE and plots were generated using the Seurat R package[28].

Mouse models

Animal procedures were approved and conducted in accordance with the SingHealth Institutional Animal Care and Use Committee (IACUC) or in accordance with local guidelines from collaborating laboratories. All mice were from a C57BL/6 genetic background and they were bred and housed in the same room and provided food and water *ad libitum*. For *in vivo* gain-of-function studies, the mice were allocated to experimental groups to ensure equal litter/sex/age across groups. Randomization was not applicable to loss-of-function animal studies owing to genotype-dependent analyses. For gain-of-function *in vivo* studies, treatment was not disclosed to investigators generating quantitative readouts after treatment. For loss-of-function studies, genotypes were not disclosed to investigators treating the animals, or generating quantitative readouts.

Il11ra1 knockout mice

Mice lacking functional alleles for Il11ra1 (*Il11ra1*$^{-/-}$, KO) and their wild-type littermates *Il11ra1*$^{+/+}$ were 10–12 weeks of age and the weights of mice did not differ significantly. *Il11ra1*$^{+/+}$ and *Il11ra1*$^{-/-}$ male mice were subcutaneously (s.c.) implanted with an osmotic minipump (Alzet model 1004, Durect) containing either angiotensin II (AngII, 2 mg kg^{-1} per day) in saline (0.9% w/v) to stimulate cardiac fibrosis or an identical volume of saline. Mice were post-operatively treated with enrofloxacin (15 mg kg^{-1}, s.c.) and buprenorphine (0.1 mg kg^{-1}, s.c.) for three consecutive days. Kidney fibrosis was induced by intraperitoneal (i.p.) injection of folic acid (180 mg kg^{-1}) in vehicle (0.3 M $NaHCO^3$) into *Il11ra1*$^{+/+}$ and *Il11ra1*$^{-/-}$ female mice; control mice were administered vehicle alone. Mice were euthanized 28 days post-implantation or post-injection, respectively. TAC was performed in *Il11ra1*$^{+/+}$ and *Il11ra1*$^{-/-}$ male mice as described previously[38]. Post-operative treatment was performed as described above. Age-matched sham mice underwent a sham operative procedure without TAC. Trans-thoracic two-dimensional Doppler echocardiography was used to confirm increased pressure gradients (> 40 mm Hg) indicative of successful TAC. Mice were euthanized at 2 weeks post-TAC for histological and molecular assessments.

Il-11 transgenic (Il-11-Tg) model

In this model, the mouse *Il11* cDNA was expressed under the control of the ubiquitous cytomegalovirus immediate early enhancer and the chicken β-actin promoter. A *loxP*-flanked STOP cassette was introduced in between the promoter and the transgene so that overexpression could be conditionally induced by Cre recombinase. The conditional transgene was introduced into the *Rosa26* gene locus of embryonic stem cells and this transgenic mouse line is referred to here as *Rosa26-Il11* mice.

To direct transgene expression in fibroblasts, heterozygous *Rosa26-Il11* mice were crossed with *Col1a2-CreER* mice[39] to create double heterozygous *Col1a2- CreER:Rosa26-Il11* progenies (referred to here as Il-11-Tg mice). Il-11-Tg mice were injected with 1mg tamoxifen (T5648, Sigma-Aldrich) i.p. at 6 weeks of age for 10 consecutive days to induce Cre-mediated recombination. Likewise, wild-type littermates were injected with 1 mg tamoxifen for 10 consecutive days as controls.

The mice were euthanized 14 days after cessation of tamoxifen administration.

In vivo Il-11 administration model

rmIl-11 was reconstituted to a concentration of 50 μg ml^{-1} in saline. Ten-week-old male mice and transgenic *Col1a1-GFP* reporter mice[23] were subjected to daily s.c. injection with either 100 μg kg^{-1} of rmIl-11 or an identical volume of saline for 21 days.

Myocardial infarction model

Wild-type male mice (10–12 weeks) underwent myocardial infarction surgery as described previously[40]. Age-matched controls underwent sham procedures without ligation of the coronary artery. In a subset of myocardial infarction mice, rmIl-11 or an identical volume of PBS were administered daily via s.c. injection for 6 successive days.

Echocardiography

Trans-thoracic echocardiography was performed on all mice using Vevo 2100 with a MS400 linear array transducer (VisualSonics), 18–38 MHz. Mice were anaesthetized with 2% isoflurane and kept warm on a heated platform (37 °C). The chest hairs were removed using depilatory cream and a layer of acoustic coupling gel was applied to the thorax. An average of 10 cardiac cycles of standard 2D and *m*-mode short axis at mid papillary muscle level according to a previously described method were obtained and stored for subsequent offline analysis[41]. Left ventricular ejection fraction and dimensions were calculated using a modified Quinone method[42].

In vivo telemetry

Before TA11PA-C10 blood pressure device (Data Sciences International) implantation, the zero offset was measured, and the unit was soaked in 0.9% NaCl. Mice were anaesthetized with isoflurane and kept warm on a heated platform. The pressure-sensing catheter was inserted into the left carotid artery and extended into the aorta, and the transmitter was placed in a subcutaneous pocket. All mice were allowed 10 days recovery from surgery before baseline blood pressure values were recorded for five days. The data from the TA11PA-C10 device were transmitted via radio frequency signals to a receiver below the home cage and sampled every 5 min for 10 s continuously day and night with a sampling rate of 1,000 Hz. Following five days baseline pressure recording, the mice received AngII as described above. Telemetry data were collected continuously for the duration of the AngII infusion.

ELISA

The level of IL-11, IL11RA, MMP-2, and TIMP-1 in equal volumes of cell culture medium were quantified using the following kits: Human IL-11 Quantikine ELISA kit (D1100, R&D Systems), Human IL11RA ELISA kit (LSF8919, Lifespan Biosciences), Total MMP-2 Quantikine ELISA kit (MMP200, R&D Systems), Human TIMP-1 Quantikine ELISA kit (DTM100, R&D Systems). Mouse plasma level of CRP, IFNγ, TGFβ1, and TNF were measured using the following kits: CRP Quantikine ELISA kit (ab157712, Abcam), and

Mouse IFNγ ELISA kit (ab100689, Abcam), Mouse TNF ELISA kit (ab208348, Abcam), and Mouse TGFβ1 ELISA kit (ab119557, Abcam).

Colourimetric assays

Quantification of total secreted collagen in the cell culture supernatant was performed using a Sirius red collagen detection kit (9062, Chondrex). The mouse plasma levels of urea and creatinine were quantified using urea assay kit (ab83362, Abcam) and creatinine assay kit (ab65340, Abcam), respectively. The amount of total collagen in the heart and kidney was quantified on the basis of colourimetric detection of hydroxyproline using a Quickzyme Total Collagen assay kit (Quickzyme Biosciences). All colourimetric assays were performed according to the manufacturer's protocol.

RT–qPCR

Total RNA was extracted from either the snap-frozen tissues or cell lysate using Trizol reagent (Invitrogen) followed by RNeasy column (Qiagen) purification. The cDNA was prepared using an iScript cDNA synthesis kit, in which each reaction contained 1 μg of total RNA, as per the manufacturer's instructions. Quantitative RT–PCR gene expression analysis was performed on triplicate samples with either TaqMan (Applied Biosystems) or fast SYBR green (Qiagen) technology using a StepOnePlus (Applied Biosystem) over 40 cycles. Expression data were normalized to *GAPDH* mRNA expression levels and we used the $2^{-\Delta\Delta C_t}$ method to calculate the fold change. Specific TaqMan probes were obtained from Applied Biosystems and are available upon request.

Immunoblotting

Western blot analysis was carried out on total protein extracts from fibroblasts or mouse tissues. Fibroblasts or frozen tissues were homogenized by gentle rocking in lysis buffer (RIPA buffer containing protease and phosphatase inhibitors (Roche)) followed by centrifugation to clear the lysate. Equal amounts of protein lysates were separated by SDS–PAGE, transferred to a PVDF membrane, and subjected to immunoblotting analysis of p-AKT (4060, CST), AKT (4691, CST), p-EIF4E (9741, CST), EIF4E (2067, CST), p-ERK1/2 (4370, CST), ERK1/2 (4695, CST), GAPDH (2118, CST), IL-11 (MAB218, R&D Systems), p-MEK1/2 (9154, CST), MEK1/2 (4694, CST), p-mTOR (2971, CST), mTOR (2972, CST), p-p38 MAPK (4511, CST), p38 MAPK (8690, CST), p-RSK1 (11989, CST), RSK (9355, CST), p-SMAD2 (5339, CST), SMAD2 (3108, CST), p-STAT3 (4113, CST) and STAT3 (4904,CST). Proteins were visualized using the ECL detection system (Pierce) with the appropriate secondary antibodies: anti-rabbit HRP (7074, CST) and anti-mouse HRP (7076, CST). Each western blot experiment was repeated independently with similar results as follows: Fig. 2c HyperIL-11 + BFA, $n = 3$; Fig. 2e various pro-fibrotic stimuli, $n = 2$; Fig. 4a, d AngII model, $n = 5$; Fig. 4e, h TAC model, $n = 3$; Fig. 4i, l folic acid (FA) model, $n = 5$; Extended Data Fig. 3c Il-11 in PLN model, $n = 3$; Extended Data Fig. 5m HypIL-11 + BFA + CHX, $n = 2$; Extended Data Fig. 5o IL-11 time course, $n = 4$; Extended Data Fig. 5p eIF4E activation, $n = 5$; Extended Data Fig. 5q TGFβ time course, $n = 3$; Extended Data Fig. 5s various pro-fibrotic stimuli, $n = 2$; Extended Data Fig. 7n Il-11-Tg model, $n = 4$; Extended Data Fig. 9e AngII model – p38, $n = 5$; Extended Data Fig. 9f TAC model – p38, $n = 3$; Extended Data Fig. 9g FA model – p38, $n = 5$.

Histology

Tissues from TAC and Col1a1–GFP mice models were subjected to cryosectioning and tissues from all other models were paraffin-embedded. Hearts were sectioned at 5 μm and kidneys at 3 μm. For paraffin sections, tissues were fixed for 24 h, at room temperature in 10% neutral-buffered formalin (Sigma-Aldrich), dehydrated and embedded in paraffin. For cryosections, freshly dissected organs were embedded with Tissue-Tek Optimal Cutting Temperature compound (VWR International). Cryomoulds were then frozen in a metal beaker with isopentane cooled in liquid nitrogen and sections were stored in − 80 °C. Total collagen was stained with Masson's trichrome stain kit (HT15, Sigma-Aldrich) according to the manufacturer's instructions. Images of the sections were captured and blue-stained fibrotic areas were semiquantitatively determined with ImageJ software (version 1.49). For immunohistochemistry, the tissue sections were incubated with anti-ACTA2 antibody (ab5694, Abcam). Primary antibody staining was visualized using an ImmPRESS HRP Anti-Rabbit IgG Polymer Detection kit (Vector Laboratories) with ImmPACT DAB Peroxidase Substrate (Vector Laboratories) as the chromogen. The sections were then counterstained with Mayer's haematoxylin (Merck). Detection of GFP expression was performed according to established protocols[43]. Each histology experiment was repeated independently with similar results as follows: Fig. 3a myocardial infarction model, $n = 5$; Fig. 3f Col1a1–GFP model, $n = 3$; Fig. 3j Il-11-Tg model, $n = 4$; Fig. 4b AngII model, $n = 4$; Fig. 4f TAC model, $n = 3$, Fig. 4j FA model, $n = 4$; Extended Data Fig. 7i rmIL-11 model, $n = 3$.

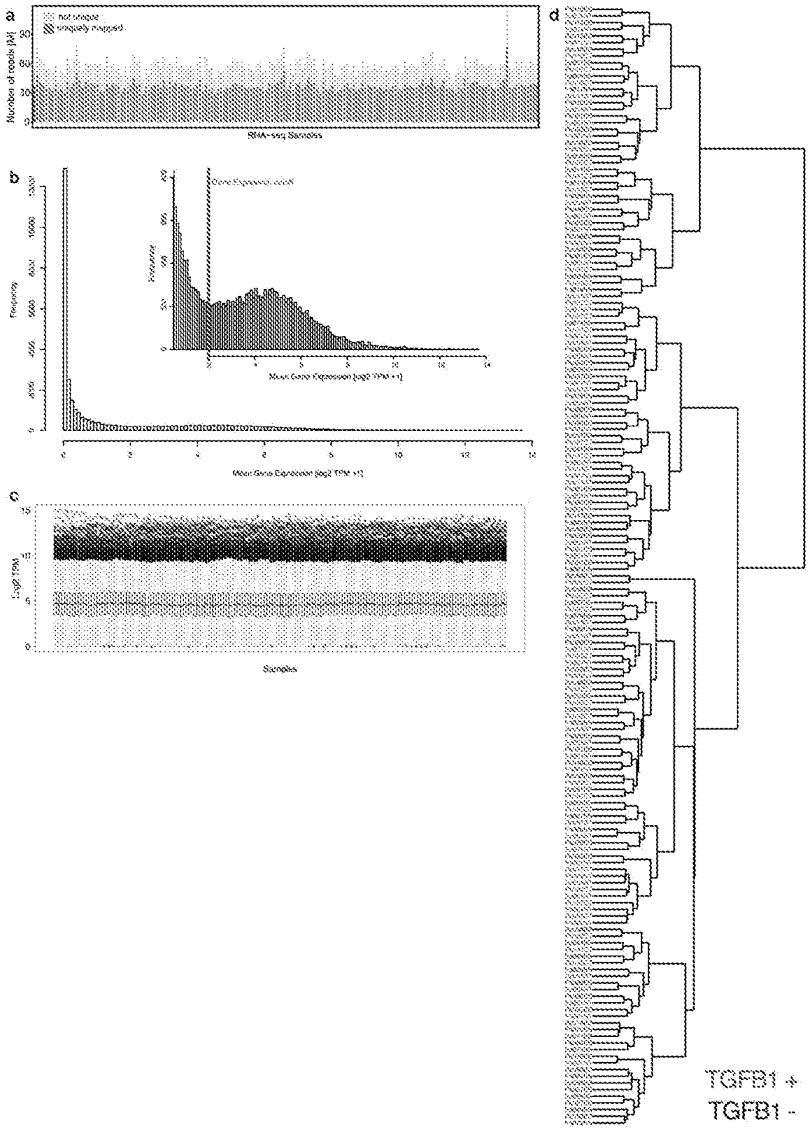
Statistical analysis

Statistical analyses of high-content imaging, qPCR and protein data were performed using GraphPad Prism software (version 6.07). Fluorescence intensity (collagen I, POSTN) was normalized to the number of cells detected in the field and recorded for seven fields per well. Cells expressing ACTA2 were quantified and the percentage of activated fibroblasts ($ACTA2^+$) was determined for each field. Outliers (ROUT 2%, GraphPad Prism software) were removed before analysis. When several experimental groups were compared to one condition (that is, to unstimulated cells), we corrected P values according to Dunnett's. When we compared several conditions within one experiment, we corrected for multiple testing according to Sidak. The criterion for statistical significance was $P < 0.05$ ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$).

Data availability

High-throughput data used throughout the manuscript (Figs 1, 2, Extended Data Figs 1, 2, 5, 7 and Supplementary Information) have been deposited into the Gene Expression Omnibus (GEO; at https://www.ncbi.nlm.nih.gov/geo/) under accession number GSE97117. The authors declare that all other data supporting the findings of this study are available within the paper and its Supplementary Information. Source data are provided with the paper as Supplementary Fig. 1 (western blots) and Source Data for Figs 3, 4 (individual data points for *in vivo* studies presented in Figs 3, 4) are included in the online version of the paper. Any additional information is available upon request from the corresponding author.

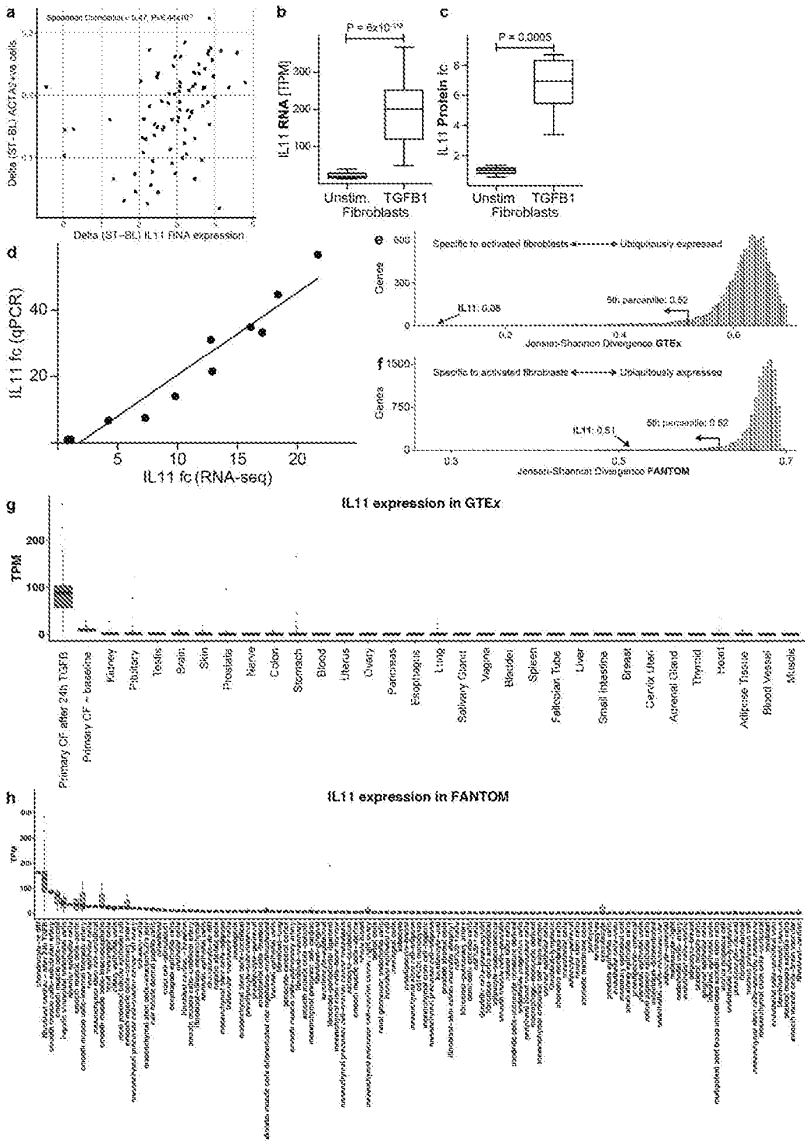
Extended Data



Extended Data Figure 1. RNA-seq of fibrotic cardiac fibroblasts

a, Mapped RNA-seq reads for human fibroblasts ($n = 84$ biologically independent samples). We aimed to generate at least 60 million reads per sample to assess RNA expression with and without stimulation of primary cardiac fibroblasts with TGFβ1. Fibroblasts were derived from the atria of 84 individuals, resulting in a total of 168 RNA-seq datasets for unstimulated and stimulated cells. After mapping, we used only reads that map to one unique location in the genome to estimate gene expression levels. **b**, Distribution of mean gene expression across all samples for each gene. We found 12,081 genes to be expressed at $\log_2(\text{TPM} + 1) > 2$. Genes below this cut-off were not considered in subsequent analyses. **c**,

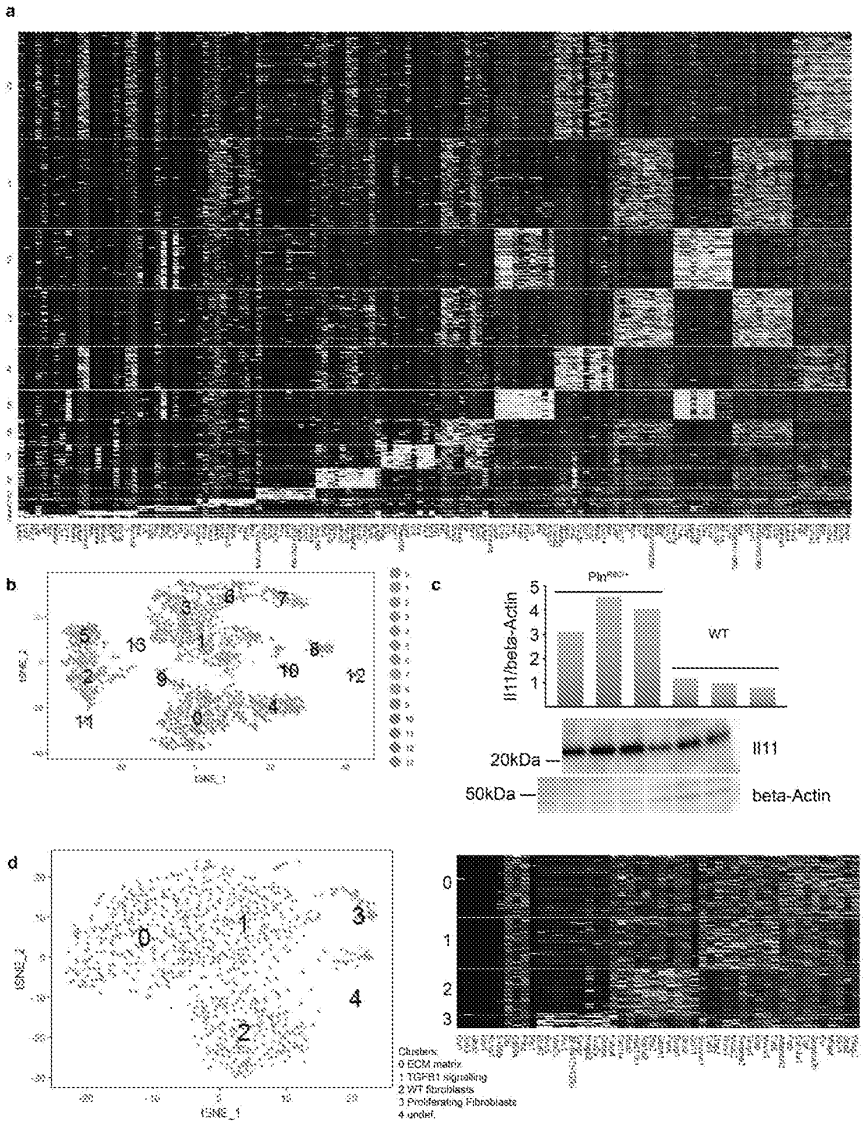
TPM distribution for each sample after filtering ($n = 84$). **d**, Ward clustering of Euclidian distance of RNA-seq samples. Sample gene expression tends to cluster mostly by genotype, indicating a strong genetic effect, beyond the effects of TGFβ1 stimulation.



Extended Data Figure 2. Characterization the TGFβ1-regulated gene, Il-11

a, We measured the amount of activated fibroblasts ($ACTA2^+$ cells) using the Operetta High-content imaging platform and IL-11 transcript levels by RNA-seq ($n = 84$ biologically independent samples). The increase in IL-11 expression correlated strongly with fibroblast activation in the cohort ($\rho = 0.47$, 95% confidence interval = 0.28–0.62). ST, stimulated cells (5 ng ml^{-1}, 24 h); BL, baseline cells (unstimulated, 24 h). **b**, TGFβ1 (5 ng ml^{-1}, 24 h)

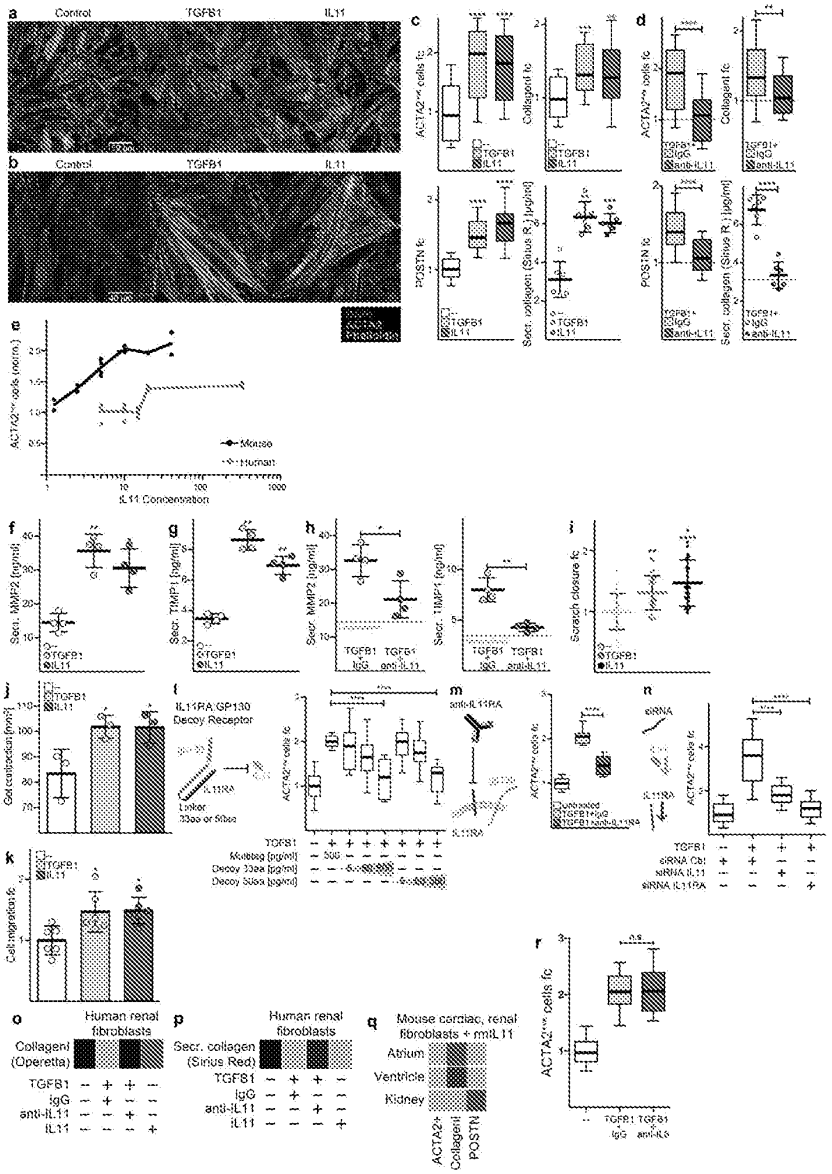
significantly upregulates *IL11* RNA (8.5-fold, $P = 6 \times 10^{-218}$) in human atrial fibroblasts according to RNA-seq analysis ($n = 84$ biologically independent samples). *P* values were calculated with DEseq2. **c**, The change in gene expression was confirmed at the protein level using an ELISA assay to measure IL-11 protein in the supernatant of cardiac fibroblasts ($n = 6$ biologically independent samples). fc, fold change. Two-tailed Student's *t*-test. **d**, RNA-seq-based expression differences in *IL11* transcript between unstimulated and TGFβ1-stimulated cardiac fibroblasts were confirmed via RT–qPCR. **e**, **f**, The JSD was calculated for all protein-coding genes expressed in activated fibroblasts. Low JSD scores indicate that a gene is highly expressed in stimulated cardiac fibroblasts and lowly expressed in healthy tissues (**e**; GTEx) or unstimulated, primary cell lines (**f**; FANTOM). **g**, **h**, *IL11* RNA expression in TGFβ1-stimulated and unstimulated cardiac fibroblasts ($n = 84$ biologically independent samples) compared to GTEx (**g**; tissues from $n = 8,723$ biologically independent samples) and FANTOM (**h**; cell types from $n = 285$ biologically independent samples) databases. **g**, RNA expression of *IL11* in TGFβ1-stimulated cardiac fibroblasts (red) and healthy tissues. **h**, RNA expression of *IL11* in TGFβ1-stimulated cardiac fibroblasts (red) and primary cells. **b**, **c**, **g**, **h**, Box-and-whisker plots show median (middle line), 25th–75th percentiles (box) and 10th–90th percentiles (whiskers).



Extended Data Figure 3. Single-cell RNA-seq of fibrotic mouse heart

Single-cell RNA-seq analysis of cells isolated from $Pln^{R9C/+}$ and wild-type adult left ventricles shown in Fig. 1. **a**, Cell types were defined according to indicated marker genes. **b**, Cells were clustered and cell type was determined using the Seurat R package (see Methods). **c**, Upregulation of Il-11 in the heart in the $Pln^{R9C/+}$ fibrosis model was confirmed using western blotting. All mice were 18 weeks old and male. **d**, Subsequently, 1,263 fibroblasts from $Pln^{R9C/+}$ and wild-type mice were re-clustered using Seurat. WNT signalling, downstream of TGFβ1 in cardiac fibroblast activation, target genes were used in this analysis of the four subsequent clusters of fibroblasts; $Il\text{-}11^{+}$ cells were primarily found in clusters 0 and 1 (16 out of 18 Il-11-expressing cells). Clusters 0 and 1 were also enriched

for $Pln^{R9C/+}$ cells compared to wild-type. Il11ra1 was expressed in all clusters. Cardiac cells were sequenced from $n = 1$ mouse, experiment was repeated one time with similar results.

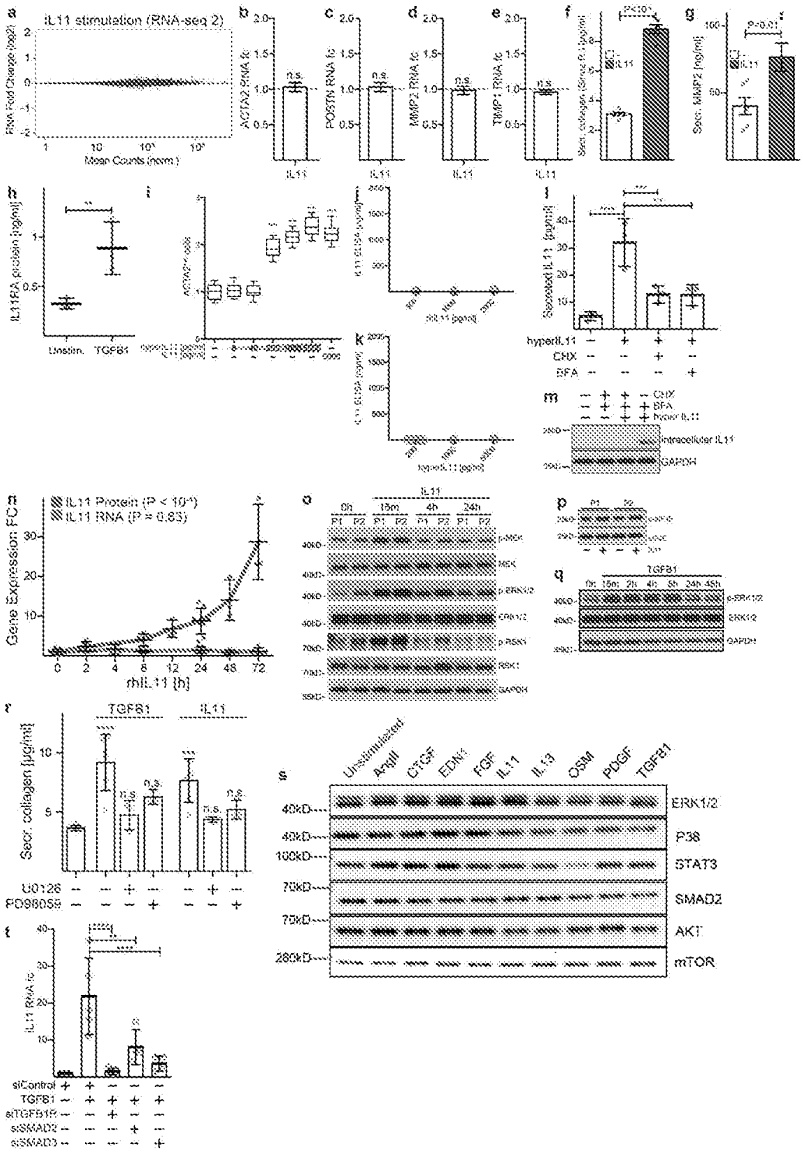


Extended Data Figure 4. IL-11 activates fibroblasts and is required for the pro-fibrotic effect of TGFβ1

a, **b**, High-resolution fluorescence imaging after TGFβ1 or IL-11 treatment (5 ng ml^{-1}, 24 h) of primary cardiac fibroblasts. Immunostaining of nuclei (DAPI, blue), ACTA2 (red) and F-actin (phalloidin, green) indicated that both TGFβ1 and IL-11 activate fibroblast stress fibre formation and increase the number of myofibroblasts *in vitro* to similar levels. Experiment was repeated four times with similar results. **c**, Automated quantification of

fluorescence (Operetta assay $n = 7$ measurements per $n = 6$ independent experiments) of primary atrial fibroblasts reveals significant fibroblast activation and ECM production induced by both TGFβ1 and IL-11 (5 ng ml^{-1}, 24 h). **d**, In addition, TGFβ1 effects can be reduced with an anti-IL-11 antibody (2 μg ml^{-1}). **c**, **d**, Collagen secretion in the supernatant ($n = 6$ independent experiments) was assessed with Sirius Red. **e**, Mouse primary fibroblasts were incubated for 24 h with indicated concentrations of recombinant human or mouse IL-11. Fibroblast activation was monitored using the Operetta High-Content Imaging platform and immunostaining for ACTA2. rhIL-11 was found to inefficiently activate mouse fibroblasts (rmIL-11, $n = 2$, rhIL-11, $n = 4$ biologically independent samples) compared to rmIL-11; this occurred for rhIL-11 treatment with rhIL-11 from two separate suppliers. **f**, **g**, MMP-2 (**f**) and TIMP-1 (**g**) concentration in the supernatant (ELISA) of cardiac fibroblasts ($n = 4$ biologically independent samples) without stimulus (−), with TGFβ1 or IL-11 (5 ng ml^{-1}, 24 h). **h**, Il-11-neutralizing antibodies (anti-IL-11, 2 μg ml^{-1}) block the increase in MMP-2 and TIMP-1 protein. **i**, *In vitro* monolayer scratch wound assay of cardiac fibroblasts. Wound closure was compared between stimulated (TGFβ1 or IL-11; 5 ng ml^{-1}, 24 h) and unstimulated cardiac fibroblasts ($n = 5$ biologically independent samples) after 24 h. **j**, Cardiac fibroblasts ($n = 3$ biologically independent samples) were seeded in collagen gel and the contraction was monitored. The area of contraction is compared between stimulated (TGFβ1 or IL-11; 5 ng ml^{-1}) and unstimulated groups after 72 h. **k**, Trans-well migration assay. After 24 h of stimulation (TGFβ1 or IL-11; 5 ng ml^{-1}), cardiac fibroblasts ($n = 6$ biologically independent samples) that crossed the membrane towards either a TGFβ1- or IL-11-containing compartment were colourimetrically quantified and compared to data from unstimulated cells. **l**–**n**, Cardiac fibroblasts were incubated with TGFβ1 (5 ng ml^{-1}, 24 h) and indicated amounts of IL11RA:gp130 decoy receptors (**l**; 33 amino acid (aa) or 50 aa linker peptide), anti-IL11RA antibody (**m**; 2 μg ml^{-1}) or siRNA pools against IL-11 or IL11RA (**n**). **l**–**n**, Fibroblast activation was monitored via immunostaining for ACTA2 on the Operetta platform. decoy receptors (**l**): $n = 7$ measurements per $n = 2$ independent experiments; anti- IL11RA (**m**): $n = 7$ measurements per $n = 2$ independent experiments; siRNA (**n**): Operetta assay $n = 7$ measurements per $n = 10$ independent experiments. **o**, Human renal fibroblasts were incubated with TGFβ1 or IL-11 (5 ng ml^{-1}, 24 h) in the presence or absence of anti-IL-11 or an IgG control antibodies (2 μg ml^{-1} each) for 24 h. ECM was assessed using the Operetta platform by staining for collagen I. Fluorescence was normalized to non-stimulated cells (black). **p**, These results were confirmed with Sirius red assay of the total collagen in the supernatant. **q**, rmIl-11 stimulation (5 ng ml^{-1}, 24 h) also activated mouse cardiac and renal fibroblasts. Myofibroblasts and ECM were assessed using the Operetta platform by staining for ACTA2, collagen I or POSTN. Fluorescence was normalized to non-stimulated cells (black). **o**–**q**, These experiments were repeated three times with similar results. **r**, Cardiac fibroblasts analysed on the Operetta high-content imaging platform with immunostaining of ACTA2 after 24 h incubation without stimulus, TGFβ1 (5 ng ml^{-1}, 24h) or TGFβ1 and IL-6-neutralizing antibody (2 μg ml^{-1}, 24h). Automated quantification of fluorescence (Operetta assay $n = 7$ measurements per $n = 6$ independent experiments) shows no significant decrease in fibroblast activation using anti-IL-6 antibodies. Data are mean and circles show individual values (**e**) or mean ± s.d. and circles show individual values (**c**, **d** bottom right, **f**–**h**, **k**); box-and-whisker plots (**c**, **d**, **l**–**n**, **r**) show median (middle line), 25th–75th percentiles (box) and 10th–90th percentiles

(whiskers). Two-tailed Dunnett's test (**c**, **f**, **g**, **i**–**k**), two-tailed Student's *t*-test (**d**, **h**, **r**) or two-tailed, Sidak-corrected Student's *t*-test (**l**–**n**). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$.

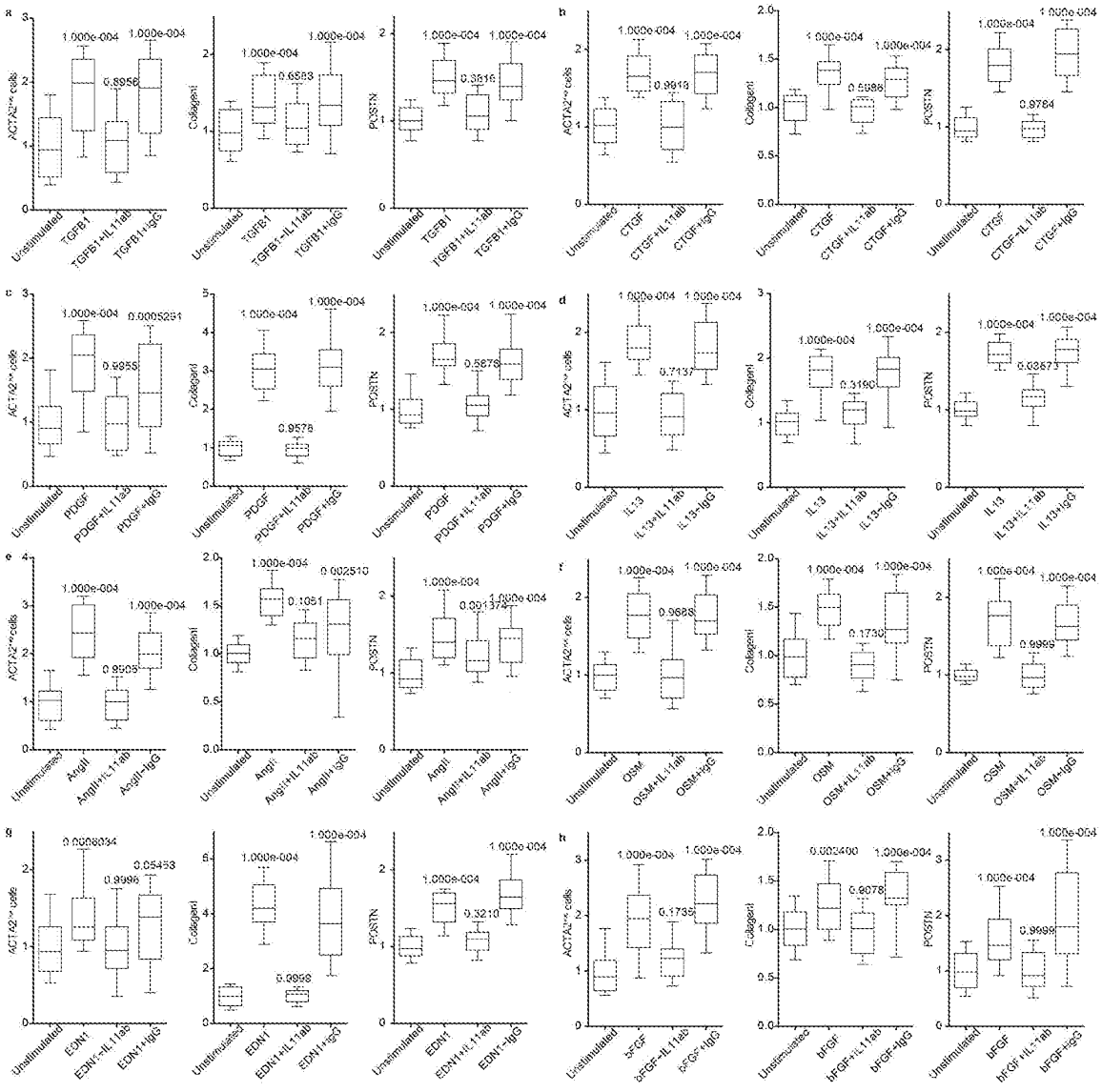


Extended Data Figure 5. IL-11 drives fibrogenic protein expression via non-canonical ERK signalling

a, Genome-wide RNA expression differences of cardiac fibroblasts in response to IL-11 (n = 4 biologically independent samples, 5 ng ml^{-1}, 24 h). Red indicates differentially expressed genes according to DEseq2. Fibrosis gene RNA is not increased by IL-11 treatment. **b**–**e**, RT–qPCR experiments for RNA expression of *ACTA2* (**b**), *POSTN* (**c**), *MMP2* (**d**) and

TIMP1 (**e**) in response to IL-11 treatment (5 ng ml^{-1}, 24 h) compared to unstimulated cells. IL-11 does not significantly upregulate these genes at the RNA level in cardiac fibroblasts (n = 4 biologically independent samples). **f**, Sirius red assay reveals significant increase in collagen protein. **g**, ELISA reveals increase in MMP-2 protein in the supernatant of the samples (shown in Fig. 2b, n = 6 biologically independent samples) that lack a change in RNA transcripts. **h**, Concentration of IL11RA (ELISA) in the supernatant of cardiac fibroblasts (n = 3 biologically independent samples) after TGFβ1 stimulation (5 ng ml^{-1}, 24 h). **i**, Cardiac fibroblasts were incubated with increasing concentrations of a fusion protein consisting of IL-11 and IL11RA connected with a linker peptide that recapitulates the features of the IL-11 *trans*-signalling complex. Concentrations as low as 200 pg ml^{-1} significantly activated cardiac fibroblasts as measured using a highcontent imaging platform and staining for ACTA2 expression (Operetta assay n = 7 measurements per n = 4 independent experiments). **j**, **k**, rhIL-11 (**j**) and hyperIL-11 (**k**) were added at indicated concentrations and subsequently measured using a commercially available IL-11 ELISA (n = 1 independent experiment). The ELISA did not detect rhIL-11 or hyperIL-11. We note that the reactivity to rhIL-11 was variable dependent on batch and provider and rhIL-11 was sometimes detected. However, in all experiments presented in the main figures, we confirmed that the rhIL-11 used was not detectable by the ELISA by additional measurements. This ELISA reliably detected native IL-11 secreted by human fibroblasts. **l**, Cardiac fibroblasts (n = 3 biologically independent samples) were incubated (8 h) with hyperIL-11 (0.2 ng ml^{-1}) in the presence of absence of the inhibitor of protein translation, cyclohexamide (CHX, 5 μg ml^{-1}), or protein secretion, brefeldin A (BFA, 1 μg ml^{-1}). Both inhibitors block the increase in IL-11 protein in the supernatant in response to hyperIL-11 treatment. **m**, Western blot and ELISA of IL-11 in cardiac fibroblasts after hyperIL-11, the inhibitor of the Golgi secretory pathway BFA and/or the translation inhibitor CHX treatment shows *de novo* protein synthesis and canonical secretion of IL-11 after stimulation. **n**, ELISA (n = 9 biologically independent samples) and RT–qPCR (n = 5 biologically independent samples) assays show an increase in endogenous IL-11 protein but not RNA over time after rhIL-11 (5 ng ml^{-1}) treatment. **o**, ERK signalling pathway activation by TGFβ1 and IL-11. Western blots show activation of the non-canonical MEK–ERK– RSK cascade in response to IL-11 stimulation of human cardiac fibroblasts. Here the response was greatest at 15 min in the two patients analysed, but more prolonged ERK activation was also seen in additional experiments. **p**, Downstream substrates of ERK, such as eIF4E, were also phosphorylated by rhIL-11. **q**, TGFβ1 also activates the ERK pathway. The time course and degree of activation was variable between patients. **r**, Collagen secretion (Sirius red) from cardiac fibroblasts (control, n = 6; TGFβ1, n = 6; TGFβ1 + U0126, n = 3; TGFβ1 + PD98059, n = 3; IL-11, n = 6; IL-11 + U0126, n = 3; IL-11 + PD98059, n = 3 biologically independent samples) induced by TGFβ1 or IL-11 (5 ng ml^{-1}, 24 h) is reduced by two separate MEK inhibitors (10 μM). **s**, Western blot of total protein levels of key signalling molecules in fibroblasts after 24 h stimulation with AngII (100 nM), CTGF (50 ng ml^{-1}), EDN1 (250 ng ml^{-1}), bFGF (10 ng ml^{-1}), IL-13 (100 ng ml^{-1}), OSM (100 ng ml^{-1}), PDGF (200 ng ml^{-1}) and TGFβ1 (5 ng ml^{-1}). The corresponding activated protein levels are shown in Fig. 2e. **t**, siRNA treatment of TGFβ1-stimulated cardiac fibroblasts. RT–qPCR shows SMAD-dependent upregulation of *IL11* RNA (control, n = 8; TGFβ1, n = 5; si*TGFB1R*, n = 5; si*SMAD2*, n = 4; si*SMAD3*, n = 4 biologically independent samples). Data are mean ±

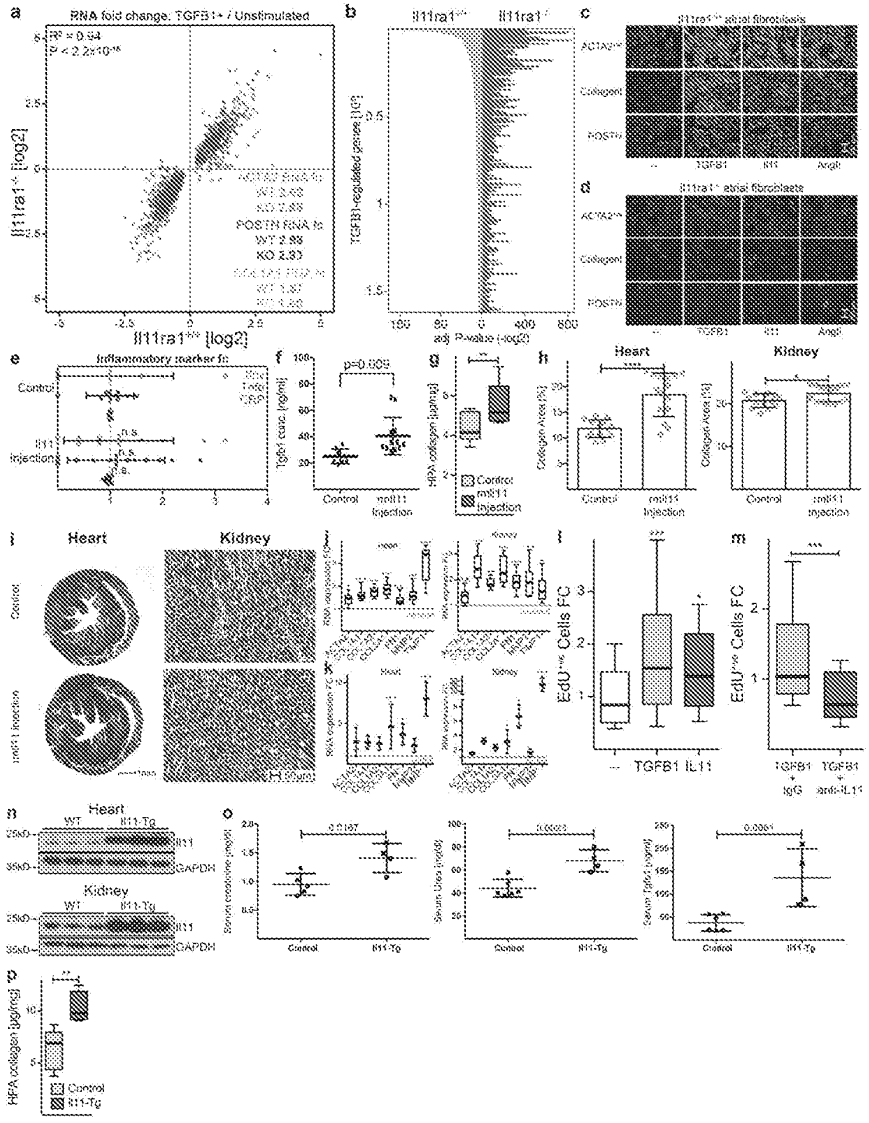
s.d. (**b**–**h**, **l**, **n**, **r**, **t**); box-and-whisker plots (**i**) show median (middle line), 25th–75th percentiles (box) and 10th–90th percentiles (whiskers). Two-tailed Student's *t*-test (**b**–**h**), two-tailed Dunnett's test (**i**, **r**) or Sidak-corrected, two-tailed Student's *t*-test (**l**, **t**) or one-way ANOVA (**n**). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$.



Extended Data Figure 6. IL-11 is required for the pro-fibrotic effects of multiple stimuli

a–**h**, Cardiac fibroblasts were incubated for 24 h with TGFβ1 (**a**; 5 ng ml^{-1}), CTGF (**b**; 50 ng ml^{-1}), PDGF (**c**; 200 ng ml^{-1}), IL-13 (**d**; 100 ng ml^{-1}), AngII (**e**; 100 nM), OSM (**f**; 100 ng ml^{-1}), EDN1 (**g**; 250 ng ml^{-1}) or bFGF (**h**; 10 ng ml^{-1}) in the presence or absence of an IL-11-neutralizing antibody (IL-11ab) or IgG control (2 μg ml^{-1}). Cells were stained for

ACTA2, collagen I and POSTN to monitor the amount of myofibroblasts and ECM production. High-content imaging and quantification of fluorescence (Operetta assay $n = 7$ measurements per $n = 6$ independent experiments for each condition and cellular phenotype) revealed that anti-IL-11 antibodies significantly reduce the pro-fibrotic effect of these stimuli on myofibroblast ratio and ECM production. Two-tailed Dunnett's test. Box-and-whisker plots show median (middle line), 25th–75th percentiles (box) and 10th–90th percentiles (whiskers).

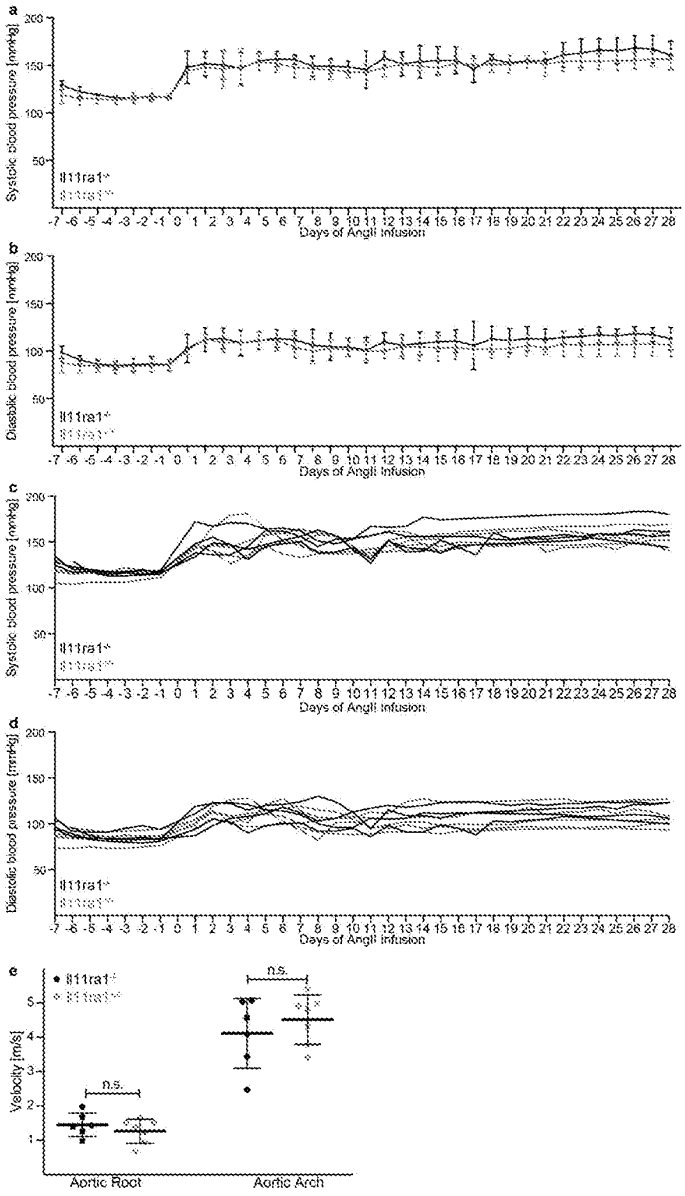


Extended Data Figure 7. Il-11 acts post-transcriptionally and causes fibrosis *in vivo*

a, RNA-seq fold change in TGFβ1-regulated genes in *Il1ra1*$^{+/+}$ cardiac fibroblasts ($n = 3$ biologically independent samples) compared to *Il1ra1*$^{-/-}$ cardiac fibroblasts ($n = 3$

biologically independent samples) after TGFβ1 stimulation (5 ng ml^{-1}, 24 h) are highly correlated. Spearman's correlation shows that RNA levels of fibrosis genes are upregulated equally in both genotypes. **b**, Wild-type and knockout fibroblasts ($n = 3$ biologically independent samples) were incubated with TGFβ1 (5 ng ml^{-1}, 24 h) and RNA-seq was performed to detect differentially expressed genes using DEseq2. All genes regulated by TGFβ1 in wild-type cells are plotted with decreasing $-\log_2(P_{adjusted})$. The P value of the same genes in stimulated *Il1ra1*$^{-/-}$ cells are plotted to the right. A similar P-value distribution suggests that TGFβ1-driven RNA expression changes are still present in the absence of IL-11 signalling showing that loss of Il1ra1 does not influence the TGFβ1-driven transcriptional response. **c**, **d**, Primary atrial fibroblasts were prepared from *Il1ra1*$^{+/+}$ (**c**) or *Il1ra1*$^{-/-}$ (**d**) mice were incubated for 24 h without stimulus or with TGFβ1 (5 ng ml^{-1}), IL-11 (5 ng ml^{-1}) or AngII (100 nM). Cells were stained with antibodies against ACTA2, collagen I or POSTN. Images were taken at low magnification (10×) on the Operetta imaging platform. As shown, fibroblasts from knockout mice do not respond to pro-fibrotic stimuli at the level of pro-fibrotic protein expression. This experiment was repeated four times with similar results. **e**, Circulating markers of inflammation after rmIl-11 injection (100 μg kg^{-1} per day, three weeks; $n = 14$ biologically independent samples). **f**, Circulating levels of Tgfβ1 (ELISA) after rmIl-11 injection (control, $n = 8$; Il-11 injection, $n = 12$ biologically independent samples). **g**, Collagen content (HPA assay) in atrium (control, $n = 7$; rmIl-11, $n = 10$ biologically independent samples) after rmIL-11 treatment. **h**, The area indicative for collagen deposition was assessed over several fields in $n = 4$ biologically independent samples and compared between samples from rmIl-11-treated and control mice. **i**, Representative histological images of the heart and kidney after rmIl-11 injection indicate increased collagen content according to Masson's trichrome staining. This experiment was repeated three times with similar results. **j**, RNA expression (RT–qPCR) of fibrosis genes in heart ($n = 12$ biologically independent samples) and kidney ($n = 11$ biologically independent samples) after rmIl-11 treatment compared to control. **k**, RNA expression (RT–qPCR) of fibrosis genes in heart (control, $n = 6$; Il-11-Tg, $n = 3$ biologically independent samples) and kidney (control, $n = 7$; Il-11-Tg, $n = 4$ biologically independent samples) of tamoxifen-treated Il-11-Tg, Col1a2–CreER and control mice. **l**, Cardiac fibroblasts were incubated with TGFβ1 or IL-11 (5 ng ml^{-1}) and EdU (10 μM ml^{-1}, 24 h), which was used to detect replicating DNA by fluorescence by automated quantification of images (Operetta assay $n = 7$ measurements per $n = 6$ independent experiments). This analysis reveals a significant increase in fibroblast proliferation (EdU^+ ells) induced by both TGFβ1 and IL-11. The percentage of EdU^+ cells was normalized to the average detected in non-stimulated cells. **m**, Cells were incubated with TGFβ1 (5 ng ml^{-1}, 24 h) and either an IgG control or anti-IL-11 antibody (2 μg ml^{-1}, 24 h). High-content imaging (Operetta assay $n = 7$ measurements per $n = 6$ independent experiments) and quantification of proliferating cells show that anti-IL-11 antibodies significantly reduce the effects of TGFβ1 on fibroblast proliferation. The percentage of EdU^+ ells was normalized to the average detected in cells stimulated with TGFβ1 and IgG control. **n**, Western blots show an increase in Il-11 protein expression in the heart and kidney after tamoxifen treatment in Il-11-Tg, Col1a2–CreER mice. **o**, Il-11 transgenic mice were crossed with a Col1a2- promoter, tamoxifen-inducible Cre mouse strain (Il-11-Tg). Six-week-old mice were treated with tamoxifen (1 mg per day, 10 consecutive days) to induce Cre-mediated recombination. Likewise, wild-type littermates

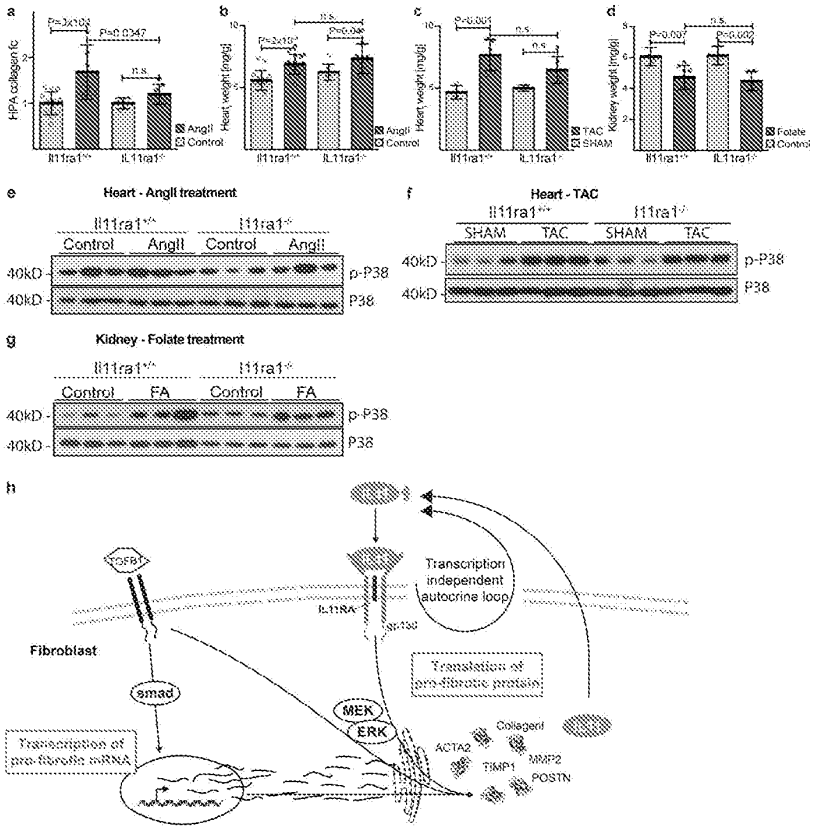
were injected with tamoxifen for 10 consecutive days as controls. The mice (control creatinine, $n = 5$; Il-11-Tg creatinine, $n = 4$; control urea, $n = 6$; Il-11-Tg urea, $n = 4$; control Tgfβ1, $n = 6$; Il-11-Tg Tgfβ1, $n = 4$ biologically independent samples) were euthanized 14 days after cessation of tamoxifen administration. Serum urea and creatinine increased and indicated renal impairment. We also observed an increase in circulating Tgfβ1 levels. **p**, Collagen content (HPA assay) in atrium (control, $n = 11$; Il-11-Tg, $n = 4$ biologically independent samples) from tamoxifentreated or control Il-11-Tg mice. Data are mean ± s.d. (**e**, **f**, **h**, **k**, **o**); box-and-whisker plots (**g**, **j**, **l**, **m**, **p**) show median (middle line), 25th–75th percentiles (box) and 10th–90th percentiles (whiskers). Sidak-corrected, two-tailed Student's *t*-test (**e**, **j**, **k**) or two-tailed Student's *t*-test (**f**–**h**, **m**, **o**, **p**) or Dunnett's test (**l**). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$.



Extended Data Figure 8. Il-11 inhibition does not alter blood pressure after AngII treatment

Il11ra1$^{+/+}$ wild-type ($n = 4$ biologically independent samples) and *Il11ra1*$^{-/-}$ knockout ($n = 5$ biologically independent samples) mice were injected with AngII (2 mg kg^{-1} per day, 28 days). **a**, **b**, Systolic (**a**) and diastolic (**b**) blood pressure was measured by *in vivo* telemetry for one week before and four weeks after the AngII infusion. Systolic (**c**) and diastolic (**d**) blood pressure for individual mice. AngII resulted in an increase in blood pressure as expected. The genotype did not have a significant effect on blood pressure. **e**, Aortic root or arch velocity of the blood. There was no significant difference in the degree of aortic

constriction in the TAC model between genotypes ($n = 6$ biologically independent samples). Two-sided Mann–Whitney *U*-test (**e**). n.s., not significant. Data are mean ± s.d. (**a**, **b**, **e**).



Extended Data Figure 9. Reduction in collagen deposition in *Il11ra1*$^{-/-}$ animals is independent of p38 MAPK signalling

Il11ra1$^{+/+}$ mice (control, $n = 13$; AngII, $n = 10$ biologically independent samples) and *Il11ra1*$^{-/-}$ mice (control, $n = 5$; AngII, $n = 7$ biologically independent samples) were injected with AngII (100 μg kg^{-1} per day, three weeks). **a**, HPA assay of ventricular tissue shows a decrease in collagen deposition in *Il11ra1*$^{-/-}$ mice after AngII infusion. **b**, Indexed heart weight of wild-type (control, $n = 17$; AngII, $n = 17$ biologically independent samples) and *Il11ra1*$^{-/-}$ (control, $n = 8$; AngII, $n = 9$ biologically independent samples) mice after AngII injection. **c**, Indexed heart weight of *Il11ra1*$^{+/+}$ (control, $n = 4$; TAC, $n = 6$ biologically independent samples) and *Il11ra1*$^{-/-}$ (control, $n = 6$; TAC, $n = 6$ biologically independent samples) mice after TAC. **d**, Kidney weight of *Il11ra1*$^{+/+}$ (control, $n = 5$; folate, $n = 8$ biologically independent samples) and *Il11ra1*$^{-/-}$ (control, $n = 6$; folate, $n = 5$ biologically independent samples) mice three weeks after folate injection (180 mg kg^{-1}). **a**–**d**, Sidak-corrected, two-tailed Student's *t*-test. Data are mean ± s.d. **e**–**g**, Western blot of p38 MAPK signalling in tissues of *Il11ra1*$^{+/+}$ and *Il11ra1*$^{-/-}$ mice after AngII infusion (e), TAC (f) or folate treatment (**g**). h, Schematic showing the proposed role of IL-11 fibroblasts. An autocrine loop of IL-11 signalling is required to feed-forward changes in pro-fibrotic mRNA

abundances to the protein level by activating translational processes that are ERK-dependent. Blocking this loop limits fibrosis caused by multiple upstream stimuli and fibrosis in preclinical models of heart and kidney disease.

Extended Data Table 1

84 patients undergoing coronary artery bypass grafting donated right atrial biopsies

DEMOGRAPHICS	
Age (years)	60.2 ± 7.2
Sex (M/F)	69/15
Race (CH/MY/IN/other)	53/12/16/3
Height (m)	1.6 ± 0.1
Weight (kg)	70.3 ± 12.0
Waist Circumference (cm)	93.6 ± 11.2
Hip Circumference (cm)	97.0 ± 9.5
Systolic BP (mmHg)	128.5 ± 17.1
Diastolic BP (mmHg)	73.0 ± 9.2
MEDICAL HISTORY	
Hypertension (Y/N)	74/8
Smoking (Y/N/Ex)	12/41/29
Myocardial infarct (Y/N)	33/40
Atrial fibrillation* (Y/N)	5/74
Diabetes (Y/N)	50/34
Diuretic (Y/N)	8/76
Beta Blocker (Y/N)	74/10
Nitrate (Y/N)	52/32
Insulin (Y/N)	13/71
Oral anti-diabetic (Y/N)	42/41
ACE or ARB (Y/N)	46/38
Statin (Y/N)	80/4
Calcium antagonist (Y/N)	26/56
ECG DATA	
PR (ms)	174 ± 29
QRS (ms)	93.8 ± 15.5
QT (ms)	415.5 ± 35.3
Heart rate (bpm)	69.0 ± 11.0
BLOOD TESTS	
Hemoglobin (g/L)	13.6 ± 1.6
Urea (mM/L)	5.7 ± 2.2
Creatinine (μM/L)	101.1 ± 70.1
Fasting glucose (mM/L)	7.9 ± 3.5

ECHO DATA	
LVEF (%)	52.3 ± 12.9
Left atrial area (cm^2)	4.1 ± 0.7
LVIDd (cm)	4.9 ± 0.7
LVIDs (cm)	3.3 ± 0.8
E/A Ratio	1.1 ± 0.7
BIOPSY	
Biopsy weight (mg)	94.6 ± 59.5

Categorical data are presented as yes (Y) or no (N), unless otherwise indicated. Echocardiography (Echo) data: left ventricular ejection fraction (LVEF), left ventricular internal diameter in diastole (LVIDd), left ventricular internal diameter in systole (LVIDs), mitral valve inflow measured as ratio of peak velocity flow in early diastole (E) compared to late diastole (A) caused by atrial contraction (E/A) ratio. Atrial fibrillation* refers to any present or previous history of atrial fibrilation. Quantitative data represented as mean ±s.d. CH, Chinese; Ex, ex-smoker; IN, Indian sub- continent; MY, Malaysian.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Rockey DC, Bell PD, Hill JA. Fibrosis--a common pathway to organ injury and failure. N Engl J Med. 2015; 372:1138–1149. [PubMed: 25785971]
2. Burstein B, Nattel S. Atrial fibrosis: mechanisms and clinical relevance in atrial fibrillation. J Am Coll Cardiol. 2008; 51:802–809. [PubMed: 18294563]
3. Leaf IA, Duffield JS. What can target kidney fibrosis? Nephrol Dial Transplant. 2017; 32(suppl 1):i89–i97. [PubMed: 28391346]
4. Davis J, Molkentin JD. Myofibroblasts: trust your heart and let fate decide. J Mol Cell Cardiol. 2014; 70:9–18. [PubMed: 24189039]
5. Akhurst RJ, Hata A. Targeting the TGFβ signalling pathway in disease. Nat Rev Drug Discov. 2012; 11:790–811. [PubMed: 23000686]
6. Bierie B, et al. Abrogation of TGF-β signaling enhances chemokine production and correlates with prognosis in human breast cancer. J Clin Invest. 2009; 119:1571–1582. [PubMed: 19451693]
7. Shull MM, et al. Targeted disruption of the mouse transforming growth factor-β1 gene results in multifocal inflammatory disease. Nature. 1992; 359:693–699. [PubMed: 1436033]
8. Wynn TA. Cellular and molecular mechanisms of fibrosis. J Pathol. 2008; 214:199–210. [PubMed: 18161745]
9. Cucoranu I, et al. NAD(P)H oxidase 4 mediates transforming growth factor-β1- induced differentiation of cardiac fibroblasts into myofibroblasts. Circ Res. 2005; 97:900–907. [PubMed: 16179589]

10. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014; 15:550. [PubMed: 25516281]
11. GTEx Consortium. The Genotype-Tissue Expression (GTEx) project. Nat Genet. 2013; 45:580–585. [PubMed: 23715323]
12. FANTOM Consortium and the RIKEN PMI and CLST (DGT). A promoter-level mammalian expression atlas. Nature. 2014; 507:462–470. [PubMed: 24670764]
13. Schmitt JP, et al. Dilated cardiomyopathy and heart failure caused by a mutation in phospholamban. Science. 2003; 299:1410–1413. [PubMed: 12610310]
14. Du XX, Neben T, Goldman S, Williams DA. Effects of recombinant human interleukin-11 on hematopoietic reconstitution in transplant mice: acceleration of recovery of peripheral blood neutrophils and platelets. Blood. 1993; 81:27–34. [PubMed: 8417798]
15. Putoczki TL, et al. Interleukin-11 is the dominant IL-6 family cytokine during gastrointestinal tumorigenesis and can be targeted therapeutically. Cancer Cell. 2013; 24:257–271. [PubMed: 23948300]
16. Obana M, et al. Therapeutic activation of signal transducer and activator of transcription 3 by interleukin-11 ameliorates cardiac fibrosis after myocardial infarction. Circulation. 2010; 121:684–691. [PubMed: 20100971]
17. Ernst M, Putoczki TL. Molecular pathways: IL11 as a tumor-promoting cytokine—translational implications for cancers. Clin Cancer Res. 2014; 20:5579–5588. [PubMed: 25074610]
18. Lokau J, et al. Proteolytic cleavage governs interleukin-11 *trans*-signaling. Cell Reports. 2016; 14:1761–1773. [PubMed: 26876177]
19. Dams-Kozlowska H, et al. A designer hyper interleukin 11 (H11) is a biologically active cytokine. BMC Biotechnol. 2012; 12:8. [PubMed: 22433466]
20. Nandurkar HH, et al. Adult mice with targeted mutation of the interleukin-11 receptor (IL11Ra) display normal hematopoiesis. Blood. 1997; 90:2148–2159. [PubMed: 9310465]
21. Duan J, et al. Wnt1/β catenin injury response activates the epicardium and cardiac fibroblasts to promote cardiac repair. EMBO J. 2012; 31:429–442. [PubMed: 22085926]
22. Kaye JA. FDA licensure of NEUMEGA to prevent severe chemotherapyinduced thrombocytopenia. Stem Cells. 1998; 16(Suppl 2):207–223. [PubMed: 11012193]
23. Moore-Morris T, et al. Resident fibroblast lineages mediate pressure overload-induced cardiac fibrosis. J Clin Invest. 2014; 124:2921–2934. [PubMed: 24937432]
24. Paul SR, et al. Molecular cloning of a cDNA encoding interleukin 11, a stromal cell-derived lymphopoietic and hematopoietic cytokine. Proc Natl Acad Sci USA. 1990; 87:7512–7516. [PubMed: 2145578]
25. Nakagawa M, et al. Four cases of investigational therapy with interleukin-11 against acute myocardial infarction. Heart Vessels. 2016; 31:1574–1578. [PubMed: 26796134]
26. Lindahl GE, et al. Microarray profiling reveals suppressed interferon stimulated gene program in fibroblasts from scleroderma-associated interstitial lung disease. Respir Res. 2013; 14:80. [PubMed: 23915349]
27. Nieminen P, et al. Inactivation of IL11 signaling causes craniosynostosis, delayed tooth eruption, and supernumerary teeth. Am J Hum Genet. 2011; 89:67–81. [PubMed: 21741611]
28. Satija R, Farrell JA, Gennert D, Schier AF, Regev A. Spatial reconstruction of single-cell gene expression data. Nat Biotechnol. 2015; 33:495–502. [PubMed: 25867923]
29. Trapnell C, Pachter L, Salzberg SL. TopHat: discovering splice junctions with RNA-seq. Bioinformatics. 2009; 25:1105–1111. [PubMed: 19289445]
30. Anders S, Pyl PT, Huber W. HTSeq—a Python framework to work with high-throughput sequencing data. Bioinformatics. 2015; 31:166–169. [PubMed: 25260700]
31. Subramanian A, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci USA. 2005; 102:15545–15550. [PubMed: 16199517]
32. Langfelder P, Horvath S. WGCNA: an R package for weighted correlation network analysis. BMC Bioinformatics. 2008; 9:559. [PubMed: 19114008]

33. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 2014; 30:2114–2120. [PubMed: 24695404]
34. Babraham Bioinformatics. FastQC, a quality control tool for high throughput sequence data. http://www.bioinformatics.babraham.ac.uk/projects/fastqc/
35. Dobin A, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics. 2013; 29:15–21. [PubMed: 23104886]
36. Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics. 2014; 30:923–930. [PubMed: 24227677]
37. Burke MA, et al. Molecular profiling of dilated cardiomyopathy that progresses to heart failure. JCI Insight. 2016; 1:e86898. [PubMed: 27239561]
38. Tarnavski O, et al. Mouse cardiac surgery: comprehensive techniques for the generation of mouse models of human diseases and their application for genomic studies. Physiol Genomics. 2004; 16:349–360. [PubMed: 14679301]
39. Zheng B, Zhang Z, Black CM, de Crombrugghe B, Denton CP. Ligand-dependent genetic recombination in fibroblasts: a potentially powerful technique for investigating gene function in fibrosis. Am J Pathol. 2002; 160:1609–1617. [PubMed: 12000713]
40. Ye L, et al. Thymosin β 4 increases the potency of transplanted mesenchymal stem cells for myocardial repair. Circulation. 2013; 128(Suppl 1):S32–S41. [PubMed: 24030419]
41. Gao S, Ho D, Vatner DE, Vatner SF. Echocardiography in mice. Curr Protoc Mouse Biol. 2011; 1:71–83. [PubMed: 21743841]
42. Tortoledo FA, Quinones MA, Fernandez GC, Waggoner AD, Winters WL Jr. Quantification of left ventricular volumes by two-dimensional echocardiography: a simplified and accurate approach. Circulation. 1983; 67:579–584. [PubMed: 6821900]
43. Mead TJ, Lefebvre V. Proliferation assays (BrdU and EdU) on skeletal tissue sections. Methods Mol Biol. 2014; 1130:233–243. [PubMed: 24482177]

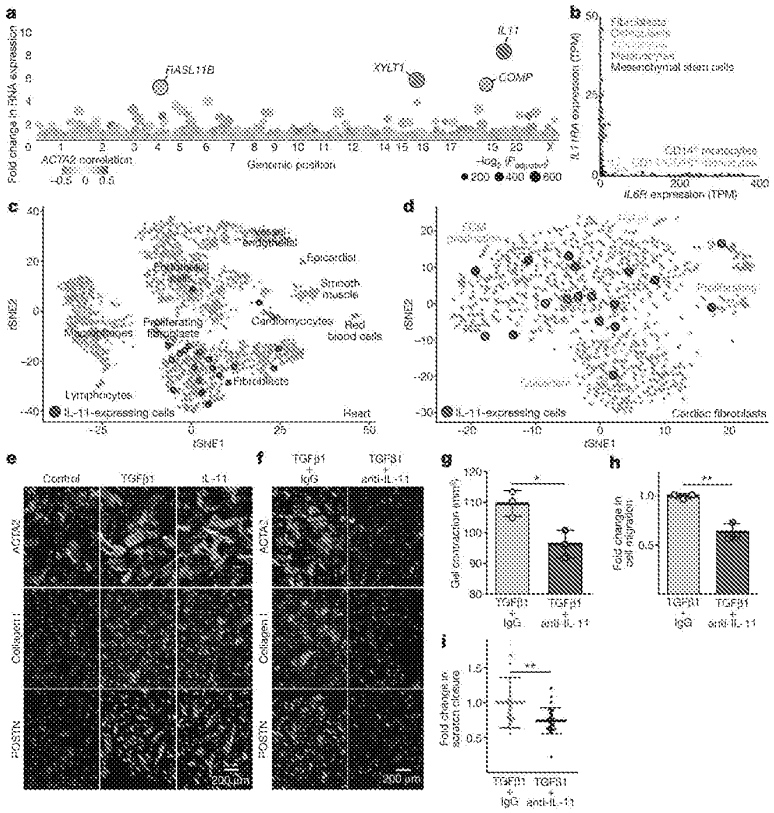

Figure 1. Fibrosis target discovery platform identifies IL-11

a, RNA-seq of primary cardiac fibroblasts ($n = 84$ biologically independent samples) with or without TGFβ1 treatment (5 ng ml^{-1}, 24 h) and Spearman's correlation of expression changes with fibroblast activation (Supplementary Table 2). DEseq2[10] fold change in expression and false-discovery rate (FDR)-adjusted *P* values are shown. **b**, RNA expression in transcripts per million (TPM) of *IL11RA* and *IL6R* across 512 cell lines from the FANTOM repository[12]. **c**, Single-cell resolution of cardiac *Il11* expression (more than 0 reads per cell). *t*-distributed stochastic neighbour embedding (*t*SNE) analysis[28] clusters cell types of the heart. *Il11* expression is highly enriched in fibroblasts. χ^2 test ($P = 5.7 \times 10^{-8}$). **d**, *t*SNE analysis of fibroblasts alone shows highest Il-11 expression in ECM-secreting and TGFβ1-activated fibroblasts. χ^2 test ($P = 0.033$). **c**, **d**, Cardiac cells were sequenced from $n = 1$ mouse, the experiment was repeated once with similar results. **e**, **f**, Representative images (chosen from 42 per condition) of cardiac fibroblasts immunostained for ACTA2, collagen I or periostin (POSTN) after a 24-h incubation without stimulus (control), TGFβ1 or IL-11 (5 ng ml^{-1}) (**e**) or with TGFβ1 (5 ng ml^{-1}) and an anti-IL-11 neutralizing antibody or an IgG control (2 μg ml^{-1}) (**f**). **g**, Cardiac fibroblasts were seeded in collagen gel and the area of contraction determined ($n = 3$ biologically independent samples) after 72 h. **h**, Trans-well migration assay (colourimetrically quantified, $n = 3$ biologically independent samples, 24 h). **i**, Scratch assay of wound closure in a monolayer of cardiac fibroblasts ($n = 5$

biologically independent samples) after 24 h. **g–i**, Two-tailed Student's *t*-test; data are mean ± s.d.; $*P < 0.05$; $**P < 0.01$.

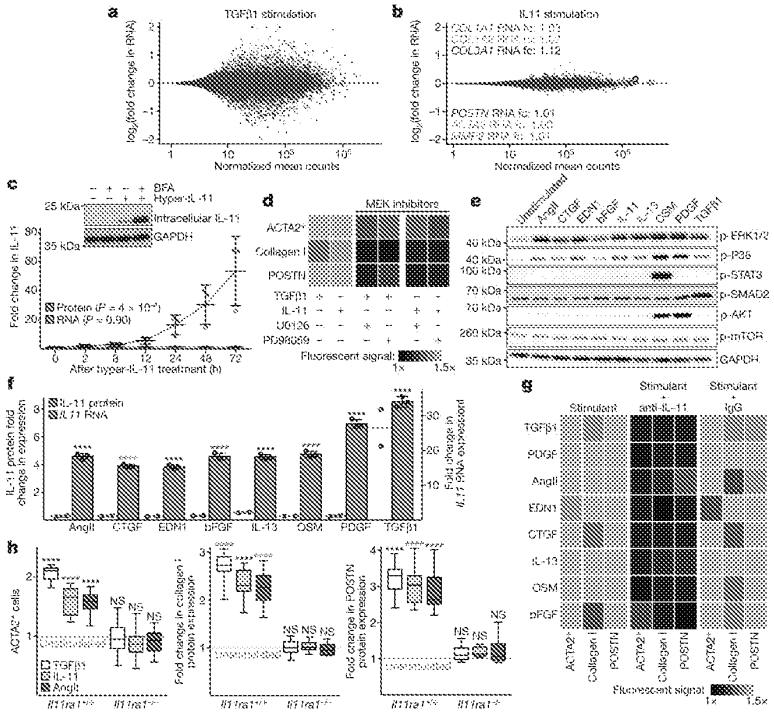

Figure 2. Non-canonical IL-11 signalling drives fibrogenic protein synthesis

a, **b**, RNA-seq of primary cardiac fibroblasts in response to TGFβ1 (**a**) or IL-11 (**b**) ($n = 6$ biologically independent samples, 5 ng ml^{-1}, 24 h). Red indicates significantly differentially expressed genes (FDR ≤ 0.05, DEseq2[10]). RNA expression of genes associated with fibrosis is not increased by IL-11 treatment. fc, fold change. **c**, ELISA and quantitative PCR with reverse transcription (RT–qPCR) assays of IL-11 expression ($n = 3$ biologically independent samples) after hyperIL-11 treatment (0.2 ng ml^{-1}). Benjamini–Hochberg corrected one-way ANOVA; data are mean ± s.d. Inset, western blot of cardiac fibroblast lysates after hyperIL-11 stimulation and brefeldin A (BFA, 1 μg ml^{-1}) treatment indicates canonical secretion of IL-11. **d**, Cardiac fibroblasts were incubated with TGFβ1, IL-11 (5 ng ml^{-1}) and MEK inhibitors U0126 or PD98059 (10 μM, 24 h). ACTA2^{+} cells and ECM production was assessed and normalized to non-stimulated cells. **e**, Western blots of phosphorylated protein (p-) expression of signalling pathways in cardiac fibroblasts in response to various pro-fibrotic stimuli (see also Extended Data Fig. 5). **f**, ELISA (supernatant, $n = 3$ biologically independent samples) and RT–qPCR ($n = 2$ biologically independent samples) of IL-11 expression in cardiac fibroblasts after 24 h stimulation with AngII (100 nM), CTGF (50 ng ml^{-1}), EDN1 (250 ng ml^{-1}), bFGF (10 ng ml^{-1}), IL-13 (100 ng ml^{-1}), OSM (100 ng ml^{-1}), PDGF (200 ng ml^{-1}) and TGFβ1 (5 ng ml^{-1}). Two-tailed Dunnett's test; Data are mean ± s.d. **g**, Cardiac fibroblasts were incubated with pro-fibrotic cytokines (24 h) and cardiac fibroblast activation was reduced by anti-IL-11 antibodies (2 μg ml^{-1}; Extended Data Fig. 6). **h**, Pro-fibrotic proteins (Operetta assay $n = 7$ measurements per $n = 2$ independent experiments) are not upregulated in cardiac fibroblasts from *Il1ra*$^{-/-}$ mice in response to TGFβ1, IL-11 (5 ng ml^{-1}) or AngII (100 nM, 24 h). Experiments were repeated twice with

similar results. Two-tailed Dunnett's test; box-and-whisker plots show median (middle line), 25th–75th percentiles (box) and 10th–90th percentiles (whiskers); **** $P < 0.0001$; NS, not significant.

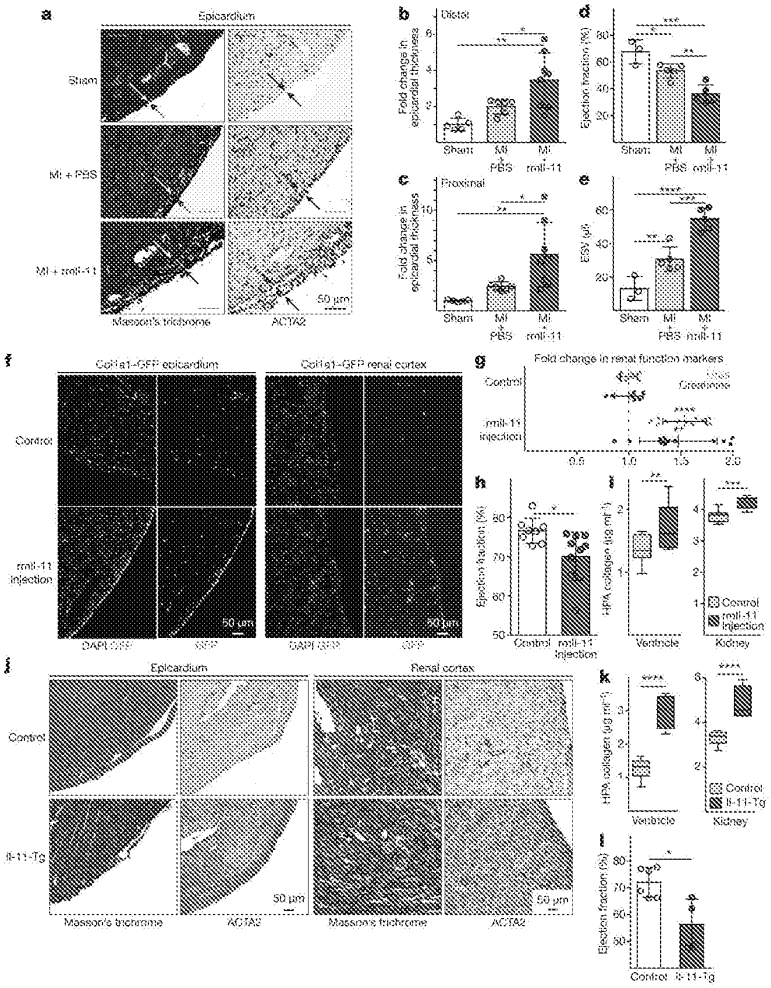

Figure 3. Il-11 causes cardiovascular fibrosis and organ failure

a, Representative histological images of the epicardium with Masson's trichrome staining and ACTA2 immunostaining in mice with myocardial infarction (MI) treated with rmIL-11 or PBS. **b**, **c**, Epicardial thickness of regions distal (**b**) or proximal (**c**) to the region of myocardial infarction. **a**–**c**, Sham, $n = 5$; myocardial infarction, $n = 6$; myocardial infarction + rmIl-11, $n = 8$ biologically independent samples. **d**, **e**, Echocardiography show a decrease in ejection fraction (**d**) and increase in end-systolic volume (ESV) (**e**) after myocardial infarction and rmIl-11 treatment (sham, $n = 3$; myocardial infarction, myocardial infarction + rmIl-11, $n = 5$ biologically independent samples). **b**–**e**, two-tailed, Holm–Sidak-corrected Student's *t*-test; Data are mean ± s.d. **f**, Representative histological images (chosen from control, $n = 3$; rmIl-11 injection, $n = 4$ biologically independent samples) of tissues from a Col1a1–GFP-reporter mouse after rmIL-11 injection (100 μg kg^{-1} per day, three weeks). **g**, Serum urea and creatinine levels after rmIL-11 injection (control urea, $n = 8$; control creatinine, $n = 7$; rmIl-11, $n = 12$ biologically independent samples). **h**, Reduced ejection fraction (echocardiography) in rmIl-11-treated mice. **i**, Hydroxyproline assay (HPA)

quantifies cardiac and renal collagen content after rmIL-11 treatment. **h**, **i**, Control, $n = 8$; rmIl-11, $n = 11$ biologically independent samples. **j**, Representative histological images of Masson's trichrome staining and ACTA2 immunostaining in the epicardium Il-11-Tg mice. **k**, HPA indicates cardiac and renal collagen content in Il-11-Tg mice. **j**, **k**, Control, $n = 12$; Il-11-Tg, $n = 4$ biologically independent samples. **i**, **k**, Two-tailed Student's *t*-test; box-and-whisker plots show median (middle line), 25th–75th percentiles (box) and 10th–90th percentiles (whiskers). **l**, Reduction in ejection fraction (echocardiography) in Il-11-Tg mice (control, $n = 6$; Il-11-Tg, $n = 4$ biologically independent samples). **g**, **h**, **l**, Two-tailed Student's *t*-test; data are mean ± s.d.; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

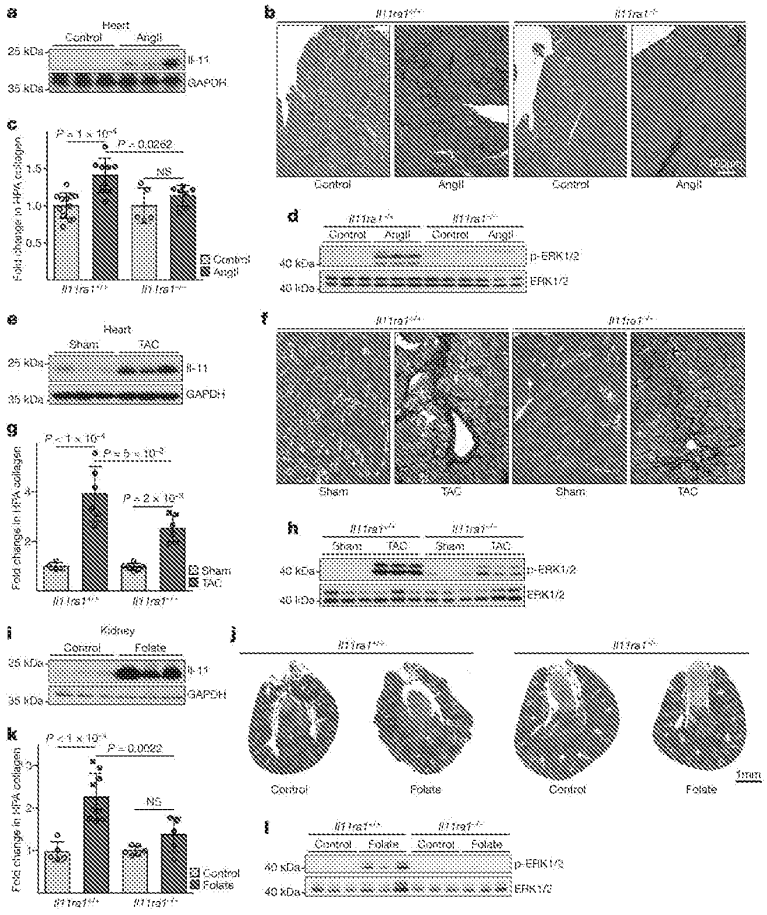

Figure 4. Inhibition of Il-11 reduces cardiovascular fibrosis

a, Western blot of cardiac Il-11 after AngII infusion (2 mg kg^{-1} per day for 28 days). **b**, **c**, Representative histological images (**b**; Masson's trichrome staining) and collagen content (**c**) in the atrium of *Il1ra1*$^{+/+}$ (control, $n = 12$; AngII, $n = 9$ biologically independent samples) and *Il1ra1*$^{-/-}$ (control, $n = 5$; AngII, $n = 7$ biologically independent samples) mice. **d**, Western blot of cardiac ERK activation after AngII infusion. **e**, Western blot of cardiac Il-11 after transverse aortic constriction (TAC). **f**, **g**, Representative histological images (**f**; Masson's trichrome staining) and collagen content. **g**; HPA of hearts from *Il1ra1*$^{+/+}$ (control, $n = 4$; TAC: $n = 6$ biologically independent samples) and *Il1ra1*$^{-/-}$ (control, $n = 6$; TAC, $n = 6$ biologically independent samples) mice after TAC or sham operations. **h**, Western blot of cardiac ERK activation after TAC. **i**, Western blot of renal Il-11 after folate treatment (180 mg kg^{-1}). **j**, **k**, Representative histological images (**j**; Masson's trichrome staining) and collagen content (**k**; HPA assay) of kidneys from *Il1ra1*$^{+/+}$ (control, $n = 5$; folate, $n = 9$ biologically independent samples) and *Il1ra1*$^{-/-}$ (control, $n = 6$; folate, $n = 5$ biologically independent samples) mice. **c**, **g**, **k**, Two-tailed, Sidak-corrected Student's *t*-test;

data are mean ± s.d. NS, not significant. **I**, Western blot of renal ERK activation after folate treatment.

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	1629
		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):
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☐ 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 5, 2018	Reg. No.	73,073

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Dated: June 5, 2018 Signature: ____/Nathaniel W. Taylor/_____ (Nathaniel W. Taylor)

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DOCKET NO.: M0546.70012US01

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:	Not Yet Assigned
Art Unit:	1629

CERTIFICATE OF ELECTRONIC FILING UNDER 37 C.F.R. § 1.8

The undersigned hereby certifies that this paper, along with any paper referred to as being attached or enclosed, is being transmitted via the Office electronic filing system in accordance with § 1.6(a)(4), on the 5th day of June, 2018.

__/Nathaniel W. Taylor/_____
Nathaniel W. Taylor

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

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.

The undersigned hereby makes the following additional information of record in the above-identified application.

The undersigned would like to bring to the Examiner's attention the following co-pending application that may contain subject matter related to this application:

Serial No.	Filing Date	Inventor(s)	Docket No.
***15/381,622**	**December 16, 2016**	**Cook et al.**	**M0546.70012US00**

*A copy of this reference is not provided as the Office has waived the requirement under 37 C.F.R. § 1.98(a)(2)(iii) for submitting a copy of a cited U.S. patent application if it is scanned to the Image File Wrapper system and is available on Private PAIR.

The undersigned would like to bring to the Examiner's attention the enclosed search report or other communication from a corresponding or related International or Foreign National Application:

Serial No.	Date of Mailing	Type(s) of Communication
PCT/EP2016/081430	**11-06-2017**	**International Preliminary Report on Patentability**
PCT/EP2016/081430	**04-18-2017**	**International Search Report and Written Opinion**

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/
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Docket No.: M0546.70012US01
Date: June 5, 2018
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DocCode – SCORE

SCORE Placeholder Sheet for IFW Content

Application Number: 15988463 Document Date: 05/24/2018

The presence of this form in the IFW record indicates that the following document type was received in electronic format on the date identified above. This content is stored in the SCORE database.

Since this was an electronic submission, there is no physical artifact folder, no artifact folder is recorded in PALM, and no paper documents or physical media exist. The TIFF images in the IFW record were created from the original documents that are stored in SCORE.

- Drawing

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- USPTO employees may access SCORE content via eDAN using the Supplemental Content tab, or via the SCORE web page.
- External customers may access SCORE content via PAIR using the Supplemental Content tab.

Form Revision Date: August 26, 2013

DocCode – SEQ.TXT

SCORE Placeholder Sheet for IFW Content

Application Number: 15988463 Document Date: 05/24/2018

The presence of this form in the IFW record indicates that the following document type was received in electronic format on the date identified above. This content is stored in the SCORE database.

Since this was an electronic submission, there is no physical artifact folder, no artifact folder is recorded in PALM, and no paper documents or physical media exist. The TIFF images in the IFW record were created from the original documents that are stored in SCORE.

- Sequence Listing

At the time of document entry (noted above):

- USPTO employees may access SCORE content via eDAN using the Supplemental Content tab, or via the SCORE web page.
- External customers may access SCORE content via PAIR using the Supplemental Content tab.

Form Revision Date: August 26, 2013

PTO/AIA/15 (10-17)
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UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.	M0546.70012US01
First Named Inventor	Stuart Alexander Cook
Title	TREATMENT OF FIBROSIS
Priority Mail Express® Label No.	

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

1. [X] **Fee Transmittal Form** (PTO/SB/17 or equivalent)
2. [ ] **Applicant asserts small entity status.** See 37 CFR 1.27
3. [ ] **Applicant certifies micro entity status.** See 37 CFR 1.29. Applicant must attach form PTO/SB/15A or B or equivalent.
4. [X] **Specification** [*Total Pages* 96] Both the claims and abstract must start on a new page. *(See MPEP § 608.01(a) for information on the preferred arrangement)*
5. [X] **Drawing(s)** (35 U.S.C. 113) [*Total Sheets* 66]
6. **Inventor's Oath or Declaration** [*Total Pages* 1] *(including substitute statements under 37 CFR 1.64 and assignments serving as an oath or declaration under 37 CFR 1.63(e))*
   a. [ ] Newly executed (original or copy)
   b. [X] A copy from a prior application (37 CFR 1.63(d))
7. [X] **Application Data Sheet** ** See note below.* See 37 CFR 1.76 (PTO/AIA/14 or equivalent)
8. **CD-ROM or CD-R** in duplicate, large table, or Computer Program (*Appendix*)
   [ ] Landscape Table on CD
9. **Nucleotide and/or Amino Acid Sequence Submission** (*if applicable, items a. – c. are required*)
   a. [ ] Computer Readable Form (CRF)
   b. [ ] Specification Sequence Listing on:
      i. [ ] CD-ROM or CD-R (2 copies); or
      ii. [ ] Paper
   c. [ ] Statements verifying identity of above copies

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P.O. Box 1450
Alexandria, VA 22313-1450

ACCOMPANYING APPLICATION PAPERS

10. [ ] **Assignment Papers** (cover sheet & document(s))
    Name of Assignee
11. [ ] **37 CFR 3.73(c) Statement** (*when there is an assignee*) [X] **Power of Attorney**
12. [ ] **English Translation Document** (*if applicable*)
13. [ ] **Information Disclosure Statement** (PTO/SB/08 or PTO-1449)
    [ ] Copies of citations attached
14. [X] **Preliminary Amendment**
15. [ ] **Return Receipt Postcard** (*MPEP § 503*) (*Should be specifically itemized*)
16. [ ] **Certified Copy of Priority Document(s)** (*if foreign priority is claimed*)
17. [ ] **Nonpublication Request** Under 35 U.S.C. 122(b)(2)(B)(i). Applicant must attach form PTO/SB/35 or equivalent.
18. [X] **Other:** Sequence Listing in Text Format
    Power of Attorney

***Note:** (1) Benefit claims under 37 CFR 1.78 and foreign priority claims under 1.55 **must** be included in an Application Data Sheet (ADS).
(2) For applications filed under 35 U.S.C. 111, the application must contain an ADS specifying the applicant if the applicant is an assignee, person to whom the inventor is under an obligation to assign, or person who otherwise shows sufficient proprietary interest in the matter. See 37 CFR 1.46(b).

19. CORRESPONDENCE ADDRESS

[X] The address associated with Customer Number: 23628 **OR** [ ] Correspondence address below

Name					
Address					
City		State		Zip Code	
Country		Telephone		Email	

Signature	/Amy J. McMahon/	Date	May 24, 2018
Name (Print/Type)	Amy J. McMahon, PhD	Registration No. (Attorney/Agent)	73,073

6332697.1

FEE TRANSMITTAL

Complete if known	
Application Number	Not Yet Assigned
Filing Date	May 24, 2018
First Named Inventor	Stuart Alexander Cook
Examiner Name	Not Yet Assigned
Art Unit	Not Yet Assigned
Practitioner Docket No.	M0546.70012US01

☐ Applicant asserts small entity status. See 37 CFR 1.27.

☐ Applicant certifies micro entity status. See 37 CFR 1.29. Form PTO/SB/15A or B or equivalent must either be enclosed or have been submitted previously.

TOTAL AMOUNT OF PAYMENT ($)2,120.00

METHOD OF PAYMENT (check all that apply)

☐ Check ☒ Credit Card ☐ Money Order ☐ None ☐ Other (please identify): _____

☒ Deposit Account Deposit Account Number: 23/2825 Deposit Account Name: Wolf, Greenfield & Sacks, P.C.

For the above-identified deposit account, the Director is hereby authorized to (check all that apply):

☐ Charge fee(s) indicated below

☐ Charge fee(s) indicated below, **except for the filing fee**

☒ Charge any additional fee(s) or underpayment of fee(s) under 37 CFR 1.16 and 1.17, **except for any excess claims fees or multiple dependent claim fee**

☐ Charge any additional fee(s) or underpayment of fee(s) under 37 CFR 1.16 and 1.17

☒ Credit any overpayment of fee(s)

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

FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES (U = undiscounted fee; S = small entity fee; M = micro entity fee)

	FILING FEES			SEARCH FEES			EXAMINATION FEES			
Application Type	**U ($)**	**S ($)**	**M ($)**	**U ($)**	**S ($)**	**M ($)**	**U ($)**	**S ($)**	**M ($)**	**Fees Paid ($)**
Utility	300	150*	75	660	330	165	760	380	190	1,720.00
Design	200	100	50	160	80	40	600	300	150	
Plant	200	100	50	420	210	105	620	310	155	
Reissue	300	150	75	660	330	165	2,200	1,100	550	
Provisional	280	140	70	0	0	0	0	0	0	

* The $150 small entity status filing fee for a utility application is further reduced to $75 for a small entity status applicant who files the application via EFS-Web.

2. EXCESS CLAIM FEES

Fee Description	**Undiscounted Fee ($)**	**Small Entity Fee ($)**	**Micro Entity Fee ($)**
Each claim over 20 (including Reissues)	100	50	25
Each independent claim over 3 (including Reissues)	460	230	115
Multiple dependent claims	820	410	205

Total Claims		**Extra Claims**	**Fee ($)**		**Fee Paid ($)**
10	- 20 or HP =		x	=	

HP = highest number of total claims paid for, if greater than 20.

Indep. Claims		**Extra Claims**	**Fee ($)**		**Fee Paid ($)**
1	- 3 or HP =		x	=	

HP = highest number of independent claims paid for, if greater than 3.

Multiple Dependent Claims	
Fee ($)	**Fee Paid ($)**

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), the application size fee due is $400 ($200 for small entity) ($100 for micro entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets		**Extra Sheets**		**Number of each additional 50 or fraction thereof**		**Fee ($)**		**Fee Paid ($)**
126	- 100 =	26	/50 =	1	(round **up** to a whole number) x	400.00	=	400.00

4. OTHER FEE(S)

	Fees Paid ($)
Non-English specification, $130 fee (no small or micro entity discount)	
Non-electronic filing fee under 37 CFR 1.16(t) for a utility application, $400 fee ($200 small or micro entity)	
Other (e.g., late filing surcharge):	

SUBMITTED BY

Signature	/Amy J. McMahon/	Registration No. (Attorney/Agent)	73,073	Telephone	617.646.8000
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ABSTRACT

Aspects of the disclosure relate to the treatment, prevention or alleviation of conditions such as fibrosis in a subject. In some embodiments, the treatment, prevention or alleviation of fibrosis in a subject through the administration of an agent capable of inhibiting the action of Interleukin 11 (IL-11) is disclosed.

CLAIMS

We claim:

1. A method of treating fibrosis in a human subject, the method comprising administering to a human subject in need of treatment a therapeutically effective amount of an anti-interleukin 11 (IL-11) antibody, wherein the anti-IL-11 antibody binds to IL-11 and inhibits IL-11 mediated signalling.

2. The method of claim 1, wherein the fibrosis is fibrosis of the heart, lung, liver, kidney, eye, or skin.

3. The method of claim 1, wherein the fibrosis is in the heart and is associated with dysfunction of the musculature or electrical properties of the heart, or thickening of the walls or valves of the heart.

4. The method of claim 1, wherein the fibrosis is in the liver and is associated with chronic liver disease or liver cirrhosis.

5. The method of claim 1, wherein the fibrosis is in the kidney and is associated with chronic kidney disease.

6. The method of claim 1, wherein the fibrosis is in the eye and is retinal fibrosis or conjunctival fibrosis.

7. The method of claim 1, wherein the subject in need of treatment has a disease or disorder selected from atrial fibrillation, ventricular fibrillation, hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), pulmonary fibrosis, cystic fibrosis, non-alcoholic steatohepatitis (NASH), cirrhosis, chronic kidney disease, systemic sclerosis, retinal fibrosis, conjunctival fibrosis, post-surgical fibrosis, scleroderma, keloid, or Chron's disease.

8. The method of claim 1, wherein the antibody is an anti-IL-11 neutralising antibody.

9. The method of claim 1, wherein the method comprises administering said anti-IL-11 antibody to a human subject in which IL-11 or Interleukin 11 receptor (IL-11R) expression is upregulated.

10. The method of claim 1, wherein the method comprises determining, optionally *in vitro*, whether IL-11 or Interleukin 11 receptor (IL-11R) expression is upregulated in the human subject and administering said anti-IL-11 antibody to a human subject in which IL-11 or IL-11R expression is upregulated.

11. A method of treating fibrosis in a subject, the method comprising:

(i) determining, optionally *in vitro*, whether IL-11 or an Interleukin 11 receptor (IL-11R) expression is upregulated in the subject; and

(ii) administering to a subject in which IL-11 or IL-11R expression is upregulated a therapeutically effective amount of an anti-IL-11 antibody, wherein the anti-IL-11 antibody binds to IL-11 and inhibits IL-11 mediated signalling.

12. The method of claim 11, wherein the fibrosis is fibrosis of the heart, lung, liver, kidney, eye or skin.

13. The method of claim 11, wherein the fibrosis is in the heart and is associated with dysfunction of the musculature or electrical properties of the heart, or thickening of the walls or valves of the heart.

14. The method of claim 11, wherein the fibrosis is in the liver and is associated with chronic liver disease or liver cirrhosis.

15. The method of claim 11, wherein the fibrosis is in the kidney and is associated with chronic kidney disease.

16. The method of claim 11, wherein the fibrosis is in the eye and is retinal fibrosis or conjunctival fibrosis .

17. The method of claim 11, wherein the subject in need of treatment has a disease or disorder selected from atrial fibrillation, ventricular fibrillation, hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), pulmonary fibrosis, cystic fibrosis, non-alcoholic steatohepatitis (NASH), cirrhosis, chronic kidney disease, systemic sclerosis, retinal fibrosis, conjunctival fibrosis, post-surgical fibrosis, scleroderma, keloid, or Chron's disease.

18. The method of claim 11, wherein the antibody is an anti-IL-11 neutralising antibody.

19. A method of treating fibrosis in a subject, the method comprising:

(i) determining, optionally *in vitro*, one or more genetic factors in the subject that are predictive of upregulation of Interleukin 11 (IL-11) or Interleukin 11 receptor (IL-11R) expression or activity;

(ii) selecting a subject for treatment based on the determination in (i); and

(ii) administering to the selected subject a therapeutically effective amount of an anti-IL-11 antibody, wherein the anti-IL-11 antibody binds to IL-11 and inhibits IL-11 mediated signalling.

20. The method of claim 19, wherein the fibrosis is fibrosis of the heart, lung, liver, kidney, eye, or skin.

TREATMENT OF FIBROSIS

RELATED APPLICATIONS

The present application claims priority under 35 USC § 119(a)-(d) to United Kingdom Application No. 1522186.4, filed December 16, 2015. The entire contents of this application is hereby incorporated by reference herein.

FIELD OF THE INVENTION

The present invention relates to the diagnosis and treatment of conditions such as fibrosis.

BACKGROUND TO THE INVENTION

Fibrosis is an essential process that is a critical part of wound healing. Excessive fibrosis is common in many rare and common disease conditions and is important in disease pathogenesis. Diseases characterized by excessive fibrosis include but are not restricted to: systemic sclerosis, scleroderma, hypertrophic cardiomyopathy, dilated cardiomyopathy (DCM), atrial fibrillation, ventricular fibrillation, myocarditis, liver cirrhosis, kidney diseases, diseases of the eye, asthma, cystic fibrosis, arthritis and idiopathic pulmonary fibrosis. Despite the large impact on human health, therapeutic and diagnostic approaches to fibrosis are still an unmet medical need.

The real physiological role of Interleukin 11 (IL-11) remains unclear. IL-11 has been most strongly linked with activation of haematopoetic cells and with platelet production but also found to be pro- as well as anti-inflammatory, pro-angiogenic and important for neoplasia. It is known that TGFβ1 or tissue injury can induce IL-11 expression (Zhu, M. et al. IL-11 Attenuates Liver Ischemia/Reperfusion Injury (IRI) through STAT3 Signaling Pathway in Mice. PLOS ONE 10, (2015); Yashiro, R. et al. Transforming growth factor-beta stimulates interleukin-11 production by human periodontal ligament and gingival fibroblasts. J. Clin. Periodontol. 33, 165–71 (2006); Obana, M. et al. Therapeutic activation of signal transducer and activator of transcription 3 by interleukin-11 ameliorates cardiac fibrosis after myocardial infarction. Circulation 121, 684–91 (2010); Tang, W., Yang, L., Yang, Y. C., Leng, S. X. & Elias, J. A. Transforming growth factor-beta stimulates interleukin-11 transcription via complex activating protein-1-dependent pathways. J. Biol. Chem. 273, 5506–13 (1998)).

The role for IL-11 in fibrosis is not clear from the published literature. IL-11 is thought to be important for fibrosis and inflammation in the lung (Tang, W. et al. Targeted expression of IL-11 in the murine airway causes lymphocytic inflammation, bronchial remodeling, and airways

obstruction. J. Clin. Invest. 98, 2845–53 (1996)) and its expression level is correlated with collagen levels in the skin (Toda, M. et al. Polarized in vivo expression of IL-11 and IL-17 between acute and chronic skin lesions. Journal of Allergy and Clinical Immunology 111, 875–881 (2003)) and the respiratory system (Molet, S., Hamid, Q. & Hamilos, D. IL-11 and IL-17 expression in nasal polyps: Relationship to collagen deposition and suppression by intranasal fluticasone propionate. The Laryngoscope 113, (2003); Minshall et al. IL-11 expression is increased in severe asthma: association with epithelial cells and eosinophils. The Journal of allergy and clinical immunology 105, (2000)).

However, the majority of studies suggest that IL-11 is anti-fibrotic: in the heart (Obana, M. et al. Therapeutic activation of signal transducer and activator of transcription 3 by interleukin-11 ameliorates cardiac fibrosis after myocardial infarction. Circulation 121, 684–91 (2010); Obana, M. et al. Therapeutic administration of IL-11 exhibits the postconditioning effects against ischemia-reperfusion injury via STAT3 in the heart. American Journal of Physiology. Heart and circulatory physiology 303, H569–77 (2012)) and kidney (Stangou, M. et al. Effect of IL-11 on glomerular expression of TGF-beta and extracellular matrix in nephrotoxic nephritis in Wistar Kyoto rats. Journal of nephrology 24, 106–11 (2011); Ham, A. et al. Critical role of interleukin-11 in isoflurane-mediated protection against ischemic acute kidney injury in mice. Anesthesiology 119, 1389–401 (2013)) and anti-inflammatory in several tissues and chronic inflammatory diseases (Trepicchio & Dorner. The therapeutic utility of Interleukin-11 in the treatment of inflammatory disease. (1998). doi:10.1517/13543784.7.9.1501). The molecular mode of action of IL-11 in general, is thought to be regulation of RNA expression of mRNA levels via STAT3-mediated transcription (Zhu, M. et al. IL-11 Attenuates Liver Ischemia/Reperfusion Injury (IRI) through STAT3 Signaling Pathway in Mice. PLOS ONE 10, (2015)).

SUMMARY OF THE INVENTION

One aspect of the present invention concerns the treatment, prevention or alleviation of fibrosis in a subject in need of treatment through the administration of an agent capable of inhibiting the action of Interleukin 11 (IL-11). The inventors have identified IL-11 to have a pro-fibrotic action. The present invention is particularly concerned with inhibiting the pro-fibrotic action of IL-11. Embodiments of the invention concern inhibition or prevention of the IL-11 mediated pro-fibrotic signal, e.g. as mediated by binding of IL-11 to an IL-11 receptor.

In some embodiments an agent capable of inhibiting the action of IL-11 may prevent or reduce the binding of IL-11 to an IL-11 receptor.

In some embodiments an agent capable of inhibiting the action of IL-11 may bind IL-11 to form a complex comprising the agent and IL-11. The complex may be a non-covalent or covalent complex. In some embodiments, the formation of the agent:IL-11 complex may prevent or reduce the ability of IL-11 to bind to an IL-11 receptor. In some embodiments such prevention or reduction may be the result of a reduction of the productive binding of IL-11 to an IL-11 receptor, i.e. reduction in the ability of IL-11 to initiate IL-11 receptor mediated signalling. In some embodiments formation of the agent:IL-11 complex may sequester IL-11 away from the IL-11 receptor, thereby preventing or reducing the contact of IL-11 with an IL-11 receptor and/or preventing or reducing the amount of IL-11 available for binding to an IL-11 receptor. In some embodiments the agent may be a decoy receptor.

In some embodiments an agent capable of inhibiting the action of IL-11 may bind to an IL-11 receptor. An agent that binds an IL-11 receptor may prevent or reduce the ability of IL-11 to bind to an IL-11 receptor (IL-11R).

Another aspect of the present invention concerns the treatment, prevention or alleviation of fibrosis in a subject in need of treatment through the administration of an agent capable of preventing or reducing the expression of IL-11 or an IL-11 receptor (IL-11R).

In one aspect of the present invention an agent capable of inhibiting the action of Interleukin 11 (IL-11) for use in a method of treating or preventing fibrosis is provided.

In another aspect of the present invention the use of an agent capable of inhibiting the action of IL-11 in the manufacture of a medicament for use in a method of treating or preventing fibrosis is provided.

In another aspect of the present invention a method of treating or preventing fibrosis is provided, the method comprising administering to a subject in need of treatment a therapeutically effective amount of an agent capable of inhibiting the action of IL-11.

In some embodiments the agent capable of inhibiting the action of IL-11 is an agent capable of preventing or reducing the binding of IL-11 to an IL-11 receptor.

In some embodiments the agent capable of inhibiting the action of IL-11 is an IL-11 binding agent. IL-11 binding agents may be selected from the group consisting of: an antibody, polypeptide, peptide, oligonucleotide, aptamer or small molecule. In some embodiments the

IL-11 binding agent is an antibody. In some embodiments the IL-11 binding agent is a decoy receptor.

In some embodiments the agent capable of inhibiting the action of IL-11 is an IL-11 receptor (IL-11R) binding agent. IL-11R binding agents may be selected from the group consisting of: an antibody, polypeptide, peptide, oligonucleotide, aptamer or small molecule. In some embodiments the IL-11R binding agent is an antibody.

In another aspect of the present invention an agent capable of preventing or reducing the expression of IL-11 or IL-11R for use in a method of treating or preventing fibrosis is provided.

In another aspect of the present invention the use of an agent capable of preventing or reducing the expression of IL-11 or IL-11R in the manufacture of a medicament for use in a method of treating or preventing fibrosis is provided.

In another aspect of the present invention a method of treating or preventing fibrosis is provided, the method comprising administering to a subject in need of treatment a therapeutically effective amount of an agent capable of preventing or reducing the expression of IL-11 or IL-11R.

In some embodiments the agent capable of preventing or reducing the expression of IL-11 or IL-11R is a small molecule or oligonucleotide.

In some embodiments the fibrosis to be treated or prevented is fibrosis of the heart, liver or kidney. In some embodiments the fibrosis to be treated or prevented is fibrosis of the eye. In some embodiments the fibrosis is in the heart and is associated with dysfunction of the musculature or electrical properties of the heart, or thickening of the walls or valves of the heart. In some embodiments the fibrosis is in the liver and is associated with chronic liver disease or liver cirrhosis. In some embodiments the fibrosis is in the kidney and is associated with chronic kidney disease.

In some embodiments the method of treating or preventing comprises administering a said agent to a subject in which IL-11 or IL-11R expression is upregulated. In some embodiments the method of treating or preventing comprises administering a said agent to a subject in which IL-11 or IL-11R expression has been determined to be upregulated. In some embodiments the method of treating or preventing comprises determining whether IL-11 or IL-

11R expression is upregulated in the subject and administering a said agent to a subject in which IL-11 or IL-11R expression is upregulated.

In another aspect of the present invention a method of determining the suitability of a subject for the treatment or prevention of fibrosis with an agent capable of inhibiting the action of IL-11 is provided, the method comprising determining, optionally *in vitro*, whether IL-11 or IL-11R expression is upregulated in the subject.

In another aspect of the present invention a method of selecting a subject for the treatment or prevention of fibrosis with an agent capable of inhibiting the action of IL-11 is provided, the method comprising determining, optionally *in vitro*, whether IL-11 or IL-11R expression is upregulated in the subject.

In another aspect of the present invention a method of diagnosing fibrosis or a risk of developing fibrosis in a subject is provided, the method comprising determining, optionally *in vitro*, the upregulation of IL-11 or IL-11R in a sample obtained from the subject.

In some embodiments the method is a method of confirming a diagnosis of fibrosis in a subject suspected of having fibrosis.

In some embodiments the method further comprises selecting the subject for treatment with an agent capable of inhibiting the action of IL-11 or with an agent capable of preventing or reducing the expression of IL-11 or IL-11R.

In another aspect of the present invention a method of providing a prognosis for a subject having, or suspected of having fibrosis, is provided, the method comprising determining, optionally *in vitro*, whether IL-11 or IL-11R is upregulated in a sample obtained from the subject and, based on the determination, providing a prognosis for treatment of the subject with an agent capable of inhibiting the action of IL-11 or with an agent capable of preventing or reducing the expression of IL-11 or IL-11R.

The method may further comprise selecting a subject determined to have upregulated IL-11 or IL-11R for treatment with an agent capable of inhibiting the action of IL-11 or with an agent capable of preventing or reducing the expression of IL-11 or IL-11R.

In another aspect of the present invention a method of diagnosing fibrosis or a risk of developing fibrosis in a subject is provided, the method comprising determining, optionally *in vitro*, one or more genetic factors in the subject that are predictive of upregulation of IL-11 or IL-11R expression, or of upregulation of IL-11 or IL-11R activity.

In some embodiments the method is a method of confirming a diagnosis of fibrosis in a subject suspected of having fibrosis.

In some embodiments the method further comprises selecting the subject for treatment with an agent capable of inhibiting the action of IL-11 or with an agent capable of preventing or reducing the expression of IL-11 or IL-11R.

In another aspect of the present invention a method of providing a prognosis for a subject having, or suspected of having, fibrosis, is provided, the method comprising determining, optionally *in vitro*, one or more genetic factors in the subject that are predictive of upregulation of IL-11 or IL-11R expression, or of upregulation of IL-11 or IL-11R activity.

The following numbered paragraphs (paras) describe further aspects and embodiments of the present invention:

1. An agent capable of inhibiting the action of Interleukin 11 (IL-11) for use in a method of treating or preventing fibrosis.

2. Use of an agent capable of inhibiting the action of Interleukin 11 (IL-11) in the manufacture of a medicament for use in a method of treating or preventing fibrosis.

3. A method of treating or preventing fibrosis, the method comprising administering to a subject in need of treatment a therapeutically effective amount of an agent capable of inhibiting the action of Interleukin 11 (IL-11).

4. The agent for use in a method of treating or preventing fibrosis according to para 1, use according to para 2 or method according to para 3, wherein the agent is an agent capable of preventing or reducing the binding of IL-11 to an IL-11 receptor.

5. The agent for use in a method of treating or preventing fibrosis according to para 1 or 4, use according to para 2 or 4, or method according to para 3 or 4, wherein the agent is an IL-11 binding agent.

6. The agent for use in a method of treating or preventing fibrosis, use or method according to para 5, wherein the IL-11 binding agent is selected from the group consisting of: an antibody, polypeptide, peptide, oligonucleotide, aptamer or small molecule.

7. The agent for use in a method of treating or preventing fibrosis, use or method according to para 5, wherein the IL-11 binding agent is an antibody.

8. The agent for use in a method of treating or preventing fibrosis, use or method according to para 5, wherein the IL-11 binding agent is a decoy receptor.

9. The agent for use in a method of treating or preventing fibrosis according to para 1 or 4, use according to para 2 or 4, or method according to para 3 or 4, wherein the agent is an IL-11 receptor (IL-11R) binding agent.

10. The agent for use in a method of treating or preventing fibrosis, use or method according to para 9, wherein the IL-11R binding agent is selected from the group consisting of: an antibody, polypeptide, peptide, oligonucleotide, aptamer or small molecule.

11. The agent for use in a method of treating or preventing fibrosis, use or method according to para 9, wherein the IL-11R binding agent is an antibody.

12. An agent capable of preventing or reducing the expression of Interleukin 11 (IL-11) or an Interleukin 11 receptor (IL-11R) for use in a method of treating or preventing fibrosis.

13. Use of an agent capable of preventing or reducing the expression of Interleukin 11 (IL-11) or an Interleukin 11 receptor (IL-11R) in the manufacture of a medicament for use in a method of treating or preventing fibrosis.

14. A method of treating or preventing fibrosis, the method comprising administering to a subject in need of treatment a therapeutically effective amount of an agent capable of preventing or reducing the expression of Interleukin 11 (IL-11) or an Interleukin 11 receptor (IL-11R).

15. The agent for use in a method of treating or preventing fibrosis according to para 12, use according to para 13 or method according to para 14, wherein the agent is a small molecule or oligonucleotide.

16. The agent for use in a method of treating or preventing fibrosis, use or method according to any one of the preceding paras, wherein the fibrosis is fibrosis of the heart, liver, kidney or eye.

17. The agent for use in a method of treating or preventing fibrosis, use or method according to any one of the preceding paras, wherein the fibrosis is in the heart and is associated with dysfunction of the musculature or electrical properties of the heart, or thickening of the walls or valves of the heart.

18. The agent for use in a method of treating or preventing fibrosis, use or method according to any one of the preceding paras, wherein the fibrosis is in the liver and is associated with chronic liver disease or liver cirrhosis.

19. The agent for use in a method of treating or preventing fibrosis, use or method according to any one of the preceding paras, wherein the fibrosis is in the kidney and is associated with chronic kidney disease.

20. The agent for use in a method of treating or preventing fibrosis, use or method according to any one of the preceding paras, wherein the fibrosis is in the eye and is retinal fibrosis, epiretinal fibrosis, or subretinal fibrosis.

21. The agent for use in a method of treating or preventing fibrosis, use or method according to any one of the preceding paras, wherein the method of treating or preventing comprises administering said agent to a subject in which IL-11 or IL-11R expression is upregulated.

22. The agent for use in a method of treating or preventing fibrosis, use or method according to any one of the preceding paras, wherein the method of treating or preventing comprises administering said agent to a subject in which IL-11 or IL-11R expression has been determined to be upregulated.

23. The agent for use in a method of treating or preventing fibrosis, use or method according to any one of the preceding paras, wherein the method of treating or preventing comprises determining whether IL-11 or IL-11R expression is upregulated in the subject and administering said agent to a subject in which IL-11 or IL-11R expression is upregulated.

24. A method of determining the suitability of a subject for the treatment or prevention of fibrosis with an agent capable of inhibiting the action of Interleukin 11 (IL-11), the method comprising determining, optionally *in vitro*, whether IL-11 or an Interleukin 11 receptor (IL-11R) expression is upregulated in the subject.

25. A method of selecting a subject for the treatment or prevention of fibrosis with an agent capable of inhibiting the action of Interleukin 11 (IL-11), the method comprising determining, optionally *in vitro*, whether IL-11 or an Interleukin 11 receptor (IL-11R) expression is upregulated in the subject.

26. A method of diagnosing fibrosis or a risk of developing fibrosis in a subject, the method comprising determining, optionally *in vitro*, the upregulation of Interleukin 11 (IL-11) or an Interleukin 11 receptor (IL-11R) in a sample obtained from the subject.

27. The method of para 26, wherein the method is a method of confirming a diagnosis of fibrosis in a subject suspected of having fibrosis.

28. The method of para 26 or 27, wherein the method further comprises selecting the subject for treatment with an agent capable of inhibiting the action of IL-11 or with an agent capable of preventing or reducing the expression of IL-11 or IL-11R.

29. A method of providing a prognosis for a subject having, or suspected of having fibrosis, the method comprising determining, optionally *in vitro*, whether Interleukin 11 (IL-11) or an Interleukin 11 receptor (IL-11R) is upregulated in a sample obtained from the subject and, based on the determination, providing a prognosis for treatment of the subject with an agent capable of inhibiting the action of IL-11 or with an agent capable of preventing or reducing the expression of IL-11 or IL-11R.

30. The method of para 29, wherein the method further comprises selecting a subject determined to have upregulated IL-11 or IL-11R for treatment with an agent capable of inhibiting the action of IL-11 or with an agent capable of preventing or reducing the expression of IL-11 or IL-11R.

31. A method of diagnosing fibrosis or a risk of developing fibrosis in a subject, the method comprising determining, optionally *in vitro*, one or more genetic factors in the subject that are

predictive of upregulation of Interleukin 11 (IL-11) or an Interleukin 11 receptor (IL-11R) expression, or of upregulation of IL-11 or IL-11R activity.

32. The method of para 31, wherein the method is a method of confirming a diagnosis of fibrosis in a subject suspected of having fibrosis.

33. The method of para 32 or 32, wherein the method further comprises selecting the subject for treatment with an agent capable of inhibiting the action of IL-11 or with an agent capable of preventing or reducing the expression of IL-11 or IL-11R.

34. A method of providing a prognosis for a subject having, or suspected of having, fibrosis, the method comprising determining, optionally *in vitro*, one or more genetic factors in the subject that are predictive of upregulation of Interleukin 11 (IL-11) or an Interleukin 11 receptor (IL-11R) expression, or of upregulation of IL-11 or IL-11R activity.

BRIEF DESCRIPTION OF THE FIGURES

Embodiments and experiments illustrating the principles of the invention will now be discussed with reference to the accompanying figures in which:

Figures 1A, 1B, 1C and 1D. TGFβ1 stimulation upregulates IL-11 in fibroblasts. Primary fibroblasts were derived from human atrial tissue of 80 individuals and incubated for 24h with and without TGFβ1 (5ng/ml). (**1A**) Chart showing IL-11 was the most upregulated gene in TGFβ1 stimulated fibroblasts compared to 11,433 expressed genes (FPKM ≥ 0.5). (**1B**) Chart showing IL-11 expression significantly increased more than 8-fold on average after fibroblast activation with TGFβ1 (FDR = 9.1×10^{-125}). (**1C**) Chart showing RT-qPCR confirmed IL-11 RNA expression-based fold changes (TGFB1+ / TGFB1-; R^2=0.94) and (**1D**) Chart showing ELISA detected a significant increase in IL-11 protein secreted by stimulated fibroblasts.

Figures 2A, 2B, 2C and 2D. Human atrial fibroblasts were incubated either with 5 ng/ml TGFβ1 or 5 ng/ml IL-11 for 24 hours. Charts show cell staining for (**2A**) α-SMA (myofibroblasts), (**2B**) EdU (proliferation), (**2C**) collagen and (**2D**) periostin to identify myofibroblasts and highly proliferative cells and to quantify the production of extracellular matrix proteins. IL-11 was found to increase the myofibroblast ratio and induce the production of collagen and periostin at a similar rate as TGFβ1 signaling. This experiment was repeated a number of times with similar results.

Figures 3A, 3B, 3C and 3D. Inhibition of IL-11 with a neutralizing antibody prevents TGFβ1-induced fibrosis. Human atrial fibroblasts were stimulated with TGFβ1 (5ng/ml), TGFβ1 and an antibody against IL-11 or TGFβ1 and an isotype control. Charts and photographs show cell stained after 24 hours for (**3A**) α-SMA, (**3A**) EdU, (**3C**) collagen and (**3D**) periostin to identify myofibroblasts and highly proliferative cells and to quantify the production of extracellular matrix proteins. Fluorescence was quantified on the Operetta platform for up to 21 fields per condition. This experiment was repeated with fibroblasts derived from different individuals with similar results. In the presence of an antibody blocking IL-11, TGFβ1-stimulated fibroblasts have a decreased ratio of myofibroblasts, are less proliferative and express less collagen and periostin compared to control cells. This shows that IL-11 is an essential component of TGFβ1 signaling pathway acting in an autocrine and/or paracrine feed forward fashion and its inhibition reduces the pro-fibrotic effects of this key regulator of fibrosis in humans.

Figures 4A and 4B. TGFβ1 stimulation upregulates IL-11 in fibroblasts. Primary fibroblasts were derived from human atrial tissue of 80 individuals and incubated for 24h with and without TGFβ1 (5ng/ml). (**4A**) Chart showing IL-11 was the most upregulated RNA transcript in TGFβ1 stimulated fibroblasts compared to 11,433 expressed genes (FPKM ≥ 0.5) across the genome as assessed by global transcriptome profiling. (**4B**) Chart showing IL-11 expression in non-stimulated (TGF-β -) and stimulated (TGF-β +) primary human fibroblasts compared to all human tissues as assessed by the GTEX project (Consortium, Gte. Human genomics. The Genotype-Tissue Expression (GTEx) pilot analysis: multitissue gene regulation in humans. *Science (New York, N.Y.)* **348,** (2015)) reveals high specificity of elevated IL-11 levels to fibroblasts and specifically activated fibroblasts, the signature of which is not appreciated at the level of the whole organ that contains multiple cell types and few, IL11-expressing, fibroblasts.

Figures 5A, 5B, 5C and 5D. IL-11 acts as an autocrine factor on fibroblasts and induces its own expression via translational regulation alone. Primary fibroblasts were stimulated with TGF-β for 24 hours. (**5A**) Chart showing IL-11 RNA expression increased significantly (FDR = $9.1x10^{-125}$) more than 8-fold on average across 80 individuals. (**5B**) Chart showing results of an ELISA assay confirming a significant increase in IL-11 protein secreted by stimulated fibroblasts (t-test). (**5C**) Chart showing incubation of primary fibroblasts with IL-11 does not increase IL-11 RNA levels (RT-qPCR). (**5D**) Chart showing incubation of primary fibroblasts with IL-11 induces IL-11 protein secretion significantly (Dunnett) as detected by ELISA. Adjusted P-values are given as **** $P < 0.0001$.

Figures 6A, 6B, 6C, 6D, 6E and 6F. IL-11 drives proliferation and activation of fibroblasts as well as extracellular matrix production and is required for the TGFβ1-mediated fibrotic response. Cardiac fibroblasts derived from 3 individuals were incubated for 24h with TGFβ1 (5 ng/ml), IL-11 (5 ng/ml) or TGFβ1 and a neutralizing IL-11/control antibody. Charts and photographs show results of cell staining following incubation for (**6A**) α-SMA content to estimate the fraction of myofibroblasts, (**6B**) EdU to track actively proliferating cells (**6C**) Periostin to estimate ECM production. Fluorescence was measured with the Operetta platform for 14 fields across 2 wells for each patient. Charts also show the secretion of fibrosis markers IL-6 (**6D**), TIMP1 (**6E**) and MMP2 (**6F**) as assessed via ELISA. Fluorescence was normalized to the control group without stimulation and the mean with standard deviation is plotted. IL-11 induces a fibrotic response at similar levels as TGFβ1 and inhibition of IL-11 rescues the TGFβ1 phenotype on the protein level. Adjusted P-values are given as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ or **** $P < 0.0001$ of experimental groups compared to unstimulated cells (Dunnett). Outliers were removed (ROUT, Q = 2%).

Figures 7A, 7B and 7C. IL-11 promotes collagen protein synthesis and stalls the pro-fibrotic effect of TGFβ1 at the RNA level. Cardiac fibroblasts derived from 3 individuals were incubated for 24h with TGFβ1 (5 ng/ml), IL-11 (5 ng/ml) or TGFβ1 and a neutralizing IL-11 antibody. Following incubation (**7A**) Chart showing results following incubation of cell staining for collagen using the Operetta assay; florescence was quantified as described above for Figure 6, (**7B**) Chart showing secreted collagen levels assessed with a Sirius Red staining and (**7C**) Chart showing collagen RNA levels measured by RT-qPCR. IL-11 induces a fibrotic response at similar levels as TGFβ1 only at the protein level. Higher expression of Collagen RNA transcripts by TGFβ1 did not lead to increased protein production if IL-11 was neutralized with an antibody. Adjusted P-values are given as * $P < 0.05$, *** $P < 0.001$ or **** $P < 0.0001$ of experimental groups compared to unstimulated cell control group (Dunnett).

Figures 8A, 8B, 8C, 8D and 8E. IL-11 is a fibrosis marker and activator across multiple tissues. Expression of IL-11 can be induced by a diverse set of upstream pro-fibrotic stimulants in addition to TGFβ1. (**8A**) Chart showing effect of TGFβ1 on IL-11 expression. (**8B**) Chart showing ET-1 (Endothelin) upregulates IL-11 in hepatic and pulmonary fibroblasts; (**8C**) Chart showing PDGF (platelet derived growth factor) induces IL-11 expression in renal fibroblasts. IL-11 RNA levels were measured by RT-qPCR; adjusted P-values are given as * $P < 0.05$, ** $P < 0.01$ or **** $P < 0.0001$ (Dunnett). To investigate the systemic effect of IL-11, saline only (grey) or recombinant IL-11 (black) was injected 6 times a week in C57BL/6 mice (200µg/kg). Collagen content in tissue was assessed with a hydroxyproline assay (QuickZyme) on the protein level and the results are shown in chart (**8D**). Tissues of animals

treated with rIL-11 have higher collagen protein content than controls (ANOVA; p= 0.012). (**8E**) Photographs of western blot showing αSMA levels are increased in the kidney and heart of IL-11 treated mice, indicating the presence of myofibroblasts.

Figure 9. Diagram illustrating role of IL-11 as an essential regulator of the fibrotic response. IL-11 is an essential regulator required for the fibrotic response. In response to tissue damage or chronic inflammation, cytokines such as TGFβ1, ET-1 or PDGF are released to upregulate the transcription of fibrosis marker genes. The autocrine agent IL-11 is then produced in response to these upstream stimuli to ensure efficient translation of upregulated transcripts into functionally relevant proteins in a cell-specific manner. Inhibition of IL-11 blocks the synthesis of key extracellular matrix and myofibroblast proteins and prevents the pro-fibrotic action of a diverse set of upstream stimuli.

Figure 10. Inhibition of IL-11 stops collagen protein synthesis in response to pro-fibrotic cytokines ANG2 (Angiotensin II), PDGF and ET-1. Cardiac fibroblasts were incubated for 24h with ANG2, PDGF or ET-1 and a neutralizing IL-11 antibody. Following incubation cells were stained for collagen and florescence was quantified. These stimuli induce a fibrotic response at similar levels to TGFβ1. However, collagen expression is not increased if IL-11 is neutralized with an antibody. P-values are given as: **** $P < 0.0001$ (t-test).

Figure 11. Nucleotide sequence of human IL-11, taken from Genbank accession number gi|391353405|ref|NM_000641.3 (Homo sapiens interleukin 11 (IL11), transcript variant 1, Mrna) [SEQ ID NO:1]. Underlined sequence encodes IL-11 mRNA. Shaded sequences were used for design of IL-11 knockdown siRNA and are shown separately as SEQ ID NOs 2 to 5. SEQ ID NOs 3 and 4 overlap with each other within SEQ ID NO:1.

Figure 12. Nucleotide sequence of human IL-11Rα, taken from Genbank accession number gi|975336|gb|U32324.1|HSU32324 (Human interleukin-11 receptor alpha chain mRNA, complete cds) [SEQ ID NO:6]. Underlined sequence encodes IL-11Rα mRNA. Shaded sequences were used for design of IL-11Rα knockdown siRNA and are shown separately as SEQ ID NOs 7 to 10.

Figure 13. Table showing siRNA sequences [SEQ ID NOs 11 to 14] for knockdown of IL-11.

Figure 14. Table showing siRNA sequences [SEQ ID NOs 15 to 18] for knockdown of IL-11Rα.

Figure 15. Chart showing siRNA knockdown of IL-11Rα in HEK cells.

Figure 16. Graph showing read depth for whole transcriptome sequencing of human atrial fibroblasts from 160 individuals with and without stimulation with TGFβ1.

Figures 17A, 17B, 17C, 17D and 17E. Graphs showing expression of endothelial, cardiomyocyte and fibroblast marker genes as determined by RNA-seq of the tissue of origin (human atrial tissues samples, n=8) and primary, unstimulated fibroblast cultures. (**17A**) PECAM1, (**17B**) MYH6 (**17C**) TNNT2, (**17D**) COL1A2, and (**17E**) ACTA2.

Figures 18A, 18B, 18C, 18D and 18E. Graphs showing upregulation of IL-11 expression in fibroblasts in response to stimulation with TGFβ1. (**18A** and **18B**) Graphs showing fold change in gene expression in fibrosis; IL-11 is the most upregulated gene in response to TGFβ1 treatment. (**18C**) IL-11 secretion by fibroblasts in response to stimulation with TGFβ1. (**18D**) Comparison of IL-11 gene expression in tissues of healthy individuals and in atrial fibroblasts, with or without TGFβ1 stimulation. (**18E**) Correspondence of fold change in IL-11 expression as determined by RNA-seq vs. qPCR.

Figures 19A, 19B, 19C and 19D. Graphs showing induction of IL-11 secretion in primary fibroblasts by various profibrotic cytokines, as determined by ELISA. (**19A**) TGFβ1, ET-1, AngII, PDGF, OSM and IL-13 induce IL-11 secretion, and IL-11 also induces IL-11 expression in a positive feedback loop. (**19B**) Graph showing that the ELISA only detects native IL-11 secreted from cells, and does not detect recombinant IL-11 used for the IL-11 stimulation condition. (**19C**) and (**19D**) Cells were stimulated with recombinant IL-11, IL-11 RNA was measured and the native IL-11 protein level was measured in the cell culture supernatant by ELISA at the indicated time points.

Figures 20A, 20B, 20C, 20D, 20E and 20F. Graphs and images showing myofibroblast generation from, and production of ECM and cytokine expression by, atrial fibroblasts in response to stimulation with TGFβ1 or IL-11. (**20A**) myofibroblast generation and ECM production by primary atrial fibroblasts following stimulation with TGFβ1 or IL-11, as measured by fluorescence microscopy following staining for a α-SMA, collagen or periostin. (**20B**) Collagen content of cell culture supernatant as determined by Sirius Red staining. Secretion of the fibrosis markers (**20C**) IL-6, (**20D**) TIMP1 and (**20E**) MMP2 as measured by

ELISA. (**20F**) Activation of murine fibroblasts by stimulation with human or mouse recombinant IL-11. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001 [Mean ± SD, Dunnett].

Figures 21A, 21B and 21C. Graphs showing the profibrotic effect of IL-11. (**21A**) Mouse fibroblasts from different tissues of origin can be activated by IL-11 and display increased ECM production. [Mean ± SD, Dunnett]. Injection of mice with recombinant IL-11 or AngII results in (**21B**) an increase in organ weight [Mean ± SEM], and (**21C**) an increase in collagen content (as determined by HPA assay). * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001 [Mean ± SD, Dunnett].

Figures 22A, 22B, 22C, 22D, 22E and 22F. Graphs and images showing that IL-11 is required the pro-fibrotic effects of TGFβ1 on fibroblasts. (**22A**) myofibroblast generation and ECM production by primary atrial fibroblasts, with or without stimulation with TGFβ1, and in the presence/absence of neutralising anti-IL-11 antibody or isotype control IgG, as measured by fluorescence microscopy following staining for (**22A**) α-SMA, (**22B**) EdU or (**22C**) Periostin. (**22D** to **22F**) Secretion of the fibrosis markers (**22D**) IL-6, (**22E**) TIMP1, and (**22F**) MMP2 was analysed by ELISA. Fluorescence was normalized to the control group without stimulation. [Mean ± SD, Dunnett] * P < 0.05, ** P < 0.01, *** P < 0.001 or **** P < 0.0001.

Figures 23A and 23B. Graphs and images showing the effect of neutralisation of IL-11 on collagen production triggered by TGFβ1. Collagen production by cardiac fibroblasts with or without stimulation with TGFβ1, and in the presence/absence of neutralising anti-IL-11 antibody or isotype control IgG, as determined by (**23A**) Operetta assay or (**23B**) Sirius Red staining. [Mean ± SD, Dunnett] * P < 0.05, ** P < 0.01, *** P < 0.001 or **** P < 0.0001.

Figure 24. Graphs showing the ability of various IL-11 and IL-11Rα antagonists to inhibit fibrosis. Human atrial fibroblasts were treated with neutralizing antibody against IL-11, neutralizing antibody against IL-11Rα, decoy IL-11 receptor molecule that binds to IL-11, siRNA that downregulates IL-11 expression or siRNA that downregulates IL-11RA expression and the effect on the TGFβ1-driven pro-fibrotic response in fibroblasts *in vitro* was analysed. [Mean ± SD, Dunnett] * P < 0.05, ** P < 0.01, *** P < 0.001 or **** P < 0.0001.

Figures 25A, 25B, 25C and 25D. Bar charts showing the response of fibroblasts from IL-11-RA knockout mice to pro-fibrotic treatment. Fibroblasts derived from IL-11RA WT (+/+), Heterozygous (+/-) and Homozygous null (-/-) mice were incubated for 24h with TGFβ1, IL-11 or AngII (5 ng/ml). (**25A**) Percentage of myofibroblasts as determined by analysis αSMA

content, (**25B**) Percentage proliferating cells as determined by staining for EdU, (**25C**) Collagen content and (**25D**) ECM production as measured by detection of periostin [Mean ± SD].

Figures 26A and 26B. Graphs showing the effect of IL-11 neutralisation on fibrosis in response to various pro-fibrotic stimuli. Fibroblasts were cultured *in vitro* in the presence/absence of various different pro-fibrotic factors, and in the presence/absence of neutralising anti-IL-11 antibody or pan anti-TGFβ antibody (**26A**) Collagen production and (**26B**) myofibroblast generation as determined by analysis of αSMA expression. [Mean ± SD, Dunnett] * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ or **** $P < 0.0001$.

Figures 27A, 27B, 27C and 27D. Bar charts showing expression of markers of fibrosis in the atrium and heart of WT and IL-11RA (-/-) animals following treatment with AngII treatment. (**27A**) Collagen content, as measured by hydroxyproline assay. (**27B**) Collagen (Col1A2) expression. (**27C**) αSMA (ACTA2) expression. (**27D**) Fibronectin (Fn1) expression.

Figure 28. Graphs showing the effect of IL-11RA knockout on folate-induced kidney fibrosis as measured by collagen content in kidney tissue.

Figures 29A, 29B and 29C. Schematics of the experimental procedures for analysing fibrosis in (**29A**) lung, (**29B**) skin and (**29C**) eye for IL-11RA -/- mice as compared to IL-11RA +/+ mice.

Figures 30A and 30B. Scatterplots showing fold change in gene expression. (**30A**) Fold changes in gene expression in fibroblasts following stimulation with TGFβ1, IL-11 or TGFβ1 and IL-11. (**30B**) Fold changes in gene expression in fibroblasts obtained from IL-11RA+/+ and IL-11RA-/- mice following stimulation with TGFβ1.

Figures 31A and 31B. Photographs showing the effect of IL-11RA knockout on wound healing and fibrosis in the eye following trabeculectomy (filtration surgery). (**31A**) Eye sections of IL-11RA+/+ (WT) and IL-11RA-/- (KO) animals 7 days after filtration surgery. (**31B**) Maturation of collagen fibres as evaluated by picro-sirius red/polarization light technique (Szendröi et al. 1984, Acta Morphol Hung 32, 47–55); more fibrosis is observed in WT mice than KO mice.

Figures 32A and 32B. Graphs showing the effect of decoy IL-11 receptors on fibrosis in response to stimulation with TGFβ1. Fibroblasts were cultured *in vitro* in the

presence/absence of TGFβ1 (5 ng/ml), in the presence or absence of (**32A**) D11R1 (Decoy Receptor 50aa Linker) or (**32B**) D11R2 (Decoy Receptor 33aa Linker), at various different concentrations. Myofibroblast generation after 24 hours (i.e. the percentage of activated fibroblasts) was determined by analysis of αSMA expression.

Figure 33. Table showing SNPs regulation of IL-11 VST_{stim} in *trans*.

Figure 34. Table showing SNPs regulation of IL-11 $VST_{stim} - VST_{unstim}$ in *cis*.

Figure 35. Table showing SNPs regulation of IL-11 $VST_{stim} - VST_{unstim}$ in *trans*.

Figures 36A, 36B, 36C and 36D Charts showing regulation of IL-11 response by local SNPs. The RNA of unstimulated and stimulated (TGFB1, 5ng/ml, 24h) fibroblasts derived from 69 genotyped individuals was sequenced. Samples were grouped according to genotype and the increase in IL-11 expression (VST_{stim}-VST_{unstim}) was compared between groups with 0, 1 or 2 minor alleles.

Figure 37. Charts showing regulation of IL-11 response by distant SNPs. The RNA of unstimulated and stimulated (TGFB1, 5ng/ml, 24h) fibroblasts derived from 69 genotyped individuals was sequenced. Samples were grouped according to genotype and the increase in IL11 expression (VST_{stim}-VST_{unstim}) was compared between groups with 0, or 1 minor allele.

Figures 38A, 38B, 38C and 38D. Graphs showing that IL-11 is required the pro-fibrotic effects of TGFβ1 in liver fibroblasts. Activation and proliferation of primary human liver fibroblasts, with or without stimulation with TGFβ1, and in the presence/absence of neutralising anti-IL-11 antibody or isotype control IgG, as measured by analysis of the proportion of (**38A**) α-SMA positive cells, (**38B**) EdU positive cells, (**38C**) Collagen positive cells and (**38D**) Periostin positive cells as compared to the unstimulated cells (Baseline). [Mean ± SD, Dunnett] * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ or **** $P < 0.0001$.

Figure 39. Bar chart showing that IL-11 is required for the pro-fibrotic effects of TGFβ1 in skin fibroblasts. Activation of mouse skin fibroblasts, with or without stimulation with TGFβ1, and in the presence/absence of neutralising anti-IL-11 antibody, as measured by analysis of the percentage of α-SMA positive cells (activated fibroblasts).

Figure 40. Bar chart showing lung fibroblast cell migration with and without IL-11 signalling. Migration of lung fibroblasts from IL-11RA+/+ (WT) and IL-11RA-/- (KO) animals was analysed in an *in vitro* scratch assay without stimulus, or in the presence of TGFβ1 or IL-11.

DETAILED DESCRIPTION OF CERTAIN EMBODIMENTS OF THE INVENTION

IL-11 and IL-11 receptor

Interleukin 11 (IL-11), also known as adipogenesis inhibitory factor, is a pleiotropic cytokine and a member of the IL-6 family of cytokines that includes IL-6, IL-11, IL-27, IL-31, oncostatin, leukemia inhibitory factor (LIF), cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC), ciliary neurotrophic factor (CNTF) and neuropoetin (NP-1).

IL-11 is transcribed with a canonical signal peptide that ensures efficient secretion from cells. The immature form of human IL-11 is a 199 amino acid polypeptide whereas the mature form of IL-11 encodes a protein of 178 amino acid residues (Garbers and Scheller., Biol. Chem. 2013; 394(9):1145-1161). The human IL-11 amino acid sequence is available under UniProt accession no. P20809 (P20809.1 GI:124294). Recombinant human IL-11 (oprelvekin) is also commercially available. IL-11 from other species, including mouse, rat, pig, cow, several species of bony fish and primates, have also been cloned and sequenced.

In this specification IL-11 refers to an IL-11 from any species and includes isoforms, fragments, variants or homologues of an IL-11 from any species. In preferred embodiments the species is human (*Homo sapiens*). Isoforms, fragments, variants or homologues of an IL-11 may optionally be characterised as having at least 70%, preferably one of 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% amino acid sequence identity to the amino acid sequence of immature or mature IL-11 from a given species, e.g. human. Isoforms, fragments, variants or homologues of an IL-11 may optionally be characterised by ability to bind IL-11Rα (preferably from the same species) and stimulate signal transduction in cells expressing IL-11Rα and gp130 (e.g. as described in Curtis et al. Blood, 1997, 90(11); or Karpovich et al. Mol. Hum. Reprod. 2003 9(2): 75-80). A fragment of IL-11 may be of any length (by number of amino acids), although may optionally be at least 25% of the length of mature IL-11 and may have a maximum length of one of 50%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% of the length of mature IL-11. A fragment of IL-11 may have a minimum length of 10 amino acids, and a maximum length of one of 15, 20, 25, 30, 40, 50, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190 or 195 amino acids

IL-11 signals through a homodimer of the ubiquitously expressed β-receptor glycoprotein 130 (gp130; also known as glycoprotein 130, IL6ST, IL6-beta or CD130). Gp130 is a transmembrane protein that forms one subunit of the type I cytokine receptor with the IL-6 receptor family. Specificity is gained through an individual IL-11 α-receptor (IL-11Rα), which does not directly participate in signal transduction, although the initial cytokine binding event to the α-receptor leads to the final complex formation with the β-receptors. IL-11 activates a downstream signaling pathway, which is predominantly the mitogen-activated protein kinase (MAPK)-cascade and the Janus kinase/signal transducer and activator of transcription (Jak/STAT) pathway (Garbers and Scheller, *supra*).

Human gp130 (including the 22 amino acid signal peptide) is a 918 amino acid protein, and the mature form is 866 amino acids, comprising a 597 amino acid extracellular domain, a 22 amino acid transmembrane domain, and a 277 amino acid intracellular domain. The extracellular domain of the protein comprises the cytokine-binding module (CBM) of gp130. The CBM of gp130 comprises the Ig-like domain D1, and the fibronectin-type III domains D2 and D3 of gp130. The amino acid sequence of human gp130 is available from Genbank accession no. NP_002175.2.

Human IL-11Rα is a 422 amino acid polypeptide (Genbank accession no. NP_001136256.1 GI:218505839) and shares ~85% nucleotide and amino acid sequence identity with the murine IL-11Rα (Du and Williams., Blood Vol, 89, No,11, June 1, 1997). Two isoforms of IL-11Rα have been reported, which differ in the cytoplasmic domain (Du and Williams, *supra*). The IL-11 receptor α-chain (IL-11Rα) shares many structural and functional similarities with the IL-6 receptor α-chain (IL-6Rα). The extracellular domain shows 24% amino acid identity including the characteristic conserved Trp-Ser-X-Trp-Ser (WSXWS) motif. The short cytoplasmic domain (34 amino acids) lacks the Box 1 and 2 regions that are required for activation of the JAK/STAT signaling pathway.

IL-11Rα binds its ligand with a low affinity (Kd ~10 nmol/L) and alone is insufficient to transduce a biological signal. The generation of a high affinity receptor (Kd ~400 to 800 pmol/L) capable of signal transduction requires co-expression of the IL-11Rα and gp130 (Curtis et al (Blood 1997 Dec 1;90 (11):4403-12; Hilton et al., EMBO J 13:4765, 1994; Nandurkar et al., Oncogene 12:585, 1996). Binding of IL-11 to cell-surface IL-11Rα induces heterodimerization, tyrosine phosphorylation, activation of gp130 and MAPK and/or Jak/STAT signalling as described above.

The receptor binding sites on murine IL-11 have been mapped and three sites – sites I, II and III - identified. Binding to gp130 is reduced by substitutions in the site II region and by substitutions in the site III region. Site III mutants show no detectable agonist activity and have IL-11Rα antagonist activity (Cytokine Inhibitors Chapter 8; edited by Gennaro Ciliberto and Rocco Savino, Marcel Dekker, Inc. 2001).

In principle, a soluble IL-11Rα can also form biologically active soluble complexes with IL-11 (Pflanz et al., 1999 FEBS Lett, 450, 117-122) raising the possibility that, similar to IL-6, IL-11 may in some instances bind soluble IL-11Rα prior to binding cell-surface gp130 (Garbers and Scheller, *supra*). Curtis et al (Blood 1997 Dec 1;90 (11):4403-12) describe expression of a soluble murine IL-11 receptor alpha chain (sIL-11R) and examined signaling in cells expressing gp130. In the presence of gp130 but not transmembrane IL-11R the sIL-11R mediated IL-11 dependent differentiation of M1 leukemic cells and proliferation in Ba/F3 cells and early intracellular events including phosphorylation of gp130, STAT3 and SHP2 similar to signalling through transmembrane IL-11R.

In this specification an IL-11 receptor (IL-11R) refers to a polypeptide capable of binding IL-11 and inducing signal transduction in cells expressing gp130. An IL-11 receptor may be from any species and includes isoforms, fragments, variants or homologues of an IL-11 receptor from any species. In preferred embodiments the species is human (*Homo sapiens*). In some embodiments the IL-11 receptor may be IL-11Rα. Isoforms, fragments, variants or homologues of an IL-11Rα may optionally be characterised as having at least 70%, preferably one of 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% amino acid sequence identity to the amino acid sequence of IL-11Rα from a given species, e.g. human. Isoforms, fragments, variants or homologues of an IL-11Rα may optionally be characterised by ability to bind IL-11 (preferably from the same species) and stimulate signal transduction in cells expressing the IL-11Rα and gp130 (e.g. as described in Curtis et al. Blood, 1997, 90(11) or Karpovich et al. Mol. Hum. Reprod. 2003 9(2): 75-80). A fragment of an IL-11 receptor may be of any length (by number of amino acids), although may optionally be at least 25% of the length of the mature IL-11Rα and have a maximum length of one of 50%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% of the length of the mature IL-11Rα. A fragment of an IL-11 receptor fragment may have a minimum length of 10 amino acids, and a maximum length of one of 15, 20, 25, 30, 40, 50, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 400, or 415 amino acids.

Agent capable of inhibiting the action of IL-11

The IL-11 signaling pathway offers multiple routes for inhibition of IL-11 signaling. For example, inhibition may be achieved by preventing or reducing the binding of IL-11 to an IL-11 receptor. As a result, suitable agents may target either IL-11 or its receptor.

In some embodiments agents capable of inhibiting the action of IL-11 may bind to IL-11 and prevent or reduce IL-11 mediated signalling, e.g. through an IL-11 receptor. In some embodiments agents capable of inhibiting the action of IL-11 may bind to the IL-11 receptor and prevent or reduce IL-11 stimulated signalling.

Agents that bind to IL-11 may inhibit IL-11 mediated signalling by blocking the binding of IL-11 to an IL-11 receptor and/or by reducing the amount of IL-11 available to bind to its receptor. Suitable IL-11 binding agents may be IL-11 inhibitors or IL-11 antagonists.

IL-11 binding agents, e.g. anti-IL-11 antibodies, according to the present invention may exhibit at least one of the following properties:

a) Bind to human IL-11 with a K_D of 1μM or less, preferably one of ≤ 1μM, ≤ 100nM, ≤10nM, ≤1nM or ≤100pM;

b) Inhibit IL-11 mediated signalling via the IL-11Rα receptor, e.g. in a cell based assay in which the cells co-express IL-11Rα and gp130. Suitable cell based assays are ^{3}H-thymidine incorporation and Ba/F3 cell proliferation assays described in e.g. Curtis et al. Blood, 1997, 90(11) and Karpovich et al. Mol. Hum. Reprod. 2003 9(2): 75-80. For example, IC_{50} for an IL-11 binding agent may be determined by culturing Ba/F3 cells expressing IL-11Rα and gp130 in the presence of human IL-11 and the IL-11 binding agent, and measuring ^{3}H-thymidine incorporation into DNA. Suitable IL-11 binding agents may exhibit an IC_{50} of 10 μg/ml or less, preferably one of ≤ 5 μg/ml, ≤ 4 μg/ml, ≤ 3.5 μg/ml, ≤ 3 μg/ml, ≤ 2 μg/ml, ≤ 1 μg/ml, ≤ 0.9 μg/ml, ≤ 0.8 μg/ml, ≤ 0.7 μg/ml, ≤ 0.6 μg/ml, or ≤ 0.5 μg/ml in such an assay.

c) Inhibit fibroblast proliferation, e.g. proliferation of cardiac/atrial fibroblasts. This can, for example, be evaluated in an assay wherein fibroblasts are stimulated with IL-11 or TGFβ1 and cell proliferation is monitored as described herein.

d) Inhibit myofibroblast generation, e.g. from cardiac/atrial fibroblasts. This can, for example, be evaluated in an assay wherein fibroblasts are stimulated with IL-11 or TGFβ1 and myofibroblast generation is monitored, e.g. by measuring αSMA levels.

e) Inhibit extracellular matrix production by fibroblasts, e.g. cardiac/atrial fibroblasts. This can, for example, be evaluated in an assay wherein fibroblasts are stimulated

with IL-11 or TGFβ1 and production of extracellular matrix components is measured.

f) Inhibit collagen and/or periostin gene or protein expression in fibroblasts, e.g. cardiac/atrial fibroblasts. This can, for example, be evaluated in an assay wherein fibroblasts are stimulated with IL-11 or TGFβ1 and collagen and/or periostin gene or protein expression is measured.

IL-11 binding agents may be of any kind, but in some embodiments an IL-11 binding agent may be an antibody, polypeptide, peptide, oligonucleotide, aptamer or small molecule.

Suitable anti-IL-11 antibodies will preferably bind to IL-11 (the antigen), preferably human IL-11, and may have a dissociation constant (K_D) of one of ≤ 1μM, ≤ 100nM, ≤10nM, ≤1nM or ≤100pM. Binding affinity of an antibody for its target is often described in terms of its dissociation constant (K_D). Binding affinity can be measured by methods known in the art, such as by Surface Plasmon Resonance (SPR), or by a radiolabeled antigen binding assay (RIA) performed with the Fab version of the antibody and antigen molecule.

Anti-IL-11 antibodies may be antagonist antibodies that inhibit or reduce a biological activity of IL-11.

Anti-IL-11 antibodies may be neutralising antibodies that neutralise the biological effect of IL-11, e.g. its ability to stimulate productive signalling via an IL-11 receptor.

Neutralising activity may be measured by ability to neutralise IL-11 induced proliferation in the T11 mouse plasmacytoma cell line (Nordan, R. P. *et al.* (1987) J. Immunol. **139**:813).

Examples of known anti-IL-11 antibodies include monoclonal antibody clone 6D9A, clone KT8 (Abbiotec), clone M3103F11 (BioLegend), clone 1F1, clone 3C6 (Abnova Corporation), clone GF1 (LifeSpan Biosciences), clone 13455 (Source BioScience) and clone 22626 (R & D Systems, used in Bockhorn et al. Nat. Commun. (2013) 4(0):1393; Monoclonal Mouse IgG_{2A}; Catalog No. MAB218; R&D Systems, MN, USA).

Antibodies may optionally be selected to exhibit substantially no cross-reactivity with one or more of human, e.g. recombinant human, IL-6, CNTF, LIF, OSM, CLC or CT-1.

Peptide or polypeptide based IL-11 binding agents may be based on the IL-11 receptor, e.g. a IL-11 binding fragment of an IL-11 receptor. In one embodiment, suitable IL-11 binding agents may comprise an IL-11 binding fragment of the IL-11Rα chain, and may preferably be soluble and/or exclude one or more, or all, of the transmembrane domain(s). Such molecules may be described as decoy receptors.

Curtis et al (Blood 1997 Dec 1;90 (11):4403-12) report that a soluble murine IL-11 receptor alpha chain (sIL-11R) was capable of antagonizing the activity of IL-11 when tested on cells expressing the transmembrane IL-11R and gp130. They proposed that the observed IL-11 antagonism by the sIL-11R depends on limiting numbers of gp130 molecules on cells already expressing the transmembrane IL-11R.

The use of soluble decoy receptors as the basis for inhibition of signal transduction and therapeutic intervention has also been reported for other signalling molecule:receptor pairs, e.g. VEGF and the VEGF receptor (De-Chao Yu et al., Molecular Therapy (2012); 20 5, 938-947; Konner and Dupont Clin Colorectal Cancer 2004 Oct;4 Suppl 2:S81-5).

As such, in some embodiments an IL-11 binding agent may be provided in the form of a decoy receptor, e.g. a soluble IL-11 receptor. Competition for IL-11 provided by a decoy receptor has been reported to lead to IL-11 antagonist action (Curtis et al., *supra*).

Decoy IL-11 receptors preferably bind IL-11 and/or IL-11 containing complexes, and thereby make these species unavailable for binding to gp130, IL-11Rα and/or gp130:IL-11Rα receptors. As such, they act as 'decoy' receptors for IL-11 and IL-11 containing complexes, much in the same way that etanercept acts as a decoy receptor for TNFα. IL-11 mediated signalling is reduced as compared to the level of signalling in the absence of the decoy receptor.

Decoy IL-11 receptors preferably bind to IL-11 through one or more cytokine binding modules (CBMs). The CBMs are, or are derived from or homologous to, the CBMs of naturally occurring receptor molecules for IL-11. For example, decoy IL-11 receptors may comprise, or consist of, one or more CBMs which are from, are derived from or homologous to the CBM of gp130 and/or IL-11Rα.

In some embodiments, a decoy IL-11 receptor may comprise, or consist of, an amino acid sequence corresponding to the cytokine binding module of gp130. In some embodiments, a

decoy IL-11 receptor may comprise an amino acid sequence corresponding to the cytokine binding module of IL-11Rα. Herein, an amino acid sequence which 'corresponds' to a reference region or sequence of a given peptide/polypeptide has at least 60%, e.g. one of at least 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to the amino acid sequence of the reference region/sequence. The gp130, IL-11Rα and IL-11 may be from any species, and include isoforms, fragments, variants or homologues from any species.

In some embodiments a decoy receptor may be able to bind IL-11, e.g. with binding affinity of at least 100μM or less, optionally one of 10μM or less, 1μM or less, 100nM or less, or about 1 to 100nM. In some embodiments a decoy receptor may comprise all or part of the IL-11 binding domain and may optionally lack all or part of the transmembrane domains. The decoy receptor may optionally be fused to an immunoglobulin constant region, e.g. IgG Fc region.

In some embodiments an IL-11 binding agent may be provided in the form of a small molecule inhibitor of IL-11, e.g. IL-11 inhibitor described in Lay et al., Int. J. Oncol. (2012); 41(2): 759-764.

Agents that bind to an IL-11 receptor (IL-11R) may inhibit IL-11 mediated signalling by blocking the binding of IL-11 to an IL-11R or by preventing signal transduction via the gp130 co-receptors. Suitable IL-11R binding agents may be IL-11R inhibitors or IL-11R antagonists. In preferred embodiments the IL-11R is IL-11Rα and suitable binding agents may bind the IL-11Rα polypeptide and may be inhibitors or antagonists of IL-11Rα.

IL-11R binding agents, e.g. anti-IL-11R antibodies, according to the present invention may exhibit at least one of the following properties:

(a) Bind to human IL-11R with a K_D of 1μM or less, preferably one of ≤ 1μM, ≤ 100nM, ≤10nM, ≤1nM or ≤100pM;

(b) Inhibit IL-11R signalling, e.g. in a cell based assay in which the cells co-express IL-11Rα and gp130. Suitable cell based assays are ^{3}H-thymidine incorporation and Ba/F3 cell proliferation assays described in e.g. Curtis et al. Blood, 1997, 90(11) and Karpovich et al. Mol. Hum. Reprod. 2003 9(2): 75-80. For example, IC_{50} for an IL-11R binding agent may be determined by culturing Ba/F3 cells expressing IL-11Rα and gp130 in the presence of human IL-11 and the IL-11R binding agent, and measuring ^{3}H-thymidine incorporation into DNA. Suitable IL-11R binding agents may exhibit an IC_{50} of 10 μg/ml or less, preferably one of ≤ 5 μg/ml, ≤ 4 μg/ml, ≤ 3.5 μg/ml, ≤ 3 μg/ml,

≤ 2 μg/ml, ≤ 1 μg/ml, ≤ 0.9 μg/ml, ≤ 0.8 μg/ml, ≤ 0.7 μg/ml, ≤ 0.6 μg/ml, or ≤ 0.5 μg/ml in such an assay.

(c) Inhibit fibroblast proliferation, e.g. proliferation of cardiac/atrial fibroblasts. This can, for example, be evaluated in an assay wherein fibroblasts are stimulated with IL-11 or TGFβ1 and cell proliferation is monitored as described herein.

(d) Inhibit myofibroblast generation, e.g. from cardiac/atrial fibroblasts. This can, for example, be evaluated in an assay wherein fibroblasts are stimulated with IL-11 or TGFβ1 and myofibroblast generation is monitored, e.g. by measuring αSMA levels.

(e) Inhibit extracellular matrix production by fibroblasts, e.g. cardiac/atrial fibroblasts. This can, for example, be evaluated in an assay wherein fibroblasts are stimulated with IL-11 or TGFβ1 and production of extracellular matrix components is measured.

(f) Inhibit collagen and/or periostin gene or protein expression in fibroblasts, e.g. cardiac/atrial fibroblasts. This can, for example, be evaluated in an assay wherein fibroblasts are stimulated with IL-11 or TGFβ1 and collagen and/or periostin gene or protein expression is measured.

IL-11R binding agents may be of any kind, but in some embodiments an IL-11R binding agent may be an antibody, polypeptide, peptide, oligonucleotide, aptamer or small molecule.

Suitable anti-IL-11R antibodies will preferably bind to IL-11R (the antigen), preferably human IL-11R, and may have a dissociation constant (K_D) of one of ≤ 1μM, ≤ 100nM, ≤10nM, ≤1nM or ≤100pM. Binding affinity of an antibody for its target is often described in terms of its dissociation constant (K_D). Binding affinity can be measured by methods known in the art, such as by Surface Plasmon Resonance (SPR), or by a radiolabeled antigen binding assay (RIA) performed with the Fab version of the antibody and antigen molecule.

Anti-IL-11R antibodies may be antagonist antibodies that inhibit or reduce a biological activity of IL-11R. Anti-IL-11R antibodies may be antagonist antibodies that inhibit or reduce any function of IL-11R, in particular signalling. For example, antagonist IL-11R antibodies may inhibit or prevent binding of IL-11 to IL-11R, or may inhibit or prevent association of IL-11Rα with gp130 to form a functional receptor complex capable of productive signalling, e.g. in response to IL-11 binding.

Anti-IL-11R antibodies may be neutralising antibodies that neutralise the biological effect of IL-11R, e.g. its ability to initiate productive signalling mediated by binding of IL-11.

Neutralising activity may be measured by ability to neutralise IL-11 induced proliferation in the T11 mouse plasmacytoma cell line (Nordan, R. P. *et al.* (1987) J. Immunol. **139**:813).

Examples of known anti-IL-11R antibodies include monoclonal antibody clone 025 (Sino Biological), clone EPR5446 (Abcam), clone 473143 (R & D Systems), clones 8E2 and 8E4 described in US 2014/0219919 A1 and the monoclonal antibodies described in Blanc et al (J. Immunol Methods. 2000 Jul 31;241(1-2);43-59).

Peptide or polypeptide based IL-11R binding agents may be based on IL-11, e.g. mutant, variant or binding fragment of IL-11. Suitable peptide or polypeptide based agents may bind to IL-11R in a manner that does not lead to initiation of signal transduction or produces sub-optimal signaling. IL-11 mutants of this kind may act as competitive inhibitors of endogenous IL-11.

For example, W147A is an IL-11 antagonist in which the amino acid 147 is mutated from a tryptophan to an alanine, which destroys the so-called 'site III' of IL-11. This mutant can bind to the IL-11R, but engagement of the gp130 homodimer fails, resulting in efficient blockade of IL-11 signaling (Underhill-Day et al., 2003; Endocrinology 2003 Aug;144(8):3406-14). Lee et al (Am J respire Cell Mol Biol. 2008 Dec; 39(6):739-746) also report the generation of an IL-11 antagonist mutant (a "mutein") capable of specifically inhibiting the binding of IL-11 to IL-11Rα.

Menkhorst et al (Biology of Reproduction May 1, 2009 vol.80 no.5 920-927) describe a PEGylated IL-11 antagonist, PEGIL11A (CSL Limited, Parkvill, Victoria, Australia) which is effective to inhibit IL-11 action in female mice.

Pasqualini et al. Cancer (2015) 121(14):2411-2421 describe a ligand-directed, peptidomimetic drug, bone metastasis-targeting peptidomimetic-11 (BMTP-11) capable of binding to IL-11Rα.

In some embodiments an IL-11R binding agent may be provided in the form of a small molecule inhibitor of IL-11R.

The inventors have identified that upregulation of IL-11 expression is consistent with the molecular mechanism of fibrosis and that inhibition of IL-11 activity leads to a reduction in the molecular basis for fibrosis. Accordingly, in some aspects of the present invention treatment, prevention or alleviation of fibrosis may be provided by administration of an agent capable of

preventing or reducing the expression of IL-11 by cells of the subject, e.g. by fibroblasts or myofibroblasts.

Suitable agents may be of any kind, but in some embodiments an agent capable of preventing or reducing the expression of IL-11 may be a small molecule or an oligonucleotide.

Taki et al (Clin Exp Immunol. 1998 Apr; 112(1): 133-138) report a reduction in the expression of IL-11 in rheumatoid synovial cells upon treatment with indomethacin, dexamethasone or interferon-gamma (IFNγ).

In some embodiments an agent capable of preventing or reducing the expression of IL-11 may be an oligonucleotide capable of repressing or silencing expression of IL-11.

Accordingly, the present invention also includes the use of techniques known in the art for the therapeutic down regulation of IL-11 expression. These include the use of antisense oligonucleotides and RNA interference (RNAi). As in other aspects of the present invention, these techniques may be used in the treatment of fibrosis.

Accordingly, in one aspect of the present invention a method of treating or preventing fibrosis is provided, the method comprising administering to a subject in need of treatment a therapeutically effective amount of an agent capable of preventing or reducing the expression of IL-11, wherein the agent comprises a vector comprising a therapeutic oligonucleotide capable of repressing or silencing expression of IL-11.

In another aspect of the present invention a method of treating or preventing fibrosis is provided, the method comprising administering to a subject in need of treatment a therapeutically effective amount of an agent capable of preventing or reducing the expression of IL-11, wherein the agent comprises an oligonucleotide vector, optionally a viral vector, encoding a therapeutic oligonucleotide capable of being expressed in cells of the subject, the expressed therapeutic oligonucleotide being capable of repressing or silencing expression of IL-11.

The ability of an agent to prevent or reduce the expression of IL-11 may be assayed by determining the ability of the agent to inhibit IL-11 gene or protein expression by fibroblasts or myofibroblasts, e.g. cardiac/atrial fibroblasts or myofibroblasts. This can, for example, be evaluated in an assay wherein fibroblasts or myofibroblasts are stimulated with IL-11 or TGFβ1, and IL-11 gene or protein expression is measured.

Reducing the amount of IL-11R available for binding to IL-11 and initiation of productive signalling provides an alternative means of reducing the level of IL-11 stimulated signalling. Accordingly, in related aspects of the present invention, treatment, prevention or alleviation of fibrosis may be provided by administration of an agent capable of preventing or reducing the expression of IL-11R by cells of the subject, e.g. by fibroblasts or myofibroblasts.

In some embodiments an agent capable of preventing or reducing the expression of IL-11R may be an oligonucleotide capable of repressing or silencing expression of IL-11R.

Accordingly, the present invention also includes the use of techniques known in the art for the therapeutic down regulation of IL-11R expression. These include the use of antisense oligonucleotides and RNA interference (RNAi). As in other aspects of the present invention, these techniques may be used in the treatment of fibrosis.

Accordingly, in one aspect of the present invention a method of treating or preventing fibrosis is provided, the method comprising administering to a subject in need of treatment a therapeutically effective amount of an agent capable of preventing or reducing the expression of IL-11R, wherein the agent comprises a vector comprising a therapeutic oligonucleotide capable of repressing or silencing expression of IL-11R.

In another aspect of the present invention a method of treating or preventing fibrosis is provided, the method comprising administering to a subject in need of treatment a therapeutically effective amount of an agent capable of preventing or reducing the expression of IL-11R, wherein the agent comprises an oligonucleotide vector, optionally a viral vector, encoding a therapeutic oligonucleotide capable of being expressed in cells of the subject, the expressed therapeutic oligonucleotide being capable of repressing or silencing expression of IL-11R.

The ability of an agent to prevent or reduce the expression of IL-11R may be assayed by determining the ability of the agent to inhibit IL-11R gene or protein expression by fibroblasts or myofibroblasts, e.g. cardiac/atrial fibroblasts or myofibroblasts. This can, for example, be evaluated in an assay wherein fibroblasts or myofibroblasts are stimulated with IL-11 or TGFβ1, and IL-11R gene or protein expression is measured.

In preferred embodiments, the IL-11R may be IL-11Rα.

Antibodies

In this specification “antibody” includes a fragment or derivative of an antibody, or a synthetic antibody or synthetic antibody fragment.

Antibodies may be provided in isolated or purified form. Antibodies may be formulated as a pharmaceutical composition or medicament.

In view of today's techniques in relation to monoclonal antibody technology, antibodies can be prepared to most antigens. The antigen-binding portion may be a part of an antibody (for example a Fab fragment) or a synthetic antibody fragment (for example a single chain Fv fragment [ScFv]). Suitable monoclonal antibodies to selected antigens may be prepared by known techniques, for example those disclosed in "Monoclonal Antibodies: A manual of techniques ", H Zola (CRC Press, 1988) and in "Monoclonal Hybridoma Antibodies: Techniques and Applications ", J G R Hurrell (CRC Press, 1982). Chimaeric antibodies are discussed by Neuberger et al (1988, 8th International Biotechnology Symposium Part 2, 792-799).

Monoclonal antibodies (mAbs) are useful in the methods of the invention and are a homogenous population of antibodies specifically targeting a single epitope on an antigen.

Polyclonal antibodies are useful in the methods of the invention. Monospecific polyclonal antibodies are preferred. Suitable polyclonal antibodies can be prepared using methods well known in the art.

Antigen binding fragments of antibodies, such as Fab and Fab_2 fragments may also be used/provided as can genetically engineered antibodies and antibody fragments. The variable heavy (V_H) and variable light (V_L) domains of the antibody are involved in antigen recognition, a fact first recognised by early protease digestion experiments. Further confirmation was found by "humanisation" of rodent antibodies. Variable domains of rodent origin may be fused to constant domains of human origin such that the resultant antibody retains the antigenic specificity of the rodent parented antibody (Morrison et al (1984) Proc. Natl. Acad. Sd. USA 81, 6851-6855).

That antigenic specificity is conferred by variable domains and is independent of the constant domains is known from experiments involving the bacterial expression of antibody fragments, all containing one or more variable domains. These molecules include Fab-like molecules (Better et al (1988) Science 240, 1041); Fv molecules (Skerra et al (1988) Science 240, 1038); single-chain Fv (ScFv) molecules where the V_H and V_L partner domains are linked via a flexible oligopeptide (Bird et al (1988) Science 242, 423; Huston et al (1988) Proc. Natl. Acad. Sd. USA 85, 5879) and single domain antibodies (dAbs) comprising isolated V domains (Ward et al (1989) Nature 341, 544). A general review of the techniques involved in the synthesis of antibody fragments which retain their specific binding sites is to be found in Winter & Milstein (1991) Nature 349, 293- 299.

By "ScFv molecules" we mean molecules wherein the V_H and V_L partner domains are covalently linked, e.g. by a flexible oligopeptide.

Fab, Fv, ScFv and dAb antibody fragments can all be expressed in and secreted from E. coli, thus allowing the facile production of large amounts of the said fragments.

Whole antibodies, and $F(ab')_2$ fragments are "bivalent". By "bivalent" we mean that the said antibodies and $F(ab')_2$ fragments have two antigen combining sites. In contrast, Fab, Fv, ScFv and dAb fragments are monovalent, having only one antigen combining site. Synthetic antibodies which bind to IL-11 or IL-11R may also be made using phage display technology as is well known in the art.

Antibodies may be produced by a process of affinity maturation in which a modified antibody is generated that has an improvement in the affinity of the antibody for antigen, compared to an unmodified parent antibody. Affinity-matured antibodies may be produced by procedures known in the art, e.g., Marks *et al.,Rio/Technology* 10:779-783 (1992); Barbas *et al. Proc Nat. Acad. Sci. USA* 91:3809-3813 (1994); Schier *et al. Gene* 169:147-155 (1995); Yelton *et al. J. Immunol.* 155:1994-2004 (1995); Jackson *et al., J. Immunol.* 154(7):331 0-15 9 (1995); and Hawkins *et al, J. Mol. Biol.* 226:889-896 (1992).

Antibodies according to the present invention preferably exhibit specific binding to IL-11 or IL-11R. An antibody that specifically binds to a target molecule preferably binds the target with greater affinity, and/or with greater duration than it binds to other targets. In one embodiment, the extent of binding of an antibody to an unrelated target is less than about 10% of the binding of the antibody to the target as measured, e.g., by ELISA, or by a radioimmunoassay (RIA). Alternatively, the binding specificity may be reflected in terms of binding affinity where the antibody binds to IL-11 or IL-11R with a K_D that is at least 0.1 order of magnitude (i.e. 0.1 x 10^n, where n is an integer representing the order of magnitude) greater than the K_D of the antibody towards another target molecule, e.g. another member of the IL-11 family such as IL-6 or the IL-6 receptor. This may optionally be one of at least 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.5, or 2.0.

Antibodies may be detectably labelled or, at least, capable of detection. Such antibodies being useful for both *in vivo* (e.g. imaging methods) and *in vitro* (e.g. assay methods) applications For example, the antibody may be labelled with a radioactive atom or a coloured molecule or a fluorescent molecule or a molecule which can be readily detected in any other way. Suitable detectable molecules include fluorescent proteins, luciferase, enzyme substrates, and radiolabels. The binding moiety may be directly labelled with a detectable label or it may be indirectly labelled. For example, the binding moiety may be an unlabelled antibody which can be detected by another antibody which is itself labelled. Alternatively, the second antibody may have bound to it biotin and binding of labelled streptavidin to the biotin is used to indirectly label the first antibody.

Aspects of the present invention include bi-specific antibodies, e.g. composed of two different fragments of two different antibodies, such that the bi-specific antibody binds two types of antigen. One of the antigens is IL-11 or IL-11R, the bi-specific antibody comprising a fragment as described herein that binds to IL-11 or IL-11R. The antibody may contain a different fragment having affinity for a second antigen, which may be any desired antigen. Techniques for the preparation of bi-specific antibodies are well known in the art, e.g. see

Mueller, D et al., (2010 *Biodrugs* **24** (2): 89–98), Wozniak-Knopp G et al., (2010 *Protein Eng Des* **23** (4): 289–297. Baeuerle, PA et al., (2009 *Cancer Res* **69** (12): 4941–4944).

In some embodiments, the bispecific antibody is provided as a fusion protein of two single-chain variable fragments (scFV) format, comprising a V_H and V_L of a IL-11 or IL-11R binding antibody or antibody fragment, and a V_H and V_L of an another antibody or antibody fragment.

Bispecific antibodies and bispecific antigen binding fragments may be provided in any suitable format, such as those formats described in Kontermann MAbs 2012, 4(2): 182-197, which is hereby incorporated by reference in its entirety. For example, a bispecific antibody or bispecific antigen binding fragment may be a bispecific antibody conjugate (e.g. an IgG2, $F(ab')_2$ or CovX-Body), a bispecific IgG or IgG-like molecule (e.g. an IgG, $scFv_4$-Ig, IgG-scFv, scFv-IgG, DVD-Ig, IgG-sVD, sVD-IgG, 2 in 1-IgG, mAb^2, or Tandemab common LC), an asymmetric bispecific IgG or IgG-like molecule (e.g. a kih IgG, kih IgG common LC, CrossMab, kih IgG-scFab, mAb-Fv, charge pair or SEED-body), a small bispecific antibody molecule (e.g. a Diabody (Db), dsDb, DART, scDb, tandAbs, tandem scFv (taFv), tandem dAb/VHH, triple body, triple head, Fab-scFv, or $F(ab')_2$-$scFv_2$), a bispecific Fc and C_H3 fusion protein (e.g. a taFv-Fc, Di-diabody, scDb-C_H3, scFv-Fc-scFv, HCAb-VHH, scFv-kih-Fc, or scFv-kih-C_H3), or a bispecific fusion protein (e.g. a $scFv_2$-albumin, scDb-albumin, taFv-toxin, DNL-Fab_3, DNL-Fab_4-IgG, DNL-Fab_4-IgG-$cytokine_2$). See in particular Figure 2 of Kontermann MAbs 2012, 4(2): 182-19.

Methods for producing bispecific antibodies include chemically crosslinking antibodies or antibody fragments, e.g. with reducible disulphide or non-reducible thioether bonds, for example as described in Segal and Bast, 2001. Production of Bispecific Antibodies. Current Protocols in Immunology. 14:IV:2.13:2.13.1–2.13.16, which is hereby incorporated by reference in its entirety. For example, *N*-succinimidyl-3-(-2-pyridyldithio)-propionate (SPDP) can be used to chemically crosslink e.g. Fab fragments via hinge region SH- groups, to create disulfide-linked bispecific $F(ab)_2$ heterodimers.

Other methods for producing bispecific antibodies include fusing antibody-producing hybridomas e.g. with polyethylene glycol, to produce a quadroma cell capable of secreting bispecific antibody, for example as described in D. M. and Bast, B. J. 2001. Production of Bispecific Antibodies. Current Protocols in Immunology. 14:IV:2.13:2.13.1–2.13.16.

Bispecific antibodies and bispecific antigen binding fragments can also be produced recombinantly, by expression from e.g. a nucleic acid construct encoding polypeptides for the

antigen binding molecules, for example as described in Antibody Engineering: Methods and Protocols, Second Edition (Humana Press, 2012), at Chapter 40: Production of Bispecific Antibodies: Diabodies and Tandem scFv (Hornig and Färber-Schwarz), or French, How to make bispecific antibodies, Methods Mol. Med. 2000; 40:333-339.

For example, a DNA construct encoding the light and heavy chain variable domains for the two antigen binding domains (i.e. the light and heavy chain variable domains for the antigen binding domain capable of binding IL-11 or IL-11R, and the light and heavy chain variable domains for the antigen binding domain capable of binding to another target protein), and including sequences encoding a suitable linker or dimerization domain between the antigen binding domains can be prepared by molecular cloning techniques. Recombinant bispecific antibody can thereafter be produced by expression (e.g. *in vitro*) of the construct in a suitable host cell (e.g. a mammalian host cell), and expressed recombinant bispecific antibody can then optionally be purified.

Aptamers

Aptamers, also called nucleic acid ligands, are nucleic acid molecules characterised by the ability to bind to a target molecule with high specificity and high affinity. Almost every aptamer identified to date is a non-naturally occurring molecule.

Aptamers to a given target (e.g. IL-11 or IL-11R) may be identified and/or produced by the method of Systematic Evolution of Ligands by EXponential enrichment (SELEX™). Aptamers and SELEX are described in Tuerk and Gold (Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science. 1990 Aug 3;249(4968):505-10) and in WO91/19813.

Aptamers may be DNA or RNA molecules and may be single stranded or double stranded. The aptamer may comprise chemically modified nucleic acids, for example in which the sugar and/or phosphate and/or base is chemically modified. Such modifications may improve the stability of the aptamer or make the aptamer more resistant to degradation and may include modification at the 2′ position of ribose.

Aptamers may be synthesised by methods which are well known to the skilled person. For example, aptamers may be chemically synthesised, e.g. on a solid support.

Solid phase synthesis may use phosphoramidite chemistry. Briefly, a solid supported nucleotide is detritylated, then coupled with a suitably activated nucleoside phosphoramidite

to form a phosphite triester linkage. Capping may then occur, followed by oxidation of the phosphite triester with an oxidant, typically iodine. The cycle may then be repeated to assemble the aptamer.

Aptamers can be thought of as the nucleic acid equivalent of monoclonal antibodies and often have K_d's in the nM or pM range, e.g. less than one of 500nM, 100nM, 50nM, 10nM, 1nM, 500pM, 100pM. As with monoclonal antibodies, they may be useful in virtually any situation in which target binding is required, including use in therapeutic and diagnostic applications, *in vitro* or *in vivo*. *In vitro* diagnostic applications may include use in detecting the presence or absence of a target molecule.

Aptamers according to the present invention may be provided in purified or isolated form. Aptamers according to the present invention may be formulated as a pharmaceutical composition or medicament.

Suitable aptamers may optionally have a minimum length of one of 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 nucleotides

Suitable aptamers may optionally have a maximum length of one of 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, or 80 nucleotides

Suitable aptamers may optionally have a length of one of 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, or 80 nucleotides.

<u>Oligonucleotide repression of IL-11 or IL-11R expression</u>

Oligonucleotide molecules, particularly RNA, may be employed to regulate gene expression. These include antisense oligonucleotides, targeted degradation of mRNAs by small interfering RNAs (siRNAs), post transcriptional gene silencing (PTGs), developmentally regulated sequence-specific translational repression of mRNA by micro-RNAs (miRNAs) and targeted transcriptional gene silencing.

An antisense oligonucleotide is an oligonucleotide, preferably single stranded, that targets and binds, by complementary sequence binding, to a target oligonucleotide, e.g. mRNA. Where the target oligonucleotide is an mRNA, binding of the antisense to the mRNA blocks translation of the mRNA and expression of the gene product. Antisense oligonucleotides may be designed to bind sense genomic nucleic acid and inhibit transcription of a target nucleotide sequence.

In view of the known nucleic acid sequences for IL-11 (e.g. the known mRNA sequences available from GenBank® under accession no.s: BC012506.1 GI:15341754 (human), BC134354.1 GI:126632002 (mouse), AF347935.1 GI:13549072 (rat)) and IL-11R (e.g. the known mRNA sequences available from GenBank® under accession no.s: NM_001142784.2 GI:391353394 (human), NM_001163401.1 GI:254281268 (mouse), NM_139116.1 GI:20806172 (rat)), oligonucleotides may be designed to repress or silence the expression of IL-11 or IL-11R. Such oligonucleotides may have any length, but may preferably be short, e.g. less than 100 nucleotides, e.g. 10-40 nucleotides, or 20-50 nucleotides, and may comprise a nucleotide sequence having complete- or near- complementarity (e.g. 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% complementarity) to a sequence of nucleotides of corresponding length in the target oligonucleotide, e.g. the IL-11 or IL-11R mRNA. The complementary region of the nucleotide sequence may have any length, but is preferably at least 5, and optionally no more than 50, nucleotides long, e.g. one of 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 nucleotides.

Repression of IL-11 or IL-11R expression will preferably result in a decrease in the quantity of IL-11 or IL-11R expressed by a cell, e.g. by a fibroblast or myofibroblast. For example, in a given cell the repression of IL-11 or IL-11R by administration of a suitable nucleic acid will result in a decrease in the quantity of IL-11 or IL-11R expressed by that cell relative to an untreated cell. Repression may be partial. Preferred degrees of repression are at least 50%, more preferably one of at least 60%, 70%, 80%, 85% or 90%. A level of repression between 90% and 100% is considered a 'silencing' of expression or function.

A role for the RNAi machinery and small RNAs in targeting of heterochromatin complexes and epigenetic gene silencing at specific chromosomal loci has been demonstrated. Double-stranded RNA (dsRNA)-dependent post transcriptional silencing, also known as RNA interference (RNAi), is a phenomenon in which dsRNA complexes can target specific genes of homology for silencing in a short period of time. It acts as a signal to promote degradation of mRNA with sequence identity. A 20-nt siRNA is generally long enough to induce gene-

specific silencing, but short enough to evade host response. The decrease in expression of targeted gene products can be extensive with 90% silencing induced by a few molecules of siRNA. RNAi based therapeutics have been progressed into Phase I, II and III clinical trials for a number of indications (Nature 2009 Jan 22; 457(7228):426-433).

In the art, these RNA sequences are termed "short or small interfering RNAs" (siRNAs) or "microRNAs" (miRNAs) depending on their origin. Both types of sequence may be used to down-regulate gene expression by binding to complementary RNAs and either triggering mRNA elimination (RNAi) or arresting mRNA translation into protein. siRNA are derived by processing of long double stranded RNAs and when found in nature are typically of exogenous origin. Micro-interfering RNAs (miRNA) are endogenously encoded small non-coding RNAs, derived by processing of short hairpins. Both siRNA and miRNA can inhibit the translation of mRNAs bearing partially complimentary target sequences without RNA cleavage and degrade mRNAs bearing fully complementary sequences.

Accordingly, the present invention provides the use of oligonucleotide sequences for down-regulating the expression of IL-11 or IL-11R.

siRNA ligands are typically double stranded and, in order to optimise the effectiveness of RNA mediated down-regulation of the function of a target gene, it is preferred that the length of the siRNA molecule is chosen to ensure correct recognition of the siRNA by the RISC complex that mediates the recognition by the siRNA of the mRNA target and so that the siRNA is short enough to reduce a host response.

miRNA ligands are typically single stranded and have regions that are partially complementary enabling the ligands to form a hairpin. miRNAs are RNA genes which are transcribed from DNA, but are not translated into protein. A DNA sequence that codes for a miRNA gene is longer than the miRNA. This DNA sequence includes the miRNA sequence and an approximate reverse complement. When this DNA sequence is transcribed into a single-stranded RNA molecule, the miRNA sequence and its reverse-complement base pair to form a partially double stranded RNA segment. The design of microRNA sequences is discussed in John et al, PLoS Biology, 11(2), 1862-1879, 2004.

Typically, the RNA ligands intended to mimic the effects of siRNA or miRNA have between 10 and 40 ribonucleotides (or synthetic analogues thereof), more preferably between 17 and 30 ribonucleotides, more preferably between 19 and 25 ribonucleotides and most preferably between 21 and 23 ribonucleotides. In some embodiments of the invention employing double-

stranded siRNA, the molecule may have symmetric 3' overhangs, e.g. of one or two (ribo)nucleotides, typically a UU of dTdT 3' overhang. Based on the disclosure provided herein, the skilled person can readily design suitable siRNA and miRNA sequences, for example using resources such the Ambion siRNA finder. siRNA and miRNA sequences can be synthetically produced and added exogenously to cause gene downregulation or produced using expression systems (e.g. vectors). In a preferred embodiment the siRNA is synthesized synthetically.

Longer double stranded RNAs may be processed in the cell to produce siRNAs (see for example Myers (2003) *Nature Biotechnology 21:324-328*). The longer dsRNA molecule may have symmetric 3' or 5' overhangs, e.g. of one or two (ribo)nucleotides, or may have blunt ends. The longer dsRNA molecules may be 25 nucleotides or longer. Preferably, the longer dsRNA molecules are between 25 and 30 nucleotides long. More preferably, the longer dsRNA molecules are between 25 and 27 nucleotides long. Most preferably, the longer dsRNA molecules are 27 nucleotides in length. dsRNAs 30 nucleotides or more in length may be expressed using the vector pDECAP (Shinagawa et al., Genes and Dev., 17, 1340-5, 2003).

Another alternative is the expression of a short hairpin RNA molecule (shRNA) in the cell. shRNAs are more stable than synthetic siRNAs. A shRNA consists of short inverted repeats separated by a small loop sequence. One inverted repeat is complimentary to the gene target. In the cell the shRNA is processed by DICER into a siRNA which degrades the target gene mRNA and suppresses expression. In a preferred embodiment the shRNA is produced endogenously (within a cell) by transcription from a vector. shRNAs may be produced within a cell by transfecting the cell with a vector encoding the shRNA sequence under control of a RNA polymerase III promoter such as the human H1 or 7SK promoter or a RNA polymerase II promoter. Alternatively, the shRNA may be synthesised exogenously (*in vitro*) by transcription from a vector. The shRNA may then be introduced directly into the cell. Preferably, the shRNA molecule comprises a partial sequence of IL-11 or IL-11R. Preferably, the shRNA sequence is between 40 and 100 bases in length, more preferably between 40 and 70 bases in length. The stem of the hairpin is preferably between 19 and 30 base pairs in length. The stem may contain G-U pairings to stabilise the hairpin structure.

siRNA molecules, longer dsRNA molecules or miRNA molecules may be made recombinantly by transcription of a nucleic acid sequence, preferably contained within a vector. Preferably, the siRNA molecule, longer dsRNA molecule or miRNA molecule comprises a partial sequence of IL-11 or IL-11R.

In one embodiment, the siRNA, longer dsRNA or miRNA is produced endogenously (within a cell) by transcription from a vector. The vector may be introduced into the cell in any of the ways known in the art. Optionally, expression of the RNA sequence can be regulated using a tissue specific (e.g. heart, liver, kidney or eye specific) promoter. In a further embodiment, the siRNA, longer dsRNA or miRNA is produced exogenously (*in vitro*) by transcription from a vector.

Suitable vectors may be oligonucleotide vectors configured to express the oligonucleotide agent capable of IL-11 or IL-11R repression. Such vectors may be viral vectors or plasmid vectors. The therapeutic oligonucleotide may be incorporated in the genome of a viral vector and be operably linked to a regulatory sequence, e.g. promoter, which drives its expression. The term "operably linked" may include the situation where a selected nucleotide sequence and regulatory nucleotide sequence are covalently linked in such a way as to place the expression of a nucleotide sequence under the influence or control of the regulatory sequence. Thus a regulatory sequence is operably linked to a selected nucleotide sequence if the regulatory sequence is capable of effecting transcription of a nucleotide sequence which forms part or all of the selected nucleotide sequence.

Viral vectors encoding promoter-expressed siRNA sequences are known in the art and have the benefit of long term expression of the therapeutic oligonucleotide. Examples include lentiviral (Nature 2009 Jan 22; 457(7228):426-433), adenovirus (Shen et al., FEBS Lett 2003 Mar 27;539(1-3)111-4) and retroviruses (Barton and Medzhitov PNAS November 12, 2002 vol.99, no.23 14943-14945).

In other embodiments a vector may be configured to assist delivery of the therapeutic oligonucleotide to the site at which repression of IL-11 or IL-11R expression is required. Such vectors typically involve complexing the oligonucleotide with a positively charged vector (e.g., cationic cell penetrating peptides, cationic polymers and dendrimers, and cationic lipids); conjugating the oligonucleotide with small molecules (e.g., cholesterol, bile acids, and lipids), polymers, antibodies, and RNAs; or encapsulating the oligonucleotide in nanoparticulate formulations (Wang et al., AAPS J. 2010 Dec; 12(4): 492-503).

In one embodiment, a vector may comprise a nucleic acid sequence in both the sense and antisense orientation, such that when expressed as RNA the sense and antisense sections will associate to form a double stranded RNA.

Alternatively, siRNA molecules may be synthesized using standard solid or solution phase synthesis techniques which are known in the art. Linkages between nucleotides may be phosphodiester bonds or alternatives, for example, linking groups of the formula P(O)S, (thioate); P(S)S, (dithioate); P(O)NR'2; P(O)R'; P(O)OR6; CO; or CONR'2 wherein R is H (or a salt) or alkyl (1-12C) and R6 is alkyl (1-9C) is joined to adjacent nucleotides through-O-or-S-.

Modified nucleotide bases can be used in addition to the naturally occurring bases, and may confer advantageous properties on siRNA molecules containing them.

For example, modified bases may increase the stability of the siRNA molecule, thereby reducing the amount required for silencing. The provision of modified bases may also provide siRNA molecules which are more, or less, stable than unmodified siRNA.

The term 'modified nucleotide base' encompasses nucleotides with a covalently modified base and/or sugar. For example, modified nucleotides include nucleotides having sugars which are covalently attached to low molecular weight organic groups other than a hydroxyl group at the 3'position and other than a phosphate group at the 5'position. Thus modified nucleotides may also include 2'substituted sugars such as 2'-O-methyl- ; 2'-O-alkyl ; 2'-O-allyl ; 2'-S-alkyl; 2'-S-allyl; 2'-fluoro- ; 2'-halo or azido-ribose, carbocyclic sugar analogues, a-anomeric sugars; epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, and sedoheptulose.

Modified nucleotides are known in the art and include alkylated purines and pyrimidines, acylated purines and pyrimidines, and other heterocycles. These classes of pyrimidines and purines are known in the art and include pseudoisocytosine, N4,N4-ethanocytosine, 8-hydroxy-N6-methyladenine, 4-acetylcytosine,5-(carboxyhydroxylmethyl) uracil, 5 fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyl uracil, dihydrouracil, inosine, N6-isopentyl-adenine, 1- methyladenine, 1-methylpseudouracil, 1-methylguanine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-methyladenine, 7-methylguanine, 5-methylaminomethyl uracil, 5-methoxy amino methyl-2-thiouracil, -D-mannosylqueosine, 5-methoxycarbonylmethyluracil, 5methoxyuracil, 2 methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid methyl ester, psueouracil, 2-thiocytosine, 5-methyl-2 thiouracil, 2-thiouracil, 4-thiouracil, 5methyluracil, N-uracil-5-oxyacetic acid methylester, uracil 5-oxyacetic acid, queosine, 2-thiocytosine, 5-propyluracil, 5-propylcytosine, 5-ethyluracil, 5ethylcytosine, 5-butyluracil, 5-pentyluracil, 5-

pentylcytosine, and 2,6,diaminopurine, methylpsuedouracil, 1-methylguanine, 1-methylcytosine.

Methods relating to the use of RNAi to silence genes in C. elegans, Drosophila, plants, and mammals are known in the art (Fire A, et al., 1998 Nature 391:806-811; Fire, A. Trends Genet. 15, 358-363 (1999); Sharp, P. A. RNA interference 2001. Genes Dev. 15, 485-490 (2001); Hammond, S. M., et al., Nature Rev. Genet. 2, 110-1119 (2001); Tuschl, T. Chem. Biochem. 2, 239-245 (2001); Hamilton, A. et al., Science 286, 950-952 (1999); Hammond, S. M., et al., Nature 404, 293-296 (2000); Zamore, P. D., et al., Cell 101, 25-33 (2000); Bernstein, E., et al., Nature 409, 363-366 (2001); Elbashir, S. M., et al., Genes Dev. 15, 188-200 (2001); WO0129058; WO9932619, and Elbashir S M, et al., 2001 Nature 411:494-498).

Accordingly, the invention provides nucleic acid that is capable, when suitably introduced into or expressed within a mammalian, e.g. human, cell that otherwise expresses IL-11 or IL-11R, of suppressing IL-11 or IL-11R expression by RNAi.

The nucleic acid may have substantial sequence identity to a portion of IL-11 or IL-11R mRNA, as defined in GenBank accession no. NM_000641.3 GI:391353405 (IL-11) or U32324.1 GI:975336 (IL-11R), or the complementary sequence to said mRNA.

The nucleic acid may be a double-stranded siRNA. (As the skilled person will appreciate, and as explained further below, a siRNA molecule may include a short 3' DNA sequence also.)

Alternatively, the nucleic acid may be a DNA (usually double-stranded DNA) which, when transcribed in a mammalian cell, yields an RNA having two complementary portions joined via a spacer, such that the RNA takes the form of a hairpin when the complementary portions hybridise with each other. In a mammalian cell, the hairpin structure may be cleaved from the molecule by the enzyme DICER, to yield two distinct, but hybridised, RNA molecules.

In some preferred embodiments, the nucleic acid is generally targeted to the sequence of one of SEQ ID NOs 2 to 5 (IL-11; Figure 11) or to one of SEQ ID NOs 7 to 10 (IL-11R; Figure 12).

Only single-stranded (i.e. non self-hybridised) regions of an mRNA transcript are expected to be suitable targets for RNAi. It is therefore proposed that other sequences very close in the IL-11 or IL-11R mRNA transcript to the sequence represented by one of SEQ ID NOs 2 to 5 or 7 to 10 may also be suitable targets for RNAi. Such target sequences are preferably 17-23 nucleotides in length and preferably overlap one of SEQ ID NOs 2 to 5 or 7 to 10 by at least 1,

2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 or all 19 nucleotides (at either end of one of SEQ ID NOs 2 to 5 or 7 to 10).

Accordingly, the invention provides nucleic acid that is capable, when suitably introduced into or expressed within a mammalian cell that otherwise expresses IL-11 or IL-11R, of suppressing IL-11 or IL-11R expression by RNAi, wherein the nucleic acid is generally targeted to the sequence of one of SEQ ID NOs 2 to 5 or 7 to 10.

By "generally targeted" the nucleic acid may target a sequence that overlaps with SEQ ID NOs 2 to 5 or 7 to 10. In particular, the nucleic acid may target a sequence in the mRNA of human IL-11 or IL-11R that is slightly longer or shorter than one of SEQ ID NOs 2 to 5 or 7 to 10 (preferably from 17-23 nucleotides in length), but is otherwise identical to one of SEQ ID NOs 2 to 5 or 7 to 10.

It is expected that perfect identity/complementarity between the nucleic acid of the invention and the target sequence, although preferred, is not essential. Accordingly, the nucleic acid of the invention may include a single mismatch compared to the mRNA of IL-11 or IL-11R. It is expected, however, that the presence of even a single mismatch is likely to lead to reduced efficiency, so the absence of mismatches is preferred. When present, 3' overhangs may be excluded from the consideration of the number of mismatches.

The term "complementarity" is not limited to conventional base pairing between nucleic acid consisting of naturally occurring ribo- and/or deoxyribonucleotides, but also includes base pairing between mRNA and nucleic acids of the invention that include non-natural nucleotides.

In one embodiment, the nucleic acid (herein referred to as double-stranded siRNA) includes the double-stranded RNA sequences shown in Figure 13 (IL-11; SEQ ID NOs 11 to 14).

In another embodiment, the nucleic acid (herein referred to as double-stranded siRNA) includes the double-stranded RNA sequences shown in Figure 14 (IL-11R; SEQ ID NOs 15 to 18).

However, it is also expected that slightly shorter or longer sequences directed to the same region of IL-11 or IL-11R mRNA will also be effective. In particular, it is expected that double-stranded sequences between 17 and 23 bp in length will also be effective.

The strands that form the double-stranded RNA may have short 3' dinucleotide overhangs, which may be DNA or RNA. The use of a 3' DNA overhang has no effect on siRNA activity compared to a 3' RNA overhang, but reduces the cost of chemical synthesis of the nucleic acid strands (Elbashir et al., 2001c). For this reason, DNA dinucleotides may be preferred.

When present, the dinucleotide overhangs may be symmetrical to each other, though this is not essential. Indeed, the 3' overhang of the sense (upper) strand is irrelevant for RNAi activity, as it does not participate in mRNA recognition and degradation (Elbashir et al., 2001a, 2001b, 2001c).

While RNAi experiments in Drosophila show that antisense 3' overhangs may participate in mRNA recognition and targeting (Elbashir et al. 2001c), 3' overhangs do not appear to be necessary for RNAi activity of siRNA in mammalian cells. Incorrect annealing of 3' overhangs is therefore thought to have little effect in mammalian cells (Elbashir et al. 2001c; Czauderna et al. 2003).

Any dinucleotide overhang may therefore be used in the antisense strand of the siRNA. Nevertheless, the dinucleotide is preferably -UU or –UG (or –TT or –TG if the overhang is DNA), more preferably -UU (or –TT). The –UU (or –TT) dinucleotide overhang is most effective and is consistent with (i.e. capable of forming part of) the RNA polymerase III end of transcription signal (the terminator signal is TTTTT). Accordingly, this dinucleotide is most preferred. The dinucleotides AA, CC and GG may also be used, but are less effective and consequently less preferred.

Moreover, the 3' overhangs may be omitted entirely from the siRNA.

The invention also provides single-stranded nucleic acids (herein referred to as single-stranded siRNAs) respectively consisting of a component strand of one of the aforementioned double-stranded nucleic acids, preferably with the 3'-overhangs, but optionally without. The invention also provides kits containing pairs of such single-stranded nucleic acids, which are capable of hybridising with each other in vitro to form the aforementioned double-stranded siRNAs, which may then be introduced into cells.

The invention also provides DNA that, when transcribed in a mammalian cell, yields an RNA (herein also referred to as an shRNA) having two complementary portions which are capable of self-hybridising to produce a double-stranded motif, e.g. including a sequence selected

from the group consisting of SEQ ID No.s 11 to 14 or 15 to 18 or a sequence that differs from any one of the aforementioned sequences by a single base pair substitution.

The complementary portions will generally be joined by a spacer, which has suitable length and sequence to allow the two complementary portions to hybridise with each other. The two complementary (i.e. sense and antisense) portions may be joined 5'-3' in either order. The spacer will typically be a short sequence, of approximately 4-12 nucleotides, preferably 4-9 nucleotides, more preferably 6-9 nucleotides.

Preferably the 5' end of the spacer (immediately 3' of the upstream complementary portion) consists of the nucleotides –UU- or –UG-, again preferably –UU- (though, again, the use of these particular dinucleotides is not essential). A suitable spacer, recommended for use in the pSuper system of OligoEngine (Seattle, Washington, USA) is UUCAAGAGA. In this and other cases, the ends of the spacer may hybridise with each other, e.g. elongating the double-stranded motif beyond the exact sequences of SEQ ID NOs 11 to 14 or 15 to 18 by a small number (e.g. 1 or 2) of base pairs.

Similarly, the transcribed RNA preferably includes a 3' overhang from the downstream complementary portion. Again, this is preferably –UU or –UG, more preferably –UU.

Such shRNA molecules may then be cleaved in the mammalian cell by the enzyme DICER to yield a double-stranded siRNA as described above, in which one or each strand of the hybridised dsRNA includes a 3' overhang.

Techniques for the synthesis of the nucleic acids of the invention are of course well known in the art.

The skilled person is well able to construct suitable transcription vectors for the DNA of the invention using well-known techniques and commercially available materials. In particular, the DNA will be associated with control sequences, including a promoter and a transcription termination sequence.

Of particular suitability are the commercially available pSuper and pSuperior systems of OligoEngine (Seattle, Washington, USA). These use a polymerase-III promoter (H1) and a T_5 transcription terminator sequence that contributes two U residues at the 3' end of the transcript (which, after DICER processing, provide a 3' UU overhang of one strand of the siRNA).

Another suitable system is described in Shin et al. (RNA, 2009 May; 15(5): 898-910), which uses another polymerase-III promoter (U6).

The double-stranded siRNAs of the invention may be introduced into mammalian cells *in vitro* or *in vivo* using known techniques, as described below, to suppress expression of IL-11 or IL-11R.

Similarly, transcription vectors containing the DNAs of the invention may be introduced into tumour cells *in vitro* or *in vivo* using known techniques, as described below, for transient or stable expression of RNA, again to suppress expression of IL-11 or IL-11R.

Accordingly, the invention also provides a method of suppressing IL-11 or IL-11R expression in a mammalian, e.g. human, cell, the method comprising administering to the cell a double-stranded siRNA of the invention or a transcription vector of the invention.

Similarly, the invention further provides a method of treating fibrosis, the method comprising administering to a subject a double-stranded siRNA of the invention or a transcription vector of the invention.

The invention further provides the double-stranded siRNAs of the invention and the transcription vectors of the invention, for use in a method of treatment, preferably a method of treating fibrosis.

The invention further provides the use of the double-stranded siRNAs of the invention and the transcription vectors of the invention in the preparation of a medicament for the treatment of fibrosis.

The invention further provides a composition comprising a double-stranded siRNA of the invention or a transcription vector of the invention in admixture with one or more pharmaceutically acceptable carriers. Suitable carriers include lipophilic carriers or vesicles, which may assist in penetration of the cell membrane.

Materials and methods suitable for the administration of siRNA duplexes and DNA vectors of the invention are well known in the art and improved methods are under development, given the potential of RNAi technology.

Generally, many techniques are available for introducing nucleic acids into mammalian cells. The choice of technique will depend on whether the nucleic acid is transferred into cultured cells *in vitro* or *in vivo* in the cells of a patient. Techniques suitable for the transfer of nucleic acid into mammalian cells *in vitro* include the use of liposomes, electroporation, microinjection, cell fusion, DEAE dextran and calcium phosphate precipitation. *In vivo* gene transfer techniques include transfection with viral (typically retroviral) vectors and viral coat protein-liposome mediated transfection (Dzau et al. (2003) Trends in Biotechnology 11, 205-210).

In particular, suitable techniques for cellular administration of the nucleic acids of the invention both *in vitro* and *in vivo* are disclosed in the following articles:

General reviews: Borkhardt, A. 2002. Blocking oncogenes in malignant cells by RNA interference--new hope for a highly specific cancer treatment? Cancer Cell. 2:167-8. Hannon, G.J. 2002. RNA interference. Nature. 418:244-51. McManus, M.T., and P.A. Sharp. 2002. Gene silencing in mammals by small interfering RNAs. Nat Rev Genet. 3:737-47. Scherr, M., M.A. Morgan, and M. Eder. 2003b. Gene silencing mediated by small interfering RNAs in mammalian cells. Curr Med Chem. 10:245-56. Shuey, D.J., D.E. McCallus, and T. Giordano. 2002. RNAi: gene-silencing in therapeutic intervention. Drug Discov Today. 7:1040-6.

Systemic delivery using liposomes: Lewis, D.L., J.E. Hagstrom, A.G. Loomis, J.A. Wolff, and H. Herweijer. 2002. Efficient delivery of siRNA for inhibition of gene expression in postnatal mice. Nat Genet. 32:107-8. Paul, C.P., P.D. Good, I. Winer, and D.R. Engelke. 2002. Effective expression of small interfering RNA in human cells. Nat Biotechnol. 20:505-8. Song, E., S.K. Lee, J. Wang, N. Ince, N. Ouyang, J. Min, J. Chen, P. Shankar, and J. Lieberman. 2003. RNA interference targeting Fas protects mice from fulminant hepatitis. Nat Med. 9:347-51. Sorensen, D.R., M. Leirdal, and M. Sioud. 2003. Gene silencing by systemic delivery of synthetic siRNAs in adult mice. J Mol Biol. 327:761-6.

Virus mediated transfer: Abbas-Terki, T., W. Blanco-Bose, N. Deglon, W. Pralong, and P. Aebischer. 2002. Lentiviral-mediated RNA interference. Hum Gene Ther. 13:2197-201. Barton, G.M., and R. Medzhitov. 2002. Retroviral delivery of small interfering RNA into primary cells. Proc Natl Acad Sci U S A. 99:14943-5. Devroe, E., and P.A. Silver. 2002. Retrovirus-delivered siRNA. BMC Biotechnol. 2:15. Lori, F., P. Guallini, L. Galluzzi, and J. Lisziewicz. 2002. Gene therapy approaches to HIV infection. Am J Pharmacogenomics. 2:245-52. Matta, H., B. Hozayev, R. Tomar, P. Chugh, and P.M. Chaudhary. 2003. Use of lentiviral vectors for delivery of small interfering RNA. Cancer Biol Ther. 2:206-10. Qin, X.F.,

D.S. An, I.S. Chen, and D. Baltimore. 2003. Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. Proc Natl Acad Sci U S A. 100:183-8. Scherr, M., K. Battmer, A. Ganser, and M. Eder. 2003a. Modulation of gene expression by lentiviral-mediated delivery of small interfering RNA. Cell Cycle. 2:251-7. Shen, C., A.K. Buck, X. Liu, M. Winkler, and S.N. Reske. 2003. Gene silencing by adenovirus-delivered siRNA. FEBS Lett. 539:111-4.

Peptide delivery: Morris, M.C., L. Chaloin, F. Heitz, and G. Divita. 2000. Translocating peptides and proteins and their use for gene delivery. Curr Opin Biotechnol. 11:461-6. Simeoni, F., M.C. Morris, F. Heitz, and G. Divita. 2003. Insight into the mechanism of the peptide-based gene delivery system MPG: implications for delivery of siRNA into mammalian cells. Nucleic Acids Res. 31:2717-24. Other technologies that may be suitable for delivery of siRNA to the target cells are based on nanoparticles or nanocapsules such as those described in US patent numbers 6,649,192B and 5,843,509B.

Formulations

In therapeutic applications, agents capable of inhibiting the action of IL-11 or agents capable of preventing or reducing the expression of IL-11 or IL-11R are preferably formulated as a medicament or pharmaceutical together with one or more other pharmaceutically acceptable ingredients well known to those skilled in the art, including, but not limited to, pharmaceutically acceptable carriers, adjuvants, excipients, diluents, fillers, buffers, preservatives, anti-oxidants, lubricants, stabilisers, solubilisers, surfactants (e.g., wetting agents), masking agents, colouring agents, flavouring agents, and sweetening agents.

The term "pharmaceutically acceptable" as used herein pertains to compounds, ingredients, materials, compositions, dosage forms, etc., which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of the subject in question (e.g., human) without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio. Each carrier, adjuvant, excipient, etc. must also be "acceptable" in the sense of being compatible with the other ingredients of the formulation.

Suitable carriers, adjuvants, excipients, etc. can be found in standard pharmaceutical texts, for example, Remington's Pharmaceutical Sciences, 18th edition, Mack Publishing Company, Easton, Pa., 1990; and Handbook of Pharmaceutical Excipients, 2nd edition, 1994.

The formulations may be prepared by any methods well known in the art of pharmacy. Such methods include the step of bringing into association the active compound with a carrier which constitutes one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association the active compound with carriers (e.g., liquid carriers, finely divided solid carrier, etc.), and then shaping the product, if necessary.

The formulations may be prepared for topical, parenteral, systemic, intravenous, intra-arterial, intramuscular, intrathecal, intraocular, intra-conjunctival, subcutaneous, oral or transdermal routes of administration which may include injection. Injectable formulations may comprise the selected agent in a sterile or isotonic medium.

Administration is preferably in a "therapeutically effective amount", this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of the disease being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of the techniques and protocols mentioned above can be found in Remington's Pharmaceutical Sciences, 20th Edition, 2000, pub. Lippincott, Williams & Wilkins.

Fibrosis

As used herein, "fibrosis" refers to the formation of excess fibrous connective tissue as a result of the excess deposition of extracellular matrix components, for example collagen. Fibrous connective tissue is characterised by having extracellular matrix (ECM) with a high collagen content. The collagen may be provided in strands or fibers, which may be arranged irregularly or aligned. The ECM of fibrous connective tissue may also include glycosaminoglycans.

As used herein, "excess fibrous connective tissue" refers to an amount of connective tissue at a given location (e.g. a given tissue or organ, or part of a given tissue or organ) which is greater than the amount of connective tissue present at that location in the absence of fibrosis, e.g. under normal, non-pathological conditions. As used herein, "excess deposition of extracellular matrix components" refers to a level of deposition of one or more extracellular matrix components which is greater than the level of deposition in the absence of fibrosis, e.g. under normal, non-pathological conditions.

The cellular and molecular mechanisms of fibrosis are described in Wynn, J. Pathol. (2008) 214(2): 199-210, and Wynn and Ramalingam, Nature Medicine (2012) 18:1028-1040, which are hereby incorporated by reference in their entirety.

The main cellular effectors of fibrosis are myofibroblasts, which produce a collagen-rich extracellular matrix.

In response to tissue injury, damaged cells and leukocytes produce pro-fibrotic factors such as TGFβ, IL-13 and PDGF, which activate fibroblasts to αSMA-expressing myofibroblasts, and recruit myofibroblasts to the site of injury. Myofibroblasts produce a large amount of extracellular matrix, and are important mediators in aiding contracture and closure of the wound. However, under conditions of persistent infection or during chronic inflammation there can be overactivation and recruitment of myofibroblasts, and thus over-production of extracellular matrix components, resulting in the formation of excess fibrous connective tissue.

In some embodiments fibrosis may be triggered by pathological conditions, e.g. conditions, infections or disease states that lead to production of pro-fibrotic factors such as TGFβ1. In some embodiments, fibrosis may be caused by physical injury/stimuli, chemical injury/stimuli or environmental injury/stimuli. Physical injury/stimuli may occur during surgery, e.g. iatrogenic causes. Chemical injury/stimuli may include drug induced fibrosis, e.g. following chronic administration of drugs such as bleomycin, cyclophosphamide, amiodarone, procainamide, penicillamine, gold and nitrofurantoin (Daba et al., Saudi Med J 2004 Jun; 25(6): 700-6). Environmental injury/stimuli may include exposure to asbestos fibres or silica.

Fibrosis can occur in many tissues of the body. For example, fibrosis can occur in the liver (e.g. cirrhosis), lungs, kidney, heart, blood vessels, eye, skin, pancreas, intestine, brain, and bone marrow. Fibrosis may also occur in multiple organs at once.

In embodiments herein, fibrosis may involve an organ of the gastrointestinal system, e.g. of the liver, small intestine, large intestine, or pancreas. In some embodiments, fibrosis may involve an organ of the respiratory system, e.g. the lungs. In embodiments, fibrosis may involve an organ of the cardiovascular system, e.g. of the heart or blood vessels. In some embodiments, fibrosis may involve the skin. In some embodiments, fibrosis may involve an organ of the nervous system, e.g. the brain. In some embodiments, fibrosis may involve an organ of the urinary system, e.g. the kidneys. In some embodiments, fibrosis may involve an organ of the musculoskeletal system, e.g. muscle tissue.

In some preferred embodiments, the fibrosis is cardiac or myocardial fibrosis, hepatic fibrosis, or renal fibrosis. In some embodiments cardiac or myocardial fibrosis is associated with dysfunction of the musculature or electrical properties of the heart, or thickening of the walls of valves of the heart. In some embodiments fibrosis is of the atrium and/or ventricles of the heart. Treatment or prevention of atrial or ventricular fibrosis may help reduce risk or onset of atrial fibrillation, ventricular fibrillation, or myocardial infarction.

In some preferred embodiments hepatic fibrosis is associated with chronic liver disease or liver cirrhosis. In some preferred embodiments renal fibrosis is associated with chronic kidney disease.

Diseases/conditions characterised by fibrosis in accordance with the present invention include but are not limited to: respiratory conditions such as pulmonary fibrosis, cystic fibrosis, idiopathic pulmonary fibrosis, progressive massive fibrosis, scleroderma, obliterative bronchiolitis, Hermansky-Pudlak syndrome, asbestosis, silicosis, chronic pulmonary hypertension, AIDS associated pulmonary hypertension, sarcoidosis, tumor stroma in lung disease, and asthma; chronic liver disease, primary biliary cirrhosis (PBC), schistosomal liver disease, liver cirrhosis; cardiovascular conditions such as hypertrophic cardiomyopathy, dilated cardiomyopathy (DCM), fibrosis of the atrium, atrial fibrillation, fibrosis of the ventricle, ventricular fibrillation, myocardial fibrosis, Brugada syndrome, myocarditis, endomyocardial fibrosis, myocardial infarction, fibrotic vascular disease, hypertensive heart disease, arrhythmogenic right ventricular cardiomyopathy (ARVC), tubulointerstitial and glomerular fibrosis, atherosclerosis, varicose veins, cerebral infarcts; neurological conditions such as gliosis and Alzheimer's disease; muscular dystrophy such as Duchenne muscular dystrophy (DMD) or Becker's muscular dystrophy (BMD); gastrointestinal conditions such as Chron's disease, microscopic colitis and primary sclerosing cholangitis (PSC); skin conditions such as scleroderma, nephrogenic systemic fibrosis and cutis keloid; arthrofibrosis; Dupuytren's contracture; mediastinal fibrosis; retroperitoneal fibrosis; myelofibrosis; Peyronie's disease; adhesive capsulitis; kidney disease (e.g., renal fibrosis, nephritic syndrome, Alport's syndrome, HIV associated nephropathy, polycystic kidney disease, Fabry's disease, diabetic nephropathy, chronic glomerulonephritis, nephritis associated with systemic lupus); progressive systemic sclerosis (PSS); chronic graft versus host disease; diseases of the eye such as Grave's opthalmopathy, epiretinal fibrosis, retinal fibrosis, subretinal fibrosis (e.g. associated with macular degeneration (e.g. wet age-related macular degeneration (AMD)), diabetic retinopathy, glaucoma, corneal fibrosis, post-surgical fibrosis (e.g. of the posterior capsule following cataract surgery, or of the bleb following trabeculectomy for glaucoma), conjunctival fibrosis, subconjunctival fibrosis; arthritis; fibrotic pre-neoplastic and fibrotic

neoplastic disease; and fibrosis induced by chemical or environmental insult (e.g., cancer chemotherapy, pesticides, radiation/cancer radiotherapy).

It will be appreciated that the many of the diseases/conditions listed above are interrelated. For example, fibrosis of the ventricle may occur post myocardial infarction, and is associated with DCM, HCM and myocarditis.

In particular embodiments, the disease/disorder may be one of pulmonary fibrosis, atrial fibrillation, ventricular fibrillation, hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), non-alcoholic steatohepatitis (NASH), cirrhosis, chronic kidney disease, scleroderma, systemic sclerosis, keloid, cystic fibrosis, Chron's disease, post-surgical fibrosis or retinal fibrosis.

Treatment, prevention or alleviation of fibrosis according to the present invention may be of fibrosis that is associated with an upregulation of IL-11, e.g. an upregulation of IL-11 in cells or tissue in which the fibrosis occurs or may occur, or upregulation of extracellular IL-11 or IL-11R.

Treatment or alleviation of fibrosis may be effective to prevent progression of the fibrosis, e.g. to prevent worsening of the condition or to slow the rate of development of the fibrosis. In some embodiments treatment or alleviation may lead to an improvement in the fibrosis, e.g. a reduction in the amount of deposited collagen fibres.

Prevention of fibrosis may refer to prevention of a worsening of the condition or prevention of the development of fibrosis, e.g. preventing an early stage fibrosis developing to a later, chronic, stage.

Subject

The subject to be treated may be any animal or human. The subject is preferably mammalian, more preferably human. The subject may be a non-human mammal, but is more preferably human. The subject may be male or female. The subject may be a patient.

Sample

A sample obtained from a subject may be of any kind. A biological sample may be taken from any tissue or bodily fluid, e.g. a blood sample, blood-derived sample, serum sample, lymph sample, semen sample, saliva sample, synovial fluid sample. A blood-derived sample may be a selected fraction of a patient's blood, e.g. a selected cell-containing fraction or a plasma or

serum fraction. A sample may comprise a tissue sample or biopsy; or cells isolated from a subject. Samples may be collected by known techniques, such as biopsy or needle aspirate. Samples may be stored and/or processed for subsequent determination of IL-11 expression levels.

Samples may be used to determine the upregulation of IL-11 or IL-11R in the subject from which the sample was taken.

In some preferred embodiments a sample may be a tissue sample, e.g. biopsy, taken from heart, liver or kidney tissue. In some embodiments a sample may be a tissue sample, e.g. biopsy, taken from the eye.

A sample may contain cells, and may preferably contain fibroblasts and/or myofibroblasts. In some embodiments, fibroblasts or myofibroblasts may be obtained from heart, liver or kidney tissue, e.g. they may be cardiac fibroblasts or cardiac myofibroblasts (e.g. *see* Colby et al., Circulation Research 2009;105:1164-1176), hepatic fibroblasts or hepatic myofibroblasts (e.g. *see* Zeisberg et al., The Journal of Biological Chemistry, August 10, 2007, 282, 23337-23347; Brenner., Fibrogenesis & Tissue Repair 2012, 5(Suppl 1):S17) or renal fibroblasts or renal myofibroblasts (e.g. *see* Strutz and Zeisberg. JASN November 2006 vol. 17 no. 11 2992-2998). In some embodiments, fibroblasts or myofibroblasts may be obtained from eye tissue, e.g. they may be corneal fibroblasts.

Upregulation of IL-11 or IL-11R expression

Some aspects and embodiments of the present invention concern detection of expression of IL-11 or IL-11R, e.g. in a sample obtained from a subject.

In some aspects and embodiments the present invention concerns the upregulation of expression (over-expression) of IL-11 or IL-11R (as a protein or oligonucleotide encoding the respective IL-11 or IL-11R) and detection of such upregulation as an indicator of suitability for treatment with an agent capable of inhibiting the action of IL-11 or with an agent capable of preventing or reducing the expression of IL-11 or IL-11R.

Upregulation of IL-11 or IL-11R expression comprises expression of IL-11 or IL-11R at a level that is greater than would normally be expected for a cell or tissue of a given type. Upregulation may be determined by determining the level of expression of IL-11 or IL-11R in a cell or tissue. A comparison may be made between the level of IL-11 or IL-11R expression in a cell or tissue sample from a subject and a reference level of IL-11 or IL-11R, e.g. a value or

range of values representing a normal level of expression of IL-11 or IL-11R for the same or corresponding cell or tissue type. In some embodiments reference levels may be determined by detecting IL-11 or IL-11R expression in a control sample, e.g. in corresponding cells or tissue from a healthy subject or from healthy tissue of the same subject. In some embodiments reference levels may be obtained from a standard curve or data set.

Levels of expression may be quantitated for absolute comparison, or relative comparisons may be made.

In some embodiments upregulation of IL-11 or IL-11R may be considered to be present when the level of expression in the test sample is at least 1.1 times that of a reference level. More preferably, the level of expression may be selected from one of at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2.0, at least 2.1, at least 2.2, at least 2.3, at least 2.4 at least 2.5, at least 2.6, at least 2.7, at least 2.8, at least 2.9, at least 3.0, at least 3.5, at least 4.0, at least 5.0, at least 6.0, at least 7.0, at least 8.0, at least 9.0, or at least 10.0 times that of the reference level.

IL-11 or IL-11R expression levels may be determined by one of a number of known *in vitro* assay techniques, such as PCR based assays, in situ hybridisation assays, flow cytometry assays, immunological or immunohistochemical assays.

By way of example suitable techniques involve a method of detecting the level of IL-11 or IL-11R in a sample by contacting the sample with an agent capable of binding IL-11 or IL-11R and detecting the formation of a complex of the agent and IL-11 or IL-11R. The agent may be any suitable binding molecule, e.g. an antibody, polypeptide, peptide, oligonucleotide, aptamer or small molecule, and may optionally be labelled to permit detection, e.g. visualisation, of the complexes formed. Suitable labels and means for their detection are well known to those in the art and include fluorescent labels (e.g. fluorescein, rhodamine, eosine and NDB, green fluorescent protein (GFP), chelates of rare earths such as europium (Eu), terbium (Tb) and samarium (Sm), tetramethyl rhodamine, Texas Red, 4-methyl umbelliferone, 7-amino-4-methyl coumarin, Cy3, Cy5), isotope markers, radioisotopes (e.g. ^{32}P, ^{33}P, ^{35}S), chemiluminescence labels (e.g. acridinium ester, luminol, isoluminol), enzymes (e.g. peroxidase, alkaline phosphatase, glucose oxidase, beta-galactosidase, luciferase), antibodies, ligands and receptors. Detection techniques are well known to those of skill in the art and can be selected to correspond with the labelling agent. Suitable techniques include PCR amplification of oligonucleotide tags, mass spectrometry, detection of fluorescence or

colour, e.g. upon enzymatic conversion of a substrate by a reporter protein, or detection of radioactivity.

Assays may be configured to quantify the amount of IL-11 or IL-11R in a sample. Quantified amounts of IL-11 or IL-11R from a test sample may be compared with reference values, and the comparison used to determine whether the test sample contains an amount of IL-11 or IL-11R that is higher or lower than that of the reference value to a selected degree of statistical significance.

Quantification of detected IL-11 or IL-11R may be used to determine up- or down-regulation or amplification of genes encoding IL-11 or IL-11R. In cases where the test sample contains fibrotic cells, such up-regulation, down-regulation or amplification may be compared to a reference value to determine whether any statistically significant difference is present.

Subject selection

A subject may be selected for treatment based on a determination that the subject has an upregulated level of IL-11 or IL-11R expression. IL-11 or IL-11R may therefore act as a marker of a fibrosis that is suitable for treatment with an agent capable of inhibiting the action of IL-11 or with an agent capable of preventing or reducing the expression of IL-11 or IL-11R.

Upregulation may be in a given tissue or in selected cells from a given tissue. A preferred tissue may be one of heart, liver or kidney. A preferred tissue may be eye. A preferred cell type may be fibroblasts or myofibroblasts. Upregulation may also be determined in a circulating fluid, e.g. blood, or in a blood derived sample. Upregulation of may be of extracellular IL-11 or IL-11R.

Determination of IL-11 or IL-11R levels may be performed by assay, preferably *in vitro*, on a sample obtained from a subject, as described herein.

Following selection, a subject may be provided with treatment for fibrosis by administration of an agent capable of inhibiting the action of IL-11 or an agent capable of preventing or reducing the expression of IL-11 or IL-11R.

In some embodiments a subject may have been diagnosed with fibrosis, be suspected of having fibrosis or be considered at risk of developing fibrosis and it is of interest whether the subject will benefit from treatment with an agent capable of inhibiting the action of IL-11 or

with an agent capable of preventing or reducing the expression of IL-11 or IL-11R. In such embodiments, the suitability of the subject for such treatment may be determined by determining whether IL-11 or IL-11R expression is upregulated in the subject. In some embodiments, IL-11 or IL-11R expression is locally or systemically upregulated in the subject.

Diagnosis and Prognosis

The detection of upregulation of IL-11 or IL-11R expression may also be used in a method of diagnosing fibrosis or the risk of developing fibrosis in a subject, and in methods of prognosing or predicting a subject's response to treatment with an agent capable of inhibiting the action of IL-11 or an agent capable of preventing or reducing the expression of IL-11 or IL-11R.

In some embodiments a subject may be suspected of having fibrosis, e.g. based on the presence of other symptoms indicative of fibrosis in the subject's body or in selected cells/tissues of the subject's body, or be considered at risk of developing fibrosis, e.g. because of genetic predisposition or exposure to environmental conditions, such as asbestos fibres.

Determination of upregulation of IL-11 or IL-11R may confirm a diagnosis or suspected diagnosis of fibrosis or may confirm that the subject is at risk of developing fibrosis. The determination may also diagnose the condition or predisposition as one suitable for treatment with an agent capable of inhibiting the action of IL-11 or an agent capable of preventing or reducing the expression of IL-11 or IL-11R.

As such, a method of providing a prognosis for a subject having, or suspected of having fibrosis may be provided, the method comprising determining whether IL-11 or IL-11R is upregulated in a sample obtained from the subject and, based on the determination, providing a prognosis for treatment of the subject with an agent capable of inhibiting the action of IL-11 or an agent capable of preventing or reducing the expression of IL-11 or IL-11R.

In some aspects methods of diagnosis or methods of prognosing or predicting a subject's response to treatment with an agent capable of inhibiting the action of IL-11 or an agent capable of preventing or reducing the expression of IL-11 or IL-11R may not require determination of IL-11 or IL-11R levels, but may be based on determining genetic factors in the subject that are predictive of upregulation of IL-11 or IL-11R expression, or upregulation of IL-11 or IL-11R activity. Such genetic factors may include the determination of genetic

mutations, single nucleotide polymorphisms (SNPs) or gene amplification in IL-11 and/or IL-11R that are correlated with and/or predictive of upregulation of IL-11 or IL-11R expression or activity or IL-11 mediated signaling activity. The use of genetic factors to predict predisposition to a disease state or response to treatment is known in the art, e.g. see Peter Stärkel *Gut* 2008;57:440-442; Wright et al., Mol. Cell. Biol. March 2010 vol. 30 no. 6 1411-1420.

Genetic factors may be assayed by methods known to those of ordinary skill in the art, including PCR based assays, e.g. quantitative PCR, competitive PCR. By determining the presence of genetic factors, e.g. in a sample obtained from a subject, a diagnosis of fibrosis may be confirmed, and/or a subject may be classified as being at risk of developing fibrosis, and/or a subject may be identified as being suitable for treatment with an agent capable of inhibiting the action of IL-11 or an agent capable of preventing or reducing the expression of IL-11 or IL-11R.

Some methods may comprise determination of the presence of one or more SNPs linked to secretion of IL-11 or susceptibility to development of fibrosis. SNPs are usually bi-allelic and therefore can be readily determined using one of a number of conventional assays known to those of skill in the art (e.g. see Anthony J. Brookes. The essence of SNPs. Gene Volume 234, Issue 2, 8 July 1999, 177-186; Fan et al., Highly Parallel SNP Genotyping. Cold Spring Harb Symp Quant Biol 2003. 68: 69-78; Matsuzaki et al., Parallel Genotyping of Over 10,000 SNPs using a one-primer assay on a high-density oligonucleotide array. Genome Res. 2004. 14: 414-425).

The methods may comprise determining which SNP allele is present in a sample obtained from a subject. In some embodiments determining the presence of the minor allele may be associated with increased IL-11 secretion or susceptibility to development of fibrosis.

Accordingly, in one aspect of the present invention a method for screening a subject is provided, the method comprising:

obtaining a nucleic acid sample from the subject;

determining which allele is present in the sample at the polymorphic nucleotide position of one or more of the SNPs listed in Figure 33, and/or Figure 34 and/or Figure 35 or an SNP in linkage disequilibrium with one of the listed SNPs with an $r^2 \geq 0.8$.

The determining step may comprise determining whether the minor allele is present in the sample at the selected polymorphic nucleotide position. It may comprise determining whether 0, 1 or 2 minor alleles are present.

The screening method may be, or form part of, a method for determining susceptibility of the subject to development of fibrosis, or a method of diagnosis or prognosis as described herein.

The method may further comprise the step of identifying the subject as having susceptibility to, or an increased risk of, developing fibrosis, e.g. if the subject is determined to have a minor allele at the polymorphic nucleotide position. The method may further comprise the step of selecting the subject for treatment with an agent capable of inhibiting the action of Interleukin 11 (IL-11) and/or administering an agent capable of inhibiting the action of Interleukin 11 (IL-11) to the subject in order to provide a treatment for fibrosis in the subject or to prevent development or progression of fibrosis in the subject.

SNPs that may be determined include one or more of the SNPs listed in Figure 33, Figure 34, or Figure 35. In some embodiments the method may comprise determining one or more of the SNPs listed in Figure 33. In some embodiments the method may comprise determining one or more of the SNPs listed in Figure 34. In some embodiments the method may comprise determining one or more of the SNPs listed in Figure 35. SNPs may be selected for determination as having a low P value or FDR (false discovery rate).

In some embodiments SNPs are selected as being good predictors of response to anti-IL-11 treatment based on regulation of VSTstim in *trans* (Figures 33). In some embodiments a method may comprise determining which allele is present for one or more of the following SNPs: rs10831850, rs4756936, rs6485827, rs7120273, and rs895468. In some embodiments SNPs are selected as being good predictors of response to anti-IL-11 treatment based on regulation VSTstim-VSTunstim in *cis* (Figure 34).

In some embodiments SNPs are selected as being good predictors of response to anti-IL-11 treatment based on regulation VSTstim-VSTunstim in *trans* (Figure 35). In some embodiments a method may comprise determining which allele is present for one or more of the following SNPs: rs7120273, rs10831850, rs4756936, rs6485827 (Figure 35).

SNPs: rs7120273, rs10831850, rs4756936, rs6485827 are in high linkage disequilibrium (LD) with one another on chromosome 11 (in a so-called LD block), and are therefore very commonly co-inherited.

The square of the correlation of gene frequencies (r^2) reflects the degree of linkage disequilibrium (LD) between two SNPs. As a result of LD between SNPs in local and therefore co-inherited regions of the genome, the genotype of a given SNP can be inferred by determining the genotype of a tagging/proxy SNP. The threshold of LD used in the art to identify pairwise tagging/proxy SNPs is an r^2 value of 0.8 (Wang et al. 2005, Nat. Rev. Genet. 6(2): 109-18; Barrett et al. 2006, Nat Genet., 38 (6): 659-662). The genotype of a given SNP can therefore be inferred by determining the genotype of a tagging/proxy SNP in linkage disequilibrium with an r^2 value ≥ 0.8.

The nucleotide sequence of SNPs is indicated using the "rs" number. The full sequence is available from the National Center for biotechnology Information (NCBI) database of single nucleotide polymorphisms (dbSNP) accessible at: https://www.ncbi.nlm.nih.gov/snp.

Methods of diagnosis or prognosis may be performed *in vitro* on a sample obtained from a subject, or following processing of a sample obtained from a subject. Once the sample is collected, the patient is not required to be present for the *in vitro* method of diagnosis or prognosis to be performed and therefore the method may be one which is not practised on the human or animal body.

Other diagnostic or prognostic tests may be used in conjunction with those described here to enhance the accuracy of the diagnosis or prognosis or to confirm a result obtained by using the tests described here.

Methods according to the present invention may be performed, or products may be present, *in vitro*, *ex vivo*, or *in vivo*. The term "*in vitro*" is intended to encompass experiments with materials, biological substances, cells and/or tissues in laboratory conditions or in culture whereas the term "*in vivo*" is intended to encompass experiments and procedures with intact multi-cellular organisms. "*Ex vivo*" refers to something present or taking place outside an organism, e.g. outside the human or animal body, which may be on tissue (e.g. whole organs) or cells taken from the organism.

The invention includes the combination of the aspects and preferred features described except where such a combination is clearly impermissible or expressly avoided.

The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described.

Aspects and embodiments of the present invention will now be illustrated, by way of example, with reference to the accompanying figures. Further aspects and embodiments will be apparent to those skilled in the art. All documents mentioned in this text are incorporated herein by reference.

Throughout this specification, including the claims which follow, unless the context requires otherwise, the word "comprise," and variations such as "comprises" and "comprising," will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Ranges may be expressed herein as from "about" one particular value, and/or to "about" another particular value. When such a range is expressed, another embodiment includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by the use of the antecedent "about," it will be understood that the particular value forms another embodiment.

Examples

Example 1

The fibrotic response is characterized by widespread molecular changes in activated resident fibroblasts. To establish the role of IL-11 as a key marker of this transition we assessed and ranked global RNA expression differences in atrial fibroblasts derived from 80 individuals before and 24 hours after Transforming growth factor beta-1 (TGFβ1) activation. We cultured primary fibroblasts derived from the atrium of 80 individuals who were undergoing cardiac surgery for coronary artery disease. Fibroblasts were studied *ex vivo* at baseline and following stimulation with TGFβ1 (a powerful pro-fibrotic stimulus) using genome-wide expression profiling (RNA-Seq) combined with phenotypic assays and genotyping.

IL-11 expression was significantly induced in response to TGFβ1 treatment with RNA levels increasing as much 30x (> 8x on average). IL-11 expression was higher than expression of all other individual genes (Figures 1a,b), meaning that of the ~11,500 genes expressed in fibroblasts IL-11 is the most markedly upregulated. This upregulation IL-11 was confirmed with RT-qPCR as well as ELISA experiments (Figures 1c,d), indicating increased production and release of IL-11 protein in activated fibroblasts is the main drivers of fibrosis.

To assess whether IL-11 acts as an autocrine signaling factor that drives fibrosis, we incubated non-stimulated atrial fibroblasts with recombinant IL-11 and monitored cell proliferation, myofibroblast generation as well as collagen and periostin expression at the protein level. We observed an increase in collagen production, cell proliferation and periostin expression at levels similar to those induced by the TGFβ1 signaling pathway. IL-11 activated fibroblasts also differentiated into α-SMA+ myofibroblasts (Figure 2).

In addition to its pro-fibrotic function, IL-11 was also found to play a critical role in the TGFβ1 induced fibrotic response itself. Inhibition of IL-11 with a neutralising anti- human IL-11 monoclonal antibody (Monoclonal Mouse IgG_{2A}; Clone #22626; Catalog No. MAB218; R&D Systems, MN, USA) reduced the activation of fibroblasts through TGFβ1. Cells incubated with TGFβ1 did not generate more extracellular matrix proteins when the IL-11 antibody was present (Figure 3).

We showed that IL-11 neutralizing antibodies prevent TGFβ1-induced fibroblast activation.

Example 2

Inflammation and tissue damage stimulates a dynamic process that involves the recruitment, proliferation and activation of fibroblasts to generate extracellular matrix and initiate wound healing and scarring. This fibrotic response is characterized by widespread molecular changes in activated resident fibroblasts that can be induced by TGFβ1, a multifunctional cytokine that is released by local and infiltrating cells.

To identify key markers of this transition we assessed and ranked global RNA expression differences via transcriptome sequencing in atrial fibroblasts derived from 80 individuals before and 24 hours after TGFβ1 treatment. As discussed in Example 1, IL-11 expression was significantly upregulated in activated fibroblasts and we showed for the first time that the IL-11 transcriptional response is higher than the transcriptional response of all other individual genes regulated in fibrosis (Figure 4a). Comparison of the IL-11 expression level in our model system to various human tissues indicated that high IL-11 levels were also very specific for the fibrotic response (Figure 4b), making it an ideal marker to assess the extent of fibrosis in the human body.

To further assess whether IL-11 acts as an autocrine signaling factor that drives fibrosis, we confirmed that an upregulation of IL-11 RNA (Figure 5a) lead to an increase in IL-11 secretion

(Figure 5b) from atrial fibroblasts. Incubation of fibroblasts with IL-11 did not increase IL-11 RNA expression (Figure 5c), but lead to an increase in IL-11 secretion from the cells (Figure 5d). This shows that IL-11 is having an autocrine effect on fibroblasts that regulates the production of IL-11 protein at the translational level.

We then incubated atrial fibroblasts with TGFβ1, recombinant IL-11 or TGFβ1 and a neutralising anti- human IL-11 monoclonal antibody (Monoclonal Mouse IgG_{2A}; Clone #22626; Catalog No. MAB218; R&D Systems, MN, USA) and monitored cell proliferation, myofibroblast generation as well as periostin expression at the protein level. We observed an increase in activated fibroblasts (αSMA-positive cells), periostin production and cell proliferation at a similar level for both TGFβ1 and IL-11 stimulated fibroblasts. In addition to its pro-fibrotic function, IL-11 was also found to play a critical role in the TGFβ1 fibrosis itself. The pro-fibrotic effect of TGFβ1 was inhibited when we neutralized IL-11 with the antibody (Figures 6a-c). The same pattern was observed when we monitored the secretion of fibrosis markers such as IL6, MMP2 and TIMP1 (Figures 6d-f).

We then monitored the deposition of collagen, the pathognomonic hallmark of the fibrotic response, using a number of assays across several regulatory levels of gene expression. TGFβ1 was found to increase intracellular collagen (Figure 7a), secreted collagen (Figure 7b) as well as collagen RNA levels (Figure 7c) as expected. The response to IL-11 was only observed at the protein level (Figure 7a,b) and not on the RNA level (Figure 7c). Stimulation with TGFβ1 in parallel to inhibiting IL-11 led to an increase in collagen RNA but this TGFβ1-driven effect was not forwarded to the protein level.

To establish further the central role of IL-11 in fibrosis downstream of multiple pro-fibrotic stimuli, we assessed IL-11 expression across fibroblast populations derived from four different tissues in response to TGFβ1 (Figure 8a), ET-1, (Figure 8b) and PDGF (Figure 8c). We also administered recombinant IL-11 systemically to C57BL/6 mice and monitored collagen and αSMA expression. Collagen production was increased across kidney, heart and liver (Figure 8d) and we also detected more activated fibroblasts in the heart and kidney, indicated by higher αSMA protein levels (Figure 8e).

Our findings demonstrate a novel and central role for IL-11 in fibrosis and, most importantly, show that IL-11 is downstream of the key pro-fibrotic stimuli across several tissues. These results show that IL-11 is required for TGFβ1 to proceed from transcriptional regulation to

protein translation. Inhibition of IL-11 stalls the pro-fibrotic effect of TGFβ1 on the transcriptome (Figure 9).

Example 3: Anti-IL-11 antibodies inhibit pro-fibrotic stimuli

In experiments similar to those described in respect of Figure 3c, atrial fibroblasts were exposed to other pro-fibrotic stimuli in the form of angiotensin II (ANG 2), platelet-derived growth factor (PDGF) and endothelin 1 (ET-1), and collagen production was measured.

In addition to induction of IL-11 mRNA expression, each of ANG2, PDGF and ET-1 induced IL-11 protein expression. Inhibition of IL-11 with a neutralising anti- human IL-11 monoclonal antibody (Monoclonal Mouse IgG_{2A}; Clone #22626; Catalog No. MAB218; R&D Systems, MN, USA) blocked the pro-fibrotic effect of each of these pro-fibrotic stimuli (Figure 10) indicating IL-11 to be the central effector of the major pro-fibrotic stimuli (TGFβ1, ANG2, PDGF and ET-1).

Example 4: IL-11R knockdown

HEK cells were transfected (24h) with non-targeting (NT) siRNA or one of four different siRNAs against the IL11RA1 receptor (siRNAs 5-8; Figure 14; SEQ ID NOs 15 to 18). RNA was extracted and assayed for IL11RA1 mRNA expression by qPCR. Data are shown in Figure 15 as mRNA expression levels relative to the control (NT).

Example 5: A role for IL-11 in fibrosis

5.1 IL-11 is upregulated in fibrosis

To understand the molecular processes underlying the transition of fibroblasts to activated myofibroblasts, atrial tissue was obtained from more than 200 patients that underwent cardiac bypass surgery at the National Heart Centre Singapore. Cells were cultured *in vitro* at low passage (passage <4), and either not stimulated or stimulated with TGFβ1 for 24h. We subsequently performed high-throughput RNA sequencing (RNA-seq) analysis of unstimulated fibroblasts and cells stimulated with the prototypic pro-fibrotic stimulus TGFβ1 across 160 individuals; average read depth was ~70M reads per sample (paired-end 100bp; Figure 16).

To ensure the purity of the atrial fibroblast cell cultures, we analysed expression of endothelial cell, cardiomyocyte and fibroblast cell type marker genes from the atrium (Hsu et al., 2012 Circulation Cardiovasc Genetics 5, 327–335) in the RNA-seq dataset.

The results are shown in Figures 17A to 17E, and confirm the purity of the atrial fibroblast cultures.

Gene expression was assessed by RNA-seq of the tissue of origin (human atrial tissues samples, n=8) and primary, unstimulated fibroblast cultures. No/very low expression of the endothelial cell marker PECAM1 (Figure 17A), and the cardiomyocyte markers MYH6 (Figure 17B) and TNNT2 (Figure 17C) was detected in the fibroblast cell culture samples. Markers for fibroblasts COL1A2 (Figure 17D) and ACTA2 (Figure 17E) were highly expressed compared to the tissue of origin.

Next, the RNA-seq data was analysed to identify genes whose expression was increased or decreased upon stimulation with TGFβ1, and this information was integrated with the large RNA-seq dataset across 35+ human tissues provided by the GTEx project (The GTEx Consortium, 2015 Science 348, 648–660). This enabled the identification of gene expression signatures that were specific to the fibroblast-myofibroblast transition.

The results are shown in Figures 18A to 18E. Across the 10000+ genes expressed in the fibroblasts, IL-11 was the most strongly upregulated gene in response to stimulation with TGFβ1, and on average across the 160 individuals was upregulated more than 10-fold (Figure 18A).

Upregulation of IL-11 expression was confirmed by ELISA analysis of the cell culture supernatant of TGFβ1 stimulated fibroblasts (Figure 18C). As compared to the level of expression level of IL-11 in other tissues of healthy individuals, this response was observed to be highly specific to activated fibroblasts (Figure 18D). Various fold changes of IL-11 RNA expression were also confirmed by qPCR analysis (Figure 18E).

Next, fibroblasts were cultured *in vitro* and stimulated with several other known pro-fibrotic factors: ET-1, ANGII, PDGF, OSM and IL-13, and also with human recombinant IL-11. For analysing upregulation of IL-11 produced in response to stimulation with IL-11, it was confirmed that the ELISA was only able to detect native IL-11 secreted from cells and does not detect recombinant IL-11 used for the stimulations (Figure 19B).

The results are shown in Figure 19A. Each factor was found to significantly induce IL-11 secretion from fibroblasts. IL-11 is shown to act in an autocrine loop in fibroblasts, which can result in an upregulation of IL-11 protein as much as 100-fold after 72 hours (Figure 19D).

Interestingly, this autocrine loop for IL-11 is similar to the autocrine production of IL-6. IL-6 is from the same cytokine family and also signals via the gp130 receptor (Garbers and Scheller, 2013 Biol Chem 394, 1145–1161), which is proposed to ensure the continued survival and growth of lung and breast cancer cells (Grivennikov and Karin, 2008 Cancer Cell 13, 7–9).

5

No increase in IL-11 RNA level was detected in response to stimulation with IL-11 (Figure 19D). Unlike TGFβ1, which increases IL-11 expression at both the RNA and protein level, therefore IL-11 seems to upregulate IL-11 expression only at the post-transcriptional level.

5.2 IL-11 has a profibrotic role in fibrosis of heart tissue

To explore whether the autocrine production of IL-11 is pro- or anti-fibrotic, fibroblasts were cultured *in vitro* with recombinant IL-11, and the fraction of myofibroblasts (αSMA-positive cells) and extracellular matrix production was analysed.

The expression of αSMA, collagen and periostin was monitored with the Operetta High-Content Imaging System in an automated, high-throughput fashion. In parallel, secretion of fibrosis marker proteins such as MMP2, TIMP1 and IL-6 was analysed by ELISA assays, and the levels of collagen were confirmed by calorimetric Sirius Red analysis of the cell culture supernatant.

Briefly, atrial fibroblasts derived from 3 individuals were incubated in 2 wells each for 24h without stimulation, with TGFβ1 (5 ng/ml), or with IL-11 (5 ng/ml). Following incubation, cells were stained to analyse α-SMA content to estimate the fraction of myofibroblasts, and for collagen and periostin to estimate ECM production. Fluorescence was measured in 7 fields per well. The supernatant of 2 wells per individual was also assessed for collagen content by Sirius Red staining. The signal was normalized to the control group without stimulation. Secretion of the fibrosis markers IL-6, TIMP1 and MMP2 was analysed via ELISA.

The results are shown in Figures 20A to 20F. TGFβ1 activated fibroblasts and increased ECM production (Figure 20A). Unexpectedly, and in contrast with the anti-fibrotic role described for IL-11 in heart tissue in the scientific literature, recombinant IL-11 caused an increase in the fraction of myofibroblasts in fibroblast cultures, and also promoted the production of extracellular matrix proteins collagen and periostin to the same extent as TGFβ1 (Figure 20A). Both of IL-11 and TGFβ1 cytokines also significantly increased the secretion of pro-fibrotic markers IL-6, TIMP1 and MMP2 (Figures 20B to 20E), and to a similar level.

The inventors hypothesized that the contradiction between the present finding that IL-11 is profibrotic in heart tissue and the antifibrotic role described in the literature might be related to the use of human IL-11 in rodents in those previous studies (Obana et al., 2010, 2012; Stangou et al., 2011; Trepicchio and Dorner, 1998).

To investigate this hypothesis, serial dilutions of both human and mouse IL-11 were performed, and the activation of human atrial fibroblasts was monitored (Figure 20F). No activation of fibroblasts was observed at low concentrations of human IL-11 on mouse cells,

suggesting that previous insights into IL-11 function may in part be due to IL-11-non-specific observations.

5.3 IL-11 has a profibrotic role in fibrosis of a variety of tissues

To test whether the profibrotic action of IL-11 was specific to atrial fibroblasts, human fibroblasts derived from several different tissues (heart, lung, skin, kidney and liver) were cultured *in vitro*, stimulated with human IL-11, and fibroblast activation and ECM production was analysed as described above. Increased fibroblast activation and production of ECM was observed as compared to non-stimulated cultures in fibroblasts derived from each of the tissues analysed.

5.3.1 Liver fibrosis

To test whether IL-11 signalling is important in liver fibrosis, human primary liver fibroblasts (Cell Biologics, Cat#: H-6019) were cultured at low passage in wells of 96-well plates and either not stimulated, stimulated with TGFβ1 (5ng/ml, 24h), IL-11 (5 ng/ml, 24h) or incubated with both TGFβ1 (5 ng/ml) and a neutralising IL-11 antibody (2 µg/ml), or TGFβ1 (5 ng/ml) and an Isotype control antibody. Fibroblast activation (αSMA positive cells), cell proliferation (EdU positive cells) and ECM production (periostin and collagen) was analysed using the Operetta platform.

The results of the experiments with primary human liver fibroblasts are shown in Figures 38A to 38D. IL-11 was found to activate liver fibroblasts, and IL-11 signalling was found to be necessary for the profibrotic action of TGFβ1 in liver fibroblasts. Both activation and proliferation of fibroblasts was inhibited by neutralising anti-IL-11 antibody.

5.3.2 Skin fibrosis

To test whether IL-11 signalling is important in skin fibrosis, primary mouse skin fibroblasts were cultured at low passage in wells of 96-well plates and either not stimulated, stimulated with TGFβ1 (5ng/ml, 24h) or incubated for 24h with both TGFβ1 (5 ng/ml) and a neutralising IL-11 antibody (2 µg/ml). Fibroblast activation (αSMA positive cells) was then analysed using the Operetta platform.

The results are shown in Figure 39. TGFβ1-mediated activation of skin fibroblasts was inhibited by neutralising anti-IL-11 antibody.

5.3.3 Fibrosis in multiple organs

Next, mouse recombinant IL-11 was injected (100µg/kg, 3 days/week, 28 days) into mice to test whether IL-11 can drive global tissue fibrosis *in vivo*.

The results are shown in Figure 21. Compared to injection of AngII (a cytokine that causes an elevation in blood pressure and hypertrophy of the heart), IL-11 also increased the heart weight but also kidney, lung and liver weight indexed to body weight (Figure 21B). Assessing collagen content in these issues by hydroxyproline assay revealed an upregulation of collagen production in these tissues, indicating fibrosis as the likely cause for the increase in organ weight (Figure 6C). Expression of fibrosis marker genes ACTA2 (= αSMA), Col1a1, Col3a1, Fn1, Mmp2 and Timp1 was also detected by qPCR analysis of RNA isolated from heart, kidney, lung and liver tissues of these animals.

Example 6: Therapeutic potential of IL-11/IL-11R antagonism

6.1 Inhibition of the fibrotic response using neutralising antagonists of IL-11/IL-11R

Next it was investigated whether the autocrine loop of IL-11 secretion was required for the pro-fibrotic effect of TGFβ1 on fibroblasts.

IL-11 was inhibited using a commercially available neutralizing antibody (Monoclonal Mouse IgG2A; Clone #22626; Catalog No. MAB218; R&D Systems, MN, USA). Fibroblasts were treated with TGFβ1 in the presence or absence of the antibody, and fibroblast activation, the proportion of proliferating cells and ECM production and markers of the fibrotic response were measured.

Briefly, atrial fibroblasts derived from 3 individuals were incubated for 24h with TGFβ1 (5 ng/ml) or TGFβ1 in the presence of neutralising anti-IL-11 antibody or isotype control antibody. Following incubation, cells were stained for αSMA to determine the fraction of myofibroblasts, the proportion of proliferating cells was determined by analysing the cells for EdU incorporation, and periostin was measured to determine ECM production. Fluorescence was measured with the Operetta platform for 14 fields across 2 wells for each individual. Secretion of the fibrosis markers IL-6, TIMP1 and MMP2 was also analysed by ELISA. Fluorescence was normalized to the control group without stimulation.

The results are shown in Figures 22A to 22F. IL-11 inhibition was found to ameliorate TGFβ1-induced fibrosis, and it was shown that IL-11 is essential for the pro-fibrotic effect of TGFβ1. Inhibition of IL-11 was found to 'rescue' the TGFβ1 phenotype at the protein level.

Collagen production was also analysed. Cardiac fibroblasts derived from 3 individuals were incubated for 24h with TGFβ1 (5 ng/ml) or TGFβ1 and a neutralizing IL-11 antibody. Following incubation the cells were stained for collagen using the Operetta assay and florescence was quantified as described above. Secreted collagen levels in the cell culture supernatant were assessed by Sirius Red staining.

The results are shown in Figures 23A and 23B, and confirm the anti-fibrotic effect of inhibition of IL-11 using a neutralising antibody.

Next, the ability of several other IL-11/IL-11R antagonists to inhibit fibrosis was analysed *in vitro* using the atrial fibroblast, TGFβ1-induced myofibroblast transition assay described herein above.

Briefly, human atrial fibroblasts cells were cultured *in vitro*, stimulated for 24h with TGFβ1 (5 ng/ml) or left unstimulated, in the presence/absence of: (i) neutralising anti-IL-11 antibody, (ii) a IL-11RA-gp130 fusion protein (iii) neutralising anti-IL-11RA antibody, (iv) treatment with siRNA directed against IL-11 or (v) treatment with siRNA directed against IL-11RA. The proportion of activated fibroblasts (myofibroblasts) was analysed by evaluating αSMA content as described above.

The results are shown in Figure 24. Each of the antagonists of IL-11/IL-11R signalling was found to be able to abrogate TGFβ1-mediated profibrotic response.

Example 7: *In vivo* confirmation of a profibrotic role for IL-11/IL-11R signalling

7.1 *In vitro* studies using cells derived from IL-11RA gene knock-out mice

All mice were bred and housed in the same room and provided food and water ad libitum. Mice lacking functional alleles for IL-11Rα (IL-11RA1 KO mice) were on C57Bl/6 genetic background. Mice were of 9-11 weeks of age and the weight of animals did not differ significantly.

To further confirm the anti-fibrotic effect of inhibition of IL-11/IL-11R signalling, primary fibroblasts were generated from IL-11RA gene knock-out mice and incubated with primary fibroblast cells harvested from IL-11RA+/+ (i.e. wildtype), IL-11RA+/- (i.e. heterozygous knockout) and IL-11RA-/- (i.e. homozygous knockout) animals with TGFβ1, IL-11 or AngII. Activation and proliferation of fibroblasts and ECM production was analysed.

Fibroblasts derived from IL-11RA+/+, IL-11RA+/- and IL-11RA-/- mice were incubated for 24 hours with TGFβ1, IL-11 or AngII (5 ng/ml). Following incubation, cells were stained for αSMA content to estimate the fraction of myofibroblasts, for EdU to identify the fraction of proliferating cells, and for collagen and periostin to estimate ECM production. Fluorescence was measured using the Operetta platform.

The results are shown in Figures 25A to 25D. IL-11RA-/- mice were found not to respond to pro-fibrotic stimuli. These results suggested that IL-11 signalling is also required for AngII-induced fibrosis.

Next, it was investigated whether this was also true for other pro-fibrotic cytokines. Briefly, fibroblasts were cultured *in vitro* in the presence/absence of various different pro-fibrotic factors (ANG2, ET-1 or PDGF), and in the presence/absence of neutralising anti-IL-11 antibody or pan anti-TGFβ antibody. After 24 hours, collagen production by the cells was determined by analysis using the Operetta system as described above, and myofibroblast generation was determined by analysis of αSMA expression as described above.

The results are shown in Figures 26A and 26B. IL-11 was found to be required for fibrosis downstream of various profibrotic stimuli, and was thus identified as a central mediator of fibrosis induced by a variety of different profibrotic factors.

In a further experiment, the role of IL-11 signalling was investigated in lung fibrosis, using an *in vitro* scratch assay of migration of lung fibroblasts. In response to pro-fibrotic stimuli, fibroblasts are activated and migrate within the fibrotic niche in the body. The migration rate of cells is a measure of cell-cell and cell-matrix interactions and a model for wound healing *in vivo* (Liang et al., 2007; Nat Protoc. 2(2):329-33).

Fibroblasts derived from lung tissue from both wild type (WT) and also homozygous IL-11RA (-/-) knockout mice were grown at low passage on a plastic surface until they formed a uniform cell monolayer. A scratch was then created in the cell layer, and cell migration close

to the scratch was monitored, either in the absence of stimulation, or in the presence of TGFβ1 or IL-11. Images captured at images at the two time points of immediately after creating the scratch and at 24h were used to determine the area covered by cells, and the rate of migration was compared between WT and KO fibroblasts. Cell migration (area in the scratch covered by cells after 24h) was normalized to the migration rate of WT cells without stimulus.

The results are shown in Figure 40. Lung fibroblasts derived from WT mice were shown to migrate faster in the presence of TGFβ1 and IL-11, indicating a pro-fibrotic effect of both cytokines in lung fibroblasts. Cells lacking IL-11 signalling derived from KO mice migrated more slowly as compared to WT cells. They also did not migrate faster in the presence of TGFβ1. The scratch assay revealed that lung fibroblasts lacking IL-11 signalling have a decrease cell migration rate both in the presence of TGFβ1 or IL-11, and at baseline. Thus, inhibition of IL-11 signalling is anti-fibrotic in the lung.

7.2 Heart fibrosis

The efficacy of IL-11 inhibition to treat fibrotic disorders was investigated *in vivo*. A mouse model for cardiac fibrosis, in which fibrosis is induced by treatment with AngII, was used to investigate whether IL-11RA -/- mice were protected from cardiac fibrosis.

Briefly, a pump was implanted, and wildtype (WT) IL-11RA(+/+) and knockout (KO) IL-11RA(-/-) mice were treated with AngII (2mg/kg/day) for 28 days. At the end of the experiment, collagen content was assessed in the atria of the mice using a calorimetric hydroxyproline-based assay kit, and the level of RNA expression of the markers or fibrosis Col1A2, αSMA (ACTA2) and fibronectin (Fn1) were analysed by qPCR.

The results are shown in Figures 27A to 27D. The IL-11RA-/- mice were found to be protected from the profibrotic effects of AngII.

7.3 Kidney fibrosis

A mouse model for kidney fibrosis was established in wildtype (WT) IL-11RA(+/+) and knockout (KO) IL-11RA(-/-) mice by intraperitoneal injection of folic acid (180mg/kg) in vehicle (0.3M $NaHCO_3$); control mice were administered vehicle alone. Kidneys were removed 28 days post-injection, weighed and either fixed in 10% neutral-buffered formalin for Masson's trichrome and Sirius staining or snap-frozen for collagen assay, RNA, and protein studies.

Total RNA was extracted from the snap-frozen kidney using Trizol reagent (Invitrogen) and Qiagen TissueLyzer method followed by RNeasy column (Qiagen) purification. The cDNA was prepared using iScriptTM cDNA synthesis kit, in which each reaction contained 1µg of total RNA, as per the manufacturer's instructions. Quantitative RT-PCR gene expression analysis was performed on triplicate samples with either TaqMan (Applied Biosystems) or fast SYBR green (Qiagen) technology using StepOnePlusTM (Applied Biosystem) over 40 cycles. Expression data were normalized to GAPDH mRNA expression level and we used the $2^{-\Delta\Delta Ct}$ method to calculate the fold-change. The snap-frozen kidneys were subjected to acid hydrolysis by heating in 6M HCl at a concentration of 50 mg/ml (95°C, 20 hours). The amount of total collagen in the hydrolysate was quantified based on the colorimetric detection of hydroxyproline using Quickzyme Total Collagen assay kit (Quickzyme Biosciences) as per the manufacturer's instructions.

The results of the analysis are shown in Figure 28. Folate-induced kidney fibrosis is shown to be dependent on IL-11 mediated signalling. A significant increase in collagen content in kidney tissue was observed in IL-11RA+/+ mice, indicative of kidney fibrosis. No significant increase in collagen content was observed in IL-11RA -/- mice. Animals deficient for IL-11 signalling had significantly less collagen deposition in kidneys after toxic injury as compared to wild type animals.

7.4 Lung fibrosis

IL-11 is confirmed as a key mediator of fibrosis in the lung, skin and eye in further *in vivo* models using the IL-11RA -/- knockout mice. Schematics of the experiments are shown in Figures 29A to 29C.

To analyse pulmonary fibrosis, IL-11RA -/- mice and IL-11RA +/+ mice are treated by intratracheal administration of bleomycin on day 0 to establish a fibrotic response in the lung (pulmonary fibrosis). Fibrosis of the lung develops by 21 days, at which point animals are sacrificed and analysed for differences in fibrosis markers between animals with and without IL-11 signalling. IL-11RA -/- mice have a reduced fibrotic response in lung tissue as compared to IL-11RA +/+ mice, as evidenced by reduced expression of markers of fibrosis.

7.5 Skin fibrosis

To analyse fibrosis of the skin, IL-11RA -/- mice and IL-11RA +/+ mice are treated by subcutaneous administration of bleomycin on day 0 to establish a fibrotic response in the skin. Fibrosis of the skin develops by 28 days, at which point animals are sacrificed and analysed for differences in fibrosis markers between animals with and without IL-11 signalling. IL-11RA

-/- mice have a reduced fibrotic response in skin tissue as compared to IL-11RA +/+ mice, as evidenced by reduced expression of markers of fibrosis.

7.6 Eye fibrosis

To analyse fibrosis in the eye, IL-11RA -/- mice and IL-11RA +/+ mice undergo trabeculectomy on day 0 to initiate a wound healing response in the eye. Fibrosis of the eye develops within 7 days. The fibrotic response is measured and compared between the IL-11RA -/- mice and IL-11RA +/+ mice. IL-11RA -/- mice have a reduced fibrotic response in eye tissue as compared to IL-11RA +/+ mice, as evidenced by reduced expression of markers of fibrosis.

7.7 Other tissues

The effect of IL-11RA knockout on fibrosis is also analysed in mouse models of fibrosis for other tissues, such as the liver, bowel, and is also analysed in a model relevant to multiorgan (i.e. systemic) fibrosis. The fibrotic response is measured and compared between the IL-11RA -/- mice and IL-11RA +/+ mice. IL-11RA -/- mice have a reduced fibrotic response as compared to IL-11RA +/+ mice, as evidenced by reduced expression of markers of fibrosis.

Example 8: Analysis of the molecular mechanisms underlying IL-11-mediated induction of fibrosis

The canonical mode of action of IL-11 is thought to be regulation of RNA expression via STAT3-mediated transcription (Zhu et al., 2015 PLoS ONE 10, e0126296), and also through activation of ERK.

STAT3 activation is observed following stimulation with IL-11. However, when fibroblasts are incubated with TGFβ1, only activation of the canonical SMAD pathway and ERK pathways is seen, and activation of STAT3 is not observed, even in spite of the fact that IL-11 is secreted in response to TGFβ1. Only ERK activation is common to both TGFβ1 and IL-11 signal transduction.

Cross-talk between TGFβ1 and IL-6 signalling has previously been described, wherein TGFβ1 blocks the activation of STAT3 by IL-6 (Walia et al., 2003 FASEB J. 17, 2130–2132). Given the close relationship between IL-6 and IL-11, similar cross-talk may be observed for IL-11 mediated signalling.

The inventors investigated by RNA-seq analysis whether regulation of RNA abundance was the underlying mechanism for the increased expression of fibrosis marker proteins in response to IL-11, which would suggest STAT3 as the underlying signalling pathway for IL-11 mediated profibrotic processes. Fibroblasts were incubated for 24 hours either without stimulus, or in the presence of TGFβ1, IL-11 or TGFβ1 and IL-11.

The results are shown in Figure 30A. TGFβ1 induced the expression of collagen, ACTA2 (αSMA) and other fibrosis marker at the RNA level. However, IL-11 did not regulate the expression of these genes, but a different set of genes.

Gene ontology analysis suggests that a pro-fibrotic effect in fibroblasts is driven by IL-11-regulated RNA expression. Both TGFβ1 and IL-11 regulate an almost completely different set of genes on the RNA level.

Whilst TGFβ1 increases IL-11 secretion, the target genes of IL-11 are not regulated when both TGFβ1 and IL-11 are present. This suggests that TGFβ1 upregulates IL-11 and simultaneously blocks the canonical IL-11-driven regulation of RNA expression via STAT3, similar to what is known about the interaction of TGFβ1 and IL-6 pathways (Walia et al., 2003 FASEB J. 17, 2130–2132).

We also analysed whether RNA expression differences induced by TGFβ1 are dependent on IL-11 signalling, by analysing changes in RNA expression in fibroblasts obtained from IL-11RA -/- mice as compared to IL-11RA +/+ mice. RNA expression regulated by TGFβ1 is still observed when IL-11RA knockout cells were stimulated with TGFβ1, and RNA levels of αSMA, collagen etc. were still upregulated in the absence of IL-11 signalling (in IL-11RA -/-

fibroblasts). When the pro-fibrotic effect of IL-11 and the anti-fibrotic effect of IL-11 inhibition was investigated *in vitro*, reduced expression of markers of fibrosis was only observed at the protein level, not at the transcriptional level as determined by qPCR.

The activation of non-canonical pathways (e.g. ERK signal transduction) is known to be crucial for the pro-fibrotic action of TGFβ1 (Guo and Wang, 2008 Cell Res 19, 71–88). It is likely that non-canonical pathways are likely to be important for signalling for all known pro-fibrotic cytokines, and that IL-11 is a post-transcriptional regulator which is essential for fibrosis.

Example 9: Human anti-human IL-11 antibodies

Fully human anti-human IL-11 antibodies were developed via phage display.

Recombinant human IL-11 (Cat. No. Z03108-1) and recombinant murine IL-11 (Cat. No. Z03052-1) were obtained from GenScript (NJ, USA). Recombinant human IL-11 was expressed in CHO cells, both as an Fc-tagged version and a tag-free version. Tag-free murine IL-11 was expressed in HEK293 cells.

IL-11 bioactivity of recombinant human IL-11 and mouse IL-11 was confirmed by *in vitro* analysis using primary fibroblast cell cultures.

Recombinant, biotinylated human IL-11 and murine IL-11 were also prepared by biotinylation of the recombinant human IL-11 and murine IL-11 molecules, according to standard methods.

Antibodies capable of binding to both human IL-11 and murine IL-11 (i.e. cross-reactive antibodies) were identified by phage display using a human naïve library by panning using biotinylated and non-biotinylated recombinant human and murine IL-11, based on 16 different panning strategies.

The phage display identified 175 scFv binders, as 'first hits'. Sequence analysis of the CDR sequences from these 175 scFv identified 86 unique scFv.

The soluble scFv were produced by recombinant expression in E. coli, and analysed for their ability to bind to human IL-11 and murine IL-11 by ELISA. Briefly, the respective antigen was coated to wells of an ELISA plate, the cell culture supernatant containing the respective scFv was added at a 1:2 dilution, and binding was detected.

The results of the ELISA analysis revealed:

- 8 scFV capable of binding only to human IL-11;
- 6 scFv capable of binding to murine IL-11 only;
- 32 scFv displaying only weak binding to human/murine IL-11, with a high signal to noise ratio, and;
- 40 scFv having cross-reactivity for both human IL-11 and murine IL-11.

From these 86 scFV, 56 candidates were selected for further functional characterisation. For further analyses, the scFV were cloned into scFV-Fc format in E. coli.

The VH and VL sequences of the antibodies were cloned into expression vectors for the generation of scFv-Fc (human IgG1) antibodies. The vectors were transiently expressed in mammalian cells cultured in serum-free media, and isolated by protein A purification.

Example 10: Functional characterisation of human anti-human IL-11 antibodies

The antibodies described in Example 9 were analysed in *in vitro* assays for their ability to (i) inhibit human IL-11-mediated signalling, and (ii) inhibit mouse IL-11-mediated signalling. The affinity of the antibodies for human IL-11 was also analysed by ELISA.

10.1 Ability to inhibit human IL-11 mediated signalling

To investigate ability to neutralise human IL-11-mediated signalling, cardiac atrial human fibroblasts were cultured in wells of 96-well plates in the presence of TGFβ1 (5 ng/ml) for 24 hours, in the presence or absence of the anti-IL-11 antibodies. TGFβ1 promotes the expression of IL-11, which in turn drives the transistion of quiescent fibroblasts to activated, αSMA-positive fibroblasts. It has previously been shown that neutralising IL-11 prevents TGFβ1-induced transition to activated, αSMA-positive fibroblasts.

Expression of αSMA was analysed with the Operetta High-Content Imaging System in an automated high-throughput fashion.

In non-stimulated cultures, ~29.7% (= 1) of the fibroblasts were αSMA-positive, activated fibroblasts at the end of the 24 hour culture period, whilst ~52% (= 1.81) of fibroblasts were αSMA-positive in cultures that were stimulated with TGFβ1 in the absence of anti-IL-11 antibodies.

Anti-IL-11 antibodies (2 µg/ml) were added to fibroblast cultures that were stimulated with TGFβ1, and at the end of the 24 hour culture period, the percentage of αSMA-positive

fibroblasts was determined. The percentages were normalised based on the percentage of αSMA-positive fibroblasts observed in cultures of fibroblasts which had not been stimulated with TGFβ1.

28 of the antibodies were demonstrated to be capable of neutralising signalling mediated by human IL-11.

A commercial monoclonal mouse anti-IL-11 antibody (Monoclonal Mouse IgG2A; Clone #22626; Catalog No. MAB218; R&D Systems, MN, USA) was also analysed for ability to inhibit signalling by human IL-11 in the experiments. This antibody was found to be able to reduce the percentage of activated fibroblasts to 28.3% (=0.99).

Several of the clones neutralised signalling by human IL-11 to a greater extent than the commercially available mouse anti-IL-11 antibody (industry standard.

10.2 Ability to inhibit mouse IL-11 mediated signalling

The ability of the human antibodies to inhibit mouse IL-11-mediated signalling was also investigated, following the same procedure as described in section 10.1 above, but using mouse dermal fibroblasts instead of human atrial fibroblasts.

After 24 hours in culture, about 31.8% (=1) of non-stimulated cells in culture were activated fibroblasts. Stimulation with TGFβ1 resulted in a ~2-fold increase in the percentage of activated fibroblasts (68.8% = 2.16) as compared to non-stimulated cultures.

The antibodies were demonstrated to be capable of neutralising signalling mediated by mouse IL-11. Monoclonal Mouse IgG2A clone #22626, catalog No. MAB218 anti-IL-11 antibody was also analysed for ability to inhibit signalling by mouse IL-11. This antibody was found to be able to reduce the percentage of activated fibroblasts to 39.4% (=1.24).

Several of the clones neutralised signalling by mouse IL-11 to a greater extent than the commercially available mouse anti-IL-11 antibody (industry standard).

10.3 Analysis of antibody affinity for human IL-11

The human anti-human IL-11 antibodies were analysed for their affinity of binding to human IL-11 by ELISA assay.

Recombinant human IL-11 was obtained from Genscript and Horseradish peroxidase (HRP)-conjugated anti-human IgG (Fc-specific) antibody was obtained from Sigma. Corning 96-well ELISA plates were obtained from Sigma. Pierce 3,3´,5,5´-tetramethylbenzidine (TMB) ELISA substrate kit was obtained from Life Technologies (0.4 g/mL TMB solution, 0.02 % hydrogen peroxide in citric acid buffer). Bovine serum albumin and sulphuric acid was obtained from Sigma. Wash buffer comprised 0.05% Tween-20 in phosphate buffered saline (PBS-T). ScFv-Fc antibodies were generated as described in above. Purified mouse and human IgG controls were purchased from Life Technologies. Tecan Infinite 200 PRO NanoQuant was used to measure absorbance.

Criss-cross serial dilution analysis was performed as described by Hornbeck et al., (2015) Curr Protoc Immunol 110, 2.1.1-23) to determine the optimal concentration of coating antigen, primary and secondary antibodies.

An indirect ELISA was performed to assess the binding affinity of primary ScFv-Fc antibodies at 50% of effective concentration (EC_{50}) as previously described (Unverdorben et al., (2016) MAbs 8, 120–128.). ELISA plates were coated with 1 µg/mL of recombinant human IL-11 overnight at 4℃ and remaining binding sites were blocked with 2 % BSA in PBS. ScFv-Fc antibodies were diluted in 1% BSA in PBS, titrated to obtain working concentrations of 800, 200, 50, 12.5, 3.125, 0.78, 0.195, and 0.049 ng/mL, and incubated in duplicates for 2 hours at room temperature. Detection of antigen-antibody binding was performed with 15.625 ng/mL of HRP-conjugated anti-human IgG (Fc-specific) antibody. Following 2 hours of incubation with the detection antibody, 100 µl of TMB substrate was added for 15 mins and chromogenic reaction stopped with 100 µl of 2 M H_2SO_4. Absorbance reading was measured at 450 nm with reference wavelength correction at 570 nm. Data were fitted with GraphPad Prism software with log transformation of antibody concentrations followed by non-linear regression analysis with the asymmetrical (five-parameter) logistic dose-response curve to determine individual EC50 values.

The same materials and procedures as described above were performed to determine the affinity of binding for the murine monoclonal anti-IL-11 antibodies, with the exception that HRP-conjugated anti-mouse IgG (H&L) was used instead of HRP-conjugated anti-human IgG.

The same materials and procedures as described above were performed to determine the affinity of binding for the human monoclonal anti-IL-11 antibodies and murine monoclonal anti-IL-11 antibodies to recombinant murine IL-11 obtained from Genscript.

The results of the ELISA assays were used to determine EC_{50} values for the antibodies.

10.4 Ability to inhibit human IL-11 mediated signalling in a variety of tissues

Ability of the antibodies to neutralise IL-11-mediated signalling in fibroblasts obtained from a variety of different tissues is investigated, essentially as described in section 10.1 except that instead of cardiac atrial human fibroblasts, human fibroblasts derived from liver, lung, kidney, eye, skin, pancreas, spleen, bowel, brain, and bone marrow are used for the experiments.

Anti-IL-11 antibodies are demonstrated to be capable of neutralising signalling in fibroblasts derived from the various different tissues, as determined by observation of a relative decrease in the proportion of αSMA-positive fibroblasts at the end of the 24 h culture period in the presence of the anti-IL-11 antibodies as compared to culture in the absence of the antibodies.

Example 11: Inhibition of fibrosis *in vivo* using anti-IL-11 antibodies

The therapeutic utility of the anti-human IL-11 antibodies is demonstrated in *in vivo* mouse models of fibrosis for various different tissues.

11.1 Heart fibrosis

A pump is implanted, and mice are treated with AngII (2mg/kg/day) for 28 days.

Neutralising anti-IL-11 antibodies, or control antibodies, are administered to different groups of mice by intravenous injection. At the end of the experiment, collagen content is assessed in the atria of the mice using a calorimetric hydroxyproline-based assay kit, and the level of RNA expression of the markers or fibrosis Col1A2, αSMA (ACTA2) and fibronectin (Fn1) were analysed by qPCR.

Mice treated with neutralising anti-IL-11 antibodies have a reduced fibrotic response in heart tissue as compared to mice treated with control antibodies, as evidenced by reduced expression of markers of fibrosis.

11.2 Kidney fibrosis

A mouse model for kidney fibrosis is established, in which fibrosis is induced by intraperitoneal injection of folic acid (180mg/kg) in vehicle (0.3M $NaHCO_3$); control mice were administered vehicle alone.

Neutralising anti-IL-11 antibodies, or control antibodies, are administered to different groups of mice by intravenous injection. Kidneys are removed at day 28, weighed and either fixed in 10% neutral-buffered formalin for Masson's trichrome and Sirius staining or snap-frozen for collagen assay, RNA, and protein studies.

Total RNA is extracted from the snap-frozen kidney using Trizol reagent (Invitrogen) and Qiagen TissueLyzer method followed by RNeasy column (Qiagen) purification. The cDNA is prepared using iScriptTM cDNA synthesis kit, in which each reaction contained 1µg of total RNA, as per the manufacturer's instructions. Quantitative RT-PCR gene expression analysis is performed on triplicate samples with either TaqMan (Applied Biosystems) or fast SYBR green (Qiagen) technology using StepOnePlusTM (Applied Biosystem) over 40 cycles. Expression data are normalized to GAPDH mRNA expression level and the $2^{-\Delta\Delta Ct}$ method is used to calculate the fold-change. The snap-frozen kidneys are subjected to acid hydrolysis by heating in 6M HCl at a concentration of 50 mg/ml (95°C,20 hours). The amount of total collagen in the hydrolysate is quantified based on the colorimetric detection of hydroxyproline using Quickzyme Total Collagen assay kit (Quickzyme Biosciences) as per the manufacturer's instructions.

Mice treated with neutralising anti-IL-11 antibodies have a reduced fibrotic response in kidney tissue as compared to mice treated with control antibodies, as evidenced by reduced expression of markers of fibrosis.

11.3 Lung fibrosis

Mice are treated by intratracheal administration of bleomycin on day 0 to establish a fibrotic response in the lung (pulmonary fibrosis).

Neutralising anti-IL-11 antibodies, or control antibodies, are administered to different groups of mice by intravenous injection. Mice are sacrificed at day 21, and analysed for differences in fibrosis markers.

Mice treated with neutralising anti-IL-11 antibodies have a reduced fibrotic response in lung tissue as compared to mice treated with control antibodies, as evidenced by reduced expression of markers of fibrosis.

11.4 Skin fibrosis

Mice are treated by subcutaneous administration of bleomycin on day 0 to establish a fibrotic response in the skin.

Neutralising anti-IL-11 antibodies, or control antibodies, are administered to different groups of mice by intravenous injection. Mice are sacrificed at day 21, and analysed for differences in fibrosis markers.

Mice treated with neutralising anti-IL-11 antibodies have a reduced fibrotic response in skin tissue as compared to mice treated with control antibodies, as evidenced by reduced expression of markers of fibrosis.

11.5 Eye fibrosis

Mice undergo trabeculectomy on day 0 to initiate a wound healing response in the eye.

Neutralising anti-IL-11 antibodies, or control antibodies, are administered to different groups of mice by intravenous injection, and fibrosis is monitored in the eye tissue.

Mice treated with neutralising anti-IL-11 antibodies have a reduced fibrotic response in eye tissue as compared to mice treated with control antibodies, as evidenced by reduced expression of markers of fibrosis.

11.6 Other tissues

The effect of treatment with neutralising anti-IL-11 antibodies on fibrosis is also analysed in mouse models of fibrosis for other tissues, such as the liver, kidney, bowel, and is also analysed in a model relevant to multiorgan (i.e. systemic) fibrosis.

Mice treated with neutralising anti-IL-11 antibodies have a reduced fibrotic response as compared to mice treated with control antibodies, as evidenced by reduced expression of markers of fibrosis.

Example 12: Anti-human IL-11Rα antibodies

Mouse monoclonal antibodies directed against human IL-11Rα protein were generated as follows.

cDNA encoding the amino acid for human IL-11Rα was cloned into expression plasmids (Aldevron GmbH, Freiburg, Germany).

Mice were immunised by intradermal application of DNA-coated gold-particles using a hand-held device for particle-bombardment ("gene gun"). Serum samples were collected from mice

after a series of immunisations, and tested in flow cytometry on HEK cells which had been transiently transfected with human IL-11Rα expression plasmids (cell surface expression of human IL-11Rα by transiently transfected HEK cells was confirmed with anti-tag antibodies recognising a tag added to the N-terminus of the IL-11Rα protein).

Antibody-producing cells were isolated from the mice and fused with mouse myeloma cells (Ag8) according to standard procedures.

Hybridomas producing antibodies specific for IL-11Rα were identified by screening for ability to bind to IL-11Rα expressing HEK cells by flow cytometry.

Cell pellets of positive hybridoma cells were prepared using an RNA protection agent (RNAlater, cat. #AM7020 by ThermoFisher Scientific) and further processed for sequencing of the variable domains of the antibodies.

Sequencing was performed using BigDye® Terminator v3.1 Cycle Sequencing kit (Life Technologies®) according to the manufacturer's instructions. All data was collected using a 3730xl DNA Analyzer system and Unified Data Collection software (Life Technologies®). Sequence assembly was performed using CodonCode Aligner (CodonCode Corporation). Mixed base calls were resolved by automatically assigning the most prevalent base call to the mixed base calls. Prevalence was determined by both frequency of a base call and the individual quality of the base calls.

In total, 17 mouse monoclonal anti-human IL-11Rα antibody clones were generated.

Example 13: Functional characterisation of anti-human IL-11Rα antibodies

13.1 Ability to inhibit human IL-11/IL-11R mediated signalling

To investigate the ability of the anti-IL-11Rα antibodies to neutralise human IL-11/IL-11R mediated signalling, cardiac atrial human fibroblasts were cultured in wells of 96-well plates in the presence of TGFβ1 (5 ng/ml) for 24 hours, in the presence or absence of the anti-IL-11Rα antibodies. This profibrotic stimulus promotes the expression of IL-11, which in turn drives the transistion of quiescent fibroblasts to activated, αSMA-positive fibroblasts. It has previously been shown that neutralising IL-11 prevents TGFβ1-induced transition to activated, αSMA-positive fibroblasts.

Anti-IL-11Rα antibodies (2 µg/ml) were added to fibroblast cultures that were stimulated with TGFβ1, and at the end of the 24 hour culture period, the percentage of αSMA-positive

fibroblasts was determined. The percentages were normalised based on the percentage of αSMA-positive fibroblasts observed in cultures of fibroblasts which had not been stimulated with TGFβ1.

Expression of αSMA was analysed with the Operetta High-Content Imaging System in an automated high-throughput fashion.

Stimulation with TGFβ1 resulted in a 1.58 fold increase in the number of αSMA-positive, activated fibroblasts at the end of the 24 hour culture period in the absence of anti-IL-11Rα antibodies.

A commercial monoclonal mouse anti-IL-11 antibody (Monoclonal Mouse IgG2A; Clone #22626; Catalog No. MAB218; R&D Systems, MN, USA) was included as a control. This antibody was found to be able to reduce the percentage of activated fibroblasts to 0.89 fold of the percentage of activated fibroblasts in unstimulated cultures (i.e. in the absence of stimulation with TGFβ1).

The anti-IL-11Rα antibodies were found to be able to inhibit IL-11/IL-11R signalling in human fibroblasts, and several were able to inhibit IL-11/IL-11R signalling to a greater extent than the monoclonal mouse anti-IL-11 antibody.

13.2 Ability to inhibit mouse IL-11 mediated signalling

The ability of the anti-IL-11Rα antibodies to inhibit mouse IL-11-mediated signalling was also investigated, following the same procedure as described in section 13.1 above, but using mouse atrial fibroblasts instead of human atrial fibroblasts.

Stimulation with TGFβ1 resulted in a 2.24 fold increase in the number of αSMA-positive, activated fibroblasts at the end of the 24 hour culture period in the absence of anti-IL-11Rα antibodies.

The commercial monoclonal mouse anti-IL-11 antibody (Monoclonal Mouse IgG2A; Clone #22626; Catalog No. MAB218; R&D Systems, MN, USA) was included as a control. This antibody was found to be able to reduce the percentage of activated fibroblasts to 1.44 fold of the percentage of activated fibroblasts in unstimulated cultures (i.e. in the absence of stimulation with TGFβ1).

The anti-IL-11Rα antibodies were found to be able to inhibit IL-11/IL-11R signalling in mouse fibroblasts, and several were able to inhibit IL-11/IL-11R signalling to a greater extent than the monoclonal mouse anti-IL-11 antibody.

13.3 Screening for ability to bind IL-11Rα

The mouse hybridomas producing anti-human IL-11Rα antibodies were sub-cloned, and cell culture supernatant from the subcloned hybridomas was analysed by "mix-and-measure" iQue assay for (i) ability to bind to human IL-11Rα, and (ii) cross reactivity for antigen other than IL-11Rα.

Briefly, labelled control cells (not expressing IL-11Rα at the cell surface) and unlabelled target cells expressing human IL-11Rα at their surface (following transient transfection with a plasmid encoding a FLAG-tagged human IL-11Rα) were mixed together with the cell culture supernatant (containing mouse-anti-IL-11Rα antibodies) and secondary detection antibodies (fluorescently-labelled anti-mouse IgG antibody).

The cells were then analysed using the HTFC Screening System (iQue) for the two labels (i.e. the cell label and the label on the secondary antibody). Detection of the secondary antibody on the unlabelled, IL-11Rα expressing cells indicated ability of the mouse-anti- IL-11Rα antibodies to bind to IL-11Rα. Detection of the secondary antibody on the labelled, control cells indicated cross-reactivity of the mouse-anti-IL-11Rα antibodies for target other than IL-11Rα.

As a positive control condition, labelled and unlabelled cells were incubated with a mouse anti-FLAG tag antibody as the primary antibody.

The majority of the subcloned hybridomas expressed antibody which was able to bind to human IL-11Rα, and which recognised this target with high specificity.

13.4 Analysis of antibody affinity for human IL-11Rα

The anti-human IL-11Rα antibodies are analysed for their affinity of binding to human IL-11Rα by ELISA assay.

Recombinant human IL-11Rα is obtained from Genscript and Horseradish peroxidase (HRP)-conjugated anti-human IgG (Fc-specific) antibody is obtained from Sigma. Corning 96-well ELISA plates are obtained from Sigma. Pierce 3,3´,5,5´-tetramethylbenzidine (TMB) ELISA substrate kit is obtained from Life Technologies (0.4 g/mL TMB solution, 0.02 % hydrogen

peroxide in citric acid buffer). Bovine serum albumin and sulphuric acid is obtained from Sigma. Wash buffer comprises 0.05% Tween-20 in phosphate buffered saline (PBS-T). Purified IgG controls are purchased from Life Technologies. Tecan Infinite 200 PRO NanoQuant is used to measure absorbance.

Criss-cross serial dilution analysis was performed as described by Hornbeck et al., (2015) Curr Protoc Immunol 110, 2.1.1-23) to determine the optimal concentration of coating antigen, primary and secondary antibodies.

An indirect ELISA is performed to assess the binding affinity of the mouse anti-IL-11Rα antibodies at 50% of effective concentration (EC_{50}) as previously described (Unverdorben et al., (2016) MAbs 8, 120–128.). ELISA plates are coated with 1 µg/mL of recombinant human IL-11Rα overnight at 4 ℃, and remaining binding sites are blocked with 2 % BSA in PBS. The antibodies are diluted in 1% BSA in PBS, titrated to obtain working concentrations of 800, 200, 50, 12.5, 3.125, 0.78, 0.195, and 0.049 ng/mL, and incubated in duplicates for 2 hours at room temperature. Detection of antigen-antibody binding is performed with 15.625 ng/mL of HRP-conjugated anti-mouse IgG antibody. Following 2 hours of incubation with the detection antibody, 100 µl of TMB substrate is added for 15 mins and chromogenic reaction stopped with 100 µl of 2 M H_2SO_4. Absorbance reading is measured at 450 nm with reference wavelength correction at 570 nm. Data are fitted with GraphPad Prism software with log transformation of antibody concentrations followed by non-linear regression analysis with the asymmetrical (five-parameter) logistic dose-response curve to determine individual EC50 values.

13.5 Ability to inhibit human IL-11/IL-11R signalling in a variety of tissues

Ability of the antibodies to neutralise IL-11/IL-11R signalling in fibroblasts obtained from a variety of different tissues is investigated, essentially as described in section 13.1 except that instead of cardiac atrial human fibroblasts, human fibroblasts derived from liver, lung, kidney, eye, skin, pancreas, spleen, bowel, brain, and bone marrow are used for the experiments.

Anti-IL-11Rα antibodies are demonstrated to be capable of neutralising IL-11/IL-11R signalling in fibroblasts derived from the various different tissues, as determined by observation of a relative decrease in the proportion of αSMA-positive fibroblasts at the end of the 24 h culture period in the presence of the anti-IL-11Rα antibodies as compared to culture in the absence of the antibodies.

Example 14: Inhibition of fibrosis *in vivo* using anti-IL-11Rα antibodies

The therapeutic utility of the anti-human IL-11Rα antibodies is demonstrated *in vivo* in mouse models of fibrosis for various different tissues.

14.1 Heart fibrosis

A pump is implanted, and mice are treated with AngII (2mg/kg/day) for 28 days.

Neutralising anti-IL-11Rα antibodies, or control antibodies, are administered to different groups of mice by intravenous injection. At the end of the experiment, collagen content is assessed in the atria of the mice using a calorimetric hydroxyproline-based assay kit, and the level of RNA expression of the markers or fibrosis Col1A2, αSMA (ACTA2) and fibronectin (Fn1) were analysed by qPCR.

Mice treated with neutralising anti-IL-11Rα antibodies have a reduced fibrotic response in heart tissue as compared to mice treated with control antibodies, as evidenced by reduced expression of markers of fibrosis.

14.2 Kidney fibrosis

A mouse model for kidney fibrosis is established, in which fibrosis is induced by intraperitoneal injection of folic acid (180mg/kg) in vehicle (0.3M $NaHCO_3$); control mice were administered vehicle alone.

Neutralising anti-IL-11Rα antibodies, or control antibodies, are administered to different groups of mice by intravenous injection. Kidneys are removed at day 28, weighed and either fixed in 10% neutral-buffered formalin for Masson's trichrome and Sirius staining or snap-frozen for collagen assay, RNA, and protein studies.

Total RNA is extracted from the snap-frozen kidney using Trizol reagent (Invitrogen) and Qiagen TissueLyzer method followed by RNeasy column (Qiagen) purification. The cDNA is prepared using iScriptTM cDNA synthesis kit, in which each reaction contained 1µg of total RNA, as per the manufacturer's instructions. Quantitative RT-PCR gene expression analysis is performed on triplicate samples with either TaqMan (Applied Biosystems) or fast SYBR green (Qiagen) technology using StepOnePlusTM (Applied Biosystem) over 40 cycles. Expression data are normalized to GAPDH mRNA expression level and the $2^{-\Delta\Delta Ct}$ method is used to calculate the fold-change. The snap-frozen kidneys are subjected to acid hydrolysis by heating in 6M HCl at a concentration of 50 mg/ml (95°C,20 hours). The amount of total collagen in the hydrolysate is quantified based on the colorimetric detection of hydroxyproline

using Quickzyme Total Collagen assay kit (Quickzyme Biosciences) as per the manufacturer's instructions.

Mice treated with neutralising anti-IL-11Rα antibodies have a reduced fibrotic response in kidney tissue as compared to mice treated with control antibodies, as evidenced by reduced expression of markers of fibrosis.

14.3 Lung fibrosis

Mice are treated by intratracheal administration of bleomycin on day 0 to establish a fibrotic response in the lung (pulmonary fibrosis).

Neutralising anti-IL-11Rα antibodies, or control antibodies, are administered to different groups of mice by intravenous injection. Mice are sacrificed at day 21, and analysed for differences in fibrosis markers.

Mice treated with neutralising anti-IL-11Rα antibodies have a reduced fibrotic response in lung tissue as compared to mice treated with control antibodies, as evidenced by reduced expression of markers of fibrosis.

14.4 Skin fibrosis

Mice are treated by subcutaneous administration of bleomycin on day 0 to establish a fibrotic response in the skin.

Neutralising anti-IL-11Rα antibodies, or control antibodies, are administered to different groups of mice by intravenous injection. Mice are sacrificed at day 21, and analysed for differences in fibrosis markers.

Mice treated with neutralising anti-IL-11Rα antibodies have a reduced fibrotic response in skin tissue as compared to mice treated with control antibodies, as evidenced by reduced expression of markers of fibrosis.

14.5 Eye fibrosis

Mice undergo trabeculectomy on day 0 to initiate a wound healing response in the eye.

Neutralising anti-IL-11Rα antibodies, or control antibodies, are administered to different groups of mice by intravenous injection, and fibrosis is monitored in the eye tissue.

Mice treated with neutralising anti-IL-11Rα antibodies have a reduced fibrotic response in eye tissue as compared to mice treated with control antibodies, as evidenced by reduced expression of markers of fibrosis.

14.6 Other tissues

The effect of treatment with neutralising anti-IL-11Rα antibodies on fibrosis is also analysed in mouse models of fibrosis for other tissues, such as the liver, kidney, bowel, and is also analysed in a model relevant to multiorgan (i.e. systemic) fibrosis.

Mice treated with neutralising anti-IL-11Rα antibodies have a reduced fibrotic response as compared to mice treated with control antibodies, as evidenced by reduced expression of markers of fibrosis.

Example 15: Decoy IL-11 Receptors

15.1 Decoy IL-11 Receptor constructs

Decoy IL-11 Receptor molecules were designed and cloned into the pTT5 vector for recombinant expression in 293-6E cells.

Briefly, an insert for the plasmid comprising cDNA encoding the ligand binding domains D1, D2 and D3 of gp130 in-frame with cDNA encoding either a 50 amino acid or 33 amino acid linker region, followed by cDNA encoding the ligand binding domains D2 and D3 of human IL-11Rα, followed by cDNA encoding the FLAG tag. The cDNA insert incorporated a leader sequence, Kozak sequences at the 5' end, and included a 5' EcoRI restriction site and a 3' HindIII restriction site (downstream of a stop codon) for insertion into the pTT5 vector.

The two constructs encoding a decoy IL-11 receptor molecule having either a 50 amino acid or 33 amino acid sequence are respectively designated Decoy IL-11 Receptor 1 (D11R1) and Decoy IL-11 Receptor 2 (D11R2).

15.2 Decoy IL-11 Receptor expression and purification

The constructs were transfected into 293-6E cells for recombinant expression and purification.

293-6E cells were grown in serum-free FreeStyle™ 293 Expression Medium (Life Technologies, Carlsbad, CA, USA). Cells were maintained in Erlenmeyer Flasks (Corning Inc., Acton, MA) at 37℃ with 5% CO_2 on an orbital shaker (VWR Scientific, Chester, PA).

One day before transfection, the cells were seeded at an appropriate density in Corning Erlenmeyer Flasks. On the day of transfection, DNA and transfection reagent were mixed at an optimal ratio and then added into the flask with cells ready for transfection. The recombinant plasmids encoding D11R1 and D11R2 were transiently transfected into suspension 293-6E cell cultures on two separate days.

Cell culture supernatants were collected on day 6 and used for purification. Briefly, cell culture broths were centrifuged and filtrated. 0.5 ml of resin was added to cell culture supernatants and incubated for 3-4 hours to capture the target protein.

After washing and elution with appropriate buffers, eluted fractions were analysed by SDS-PAGE and Western blot using Rabbit anti-FLAG polyclonal Ab (GenScript, Cat.No.A00170) to confirm expression of the FLAG-tagged decoy IL-11 receptor molecules.

The purified species were quantified and stored at -80℃.

Example 16: Functional characterisation of Decoy IL-11 Receptors

16.1 Ability to inhibit human IL-11 mediated signalling

To investigate ability to neutralise human IL-11-mediated signalling, cardiac atrial human fibroblasts were cultured in wells of 96-well plates in the presence of TGFβ1 (5 ng/ml) for 24 hours, in the presence or absence of various concentrations of D11R1 or D11R2.

TGFβ1 promotes the expression of IL-11, which in turn drives the transition of quiescent fibroblasts to activated, αSMA-positive fibroblasts. It has previously been shown that neutralising IL-11 prevents TGFβ1-induced transition to activated, αSMA-positive fibroblasts.

Expression of αSMA was analysed with the Operetta High-Content Imaging System in an automated high-throughput fashion.

D11R1 or D11R2 were added to fibroblast cultures that were stimulated with TGFβ1 at final concentrations of 5 ng/ml, 50 ng/ml and 500 ng/ml, and at the end of the 24 hour culture period, the percentage of αSMA-positive fibroblasts in the culture was determined.

Both D11R1 and D11R2 were demonstrated to be capable of neutralising signalling mediated by human IL-11 in a dose-dependent manner.

The results of the experiments are shown in Figures 32A and 32B. Both D11R1 and D11R2 were demonstrated to be capable of neutralising signalling mediated by human IL-11 in a dose-dependent manner.

The IC_{50} for the D11R1 and D11R2 molecules was determined to be ~1 nM.

16.2 Ability to inhibit mouse IL-11 mediated signalling

The ability of D11R1 and D11R2 to inhibit mouse IL-11-mediated signalling is investigated, following the same procedure as described in section 16.1 above, but using mouse dermal fibroblasts instead of human atrial fibroblasts.

D11R1 and D11R2 are demonstrated to be capable of neutralising IL-11/IL-11R signalling in mouse dermal fibroblasts, as determined by observation of a relative decrease in the proportion of αSMA-positive fibroblasts at the end of the 24 h culture period in the presence of D11R1 or D11R2 as compared to culture in the absence of the decoy IL-11 receptors.

16.3 Analysis of decoy IL-11 receptor affinity for IL-11

D11R1 and D11R2 are analysed for their affinity of binding to human IL-11 by ELISA assay.

Recombinant human IL-11 was obtained from Genscript and Horseradish peroxidase (HRP)-conjugated anti-FLAG antibody is obtained. Corning 96-well ELISA plates were obtained from Sigma. Pierce 3,3´,5,5´-tetramethylbenzidine (TMB) ELISA substrate kit was obtained from Life Technologies (0.4 g/mL TMB solution, 0.02 % hydrogen peroxide in citric acid buffer). Bovine serum albumin and sulphuric acid was obtained from Sigma. Wash buffer comprised 0.05% Tween-20 in phosphate buffered saline (PBS-T). Tecan Infinite 200 PRO NanoQuant is used to measure absorbance.

An indirect ELISA is performed to assess the binding affinity of D11R1 and D11R2 at 50% of effective concentration (EC_{50}) as previously described (Unverdorben et al., (2016) MAbs 8, 120–128.). ELISA plates are coated with 1 µg/mL of recombinant human IL-11 overnight at 4℃ and remaining binding sites were blocked with 2 % BSA in PBS. D11R1 and D11R1 are diluted in 1% BSA in PBS, titrated to obtain working concentrations of 800, 200, 50, 12.5, 3.125, 0.78, 0.195, and 0.049 ng/mL, and incubated in duplicates for 2 hours at room temperature. Detection of antigen-decoy IL-11 receptor binding is performed with HRP-conjugated anti-FLAG antibody. Following 2 hours of incubation with the detection antibody, 100 µl of TMB substrate is added for 15 mins and chromogenic reaction stopped with 100 µl of 2 M H_2SO_4. Absorbance reading is measured at 450 nm with reference wavelength

correction at 570 nm. Data are fitted with GraphPad Prism software with log transformation of decoy IL-11 receptor concentrations followed by non-linear regression analysis with the asymmetrical (five-parameter) logistic dose-response curve to determine EC50 values.

The same materials and procedures as described above were performed to determine the affinity of binding to recombinant murine IL-11 obtained from Genscript.

16.4 Ability to inhibit human IL-11 mediated signalling in a variety of tissues

Ability of the decoy IL-11 receptors D11R1 and D11R2 to neutralise IL-11-mediated signalling in fibroblasts obtained from a variety of different tissues is investigated, essentially as described in sections 18.1 except that instead of cardiac atrial human fibroblasts, human fibroblasts derived from liver, lung, kidney, eye, skin, pancreas, spleen, bowel, brain, and bone marrow are used for the experiments.

D11R1 and D11R2 are demonstrated to be capable of neutralising signalling in fibroblasts derived from the various different tissues, as determined by observation of a relative decrease in the proportion of αSMA-positive fibroblasts at the end of the 24 h culture period in the presence of the decoy IL-11 receptors as compared to culture in the absence of the decoy IL-11 receptors.

Example 17: Inhibition of fibrosis *in vivo* using decoy IL-11 receptors

The therapeutic utility of the decoy IL-11 receptors is demonstrated in *in vivo* mouse models of fibrosis for various different tissues.

17.1 Heart fibrosis

A pump is implanted, and mice are treated with AngII (2mg/kg/day) for 28 days.

Decoy IL-11 receptors D11R1 or D11R2 are administered to different groups of mice by intravenous injection. At the end of the experiment, collagen content is assessed in the atria of the mice using a calorimetric hydroxyproline-based assay kit, and the level of RNA expression of the markers or fibrosis Col1A2, αSMA (ACTA2) and fibronectin (Fn1) were analysed by qPCR.

Mice treated with decoy IL-11 receptors have a reduced fibrotic response in heart tissue as compared to untreated/vehicle treated controls, as evidenced by reduced expression of markers of fibrosis.

17.2 Kidney fibrosis

A mouse model for kidney fibrosis is established, in which fibrosis is induced by intraperitoneal injection of folic acid (180mg/kg) in vehicle (0.3M $NaHCO_3$); control mice were administered vehicle alone.

Decoy IL-11 receptors D11R1 or D11R2 are administered to different groups of mice by intravenous injection. Kidneys are removed at day 28, weighed and either fixed in 10% neutral-buffered formalin for Masson's trichrome and Sirius staining or snap-frozen for collagen assay, RNA, and protein studies.

Total RNA is extracted from the snap-frozen kidney using Trizol reagent (Invitrogen) and Qiagen TissueLyzer method followed by RNeasy column (Qiagen) purification. The cDNA is prepared using iScriptTM cDNA synthesis kit, in which each reaction contained 1µg of total RNA, as per the manufacturer's instructions. Quantitative RT-PCR gene expression analysis is performed on triplicate samples with either TaqMan (Applied Biosystems) or fast SYBR green (Qiagen) technology using StepOnePlusTM (Applied Biosystem) over 40 cycles. Expression data are normalized to GAPDH mRNA expression level and the $2^{-\Delta\Delta Ct}$ method is used to calculate the fold-change. The snap-frozen kidneys are subjected to acid hydrolysis by heating in 6M HCl at a concentration of 50 mg/ml (95°C,20 hours). The amount of total collagen in the hydrolysate is quantified based on the colorimetric detection of hydroxyproline using Quickzyme Total Collagen assay kit (Quickzyme Biosciences) as per the manufacturer's instructions.

Mice treated with decoy IL-11 receptors have a reduced fibrotic response in kidney tissue as compared to untreated/vehicle treated controls, as evidenced by reduced expression of markers of fibrosis.

17.3 Lung fibrosis

Mice are treated by intratracheal administration of bleomycin on day 0 to establish a fibrotic response in the lung (pulmonary fibrosis).

Decoy IL-11 receptors D11R1 or D11R2 are administered to different groups of mice by intravenous injection. Mice are sacrificed at day 21, and analysed for differences in fibrosis markers.

Mice treated with decoy IL-11 receptors have a reduced fibrotic response in lung tissue as compared to untreated/vehicle treated controls, as evidenced by reduced expression of markers of fibrosis.

17.4 Skin fibrosis

Mice are treated by subcutaneous administration of bleomycin on day 0 to establish a fibrotic response in the skin.

Decoy IL-11 receptors D11R1 or D11R2 are administered to different groups of mice by intravenous injection. Mice are sacrificed at day 21, and analysed for differences in fibrosis markers.

Mice treated with decoy IL-11 receptors have a reduced fibrotic response in skin tissue as compared to untreated/vehicle treated controls, as evidenced by reduced expression of markers of fibrosis.

17.5 Eye fibrosis

Mice undergo trabeculectomy procedure as described in Example 7.6 above to initiate a wound healing response in the eye.

Decoy IL-11 receptors D11R1 or D11R2 are administered to different groups of mice by intravenous injection, and fibrosis is monitored in the eye tissue.

Mice treated with decoy IL-11 receptors have a reduced fibrotic response in eye tissue as compared to untreated/vehicle treated controls, as evidenced by reduced expression of markers of fibrosis.

17.6 Other tissues

The effect of treatment with decoy IL-11 receptors D11R1 or D11R2 on fibrosis is also analysed in mouse models of fibrosis for other tissues, such as the liver, kidney, bowel, and is also analysed in a model relevant to multiorgan (i.e. systemic) fibrosis.

The fibrotic response is measured and compared between mice treated with decoy IL-11 receptors and untreated mice, or vehicle treated controls. . Mice treated with decoy IL-11 receptors have a reduced fibrotic response as compared to untreated/vehicle treated controls, as evidenced by reduced expression of markers of fibrosis.

Example 18: Genetic biomarkers for IL-11 response

In addition to measuring IL-11 protein as a potential biomarker for fibrosis, we developed an assay that can predict IL-11 secretion status in humans. This assay could be used as a companion diagnostic in IL-11-related clinical trials.

We first generated RNA-seq data (Figure 16**Error! Reference source not found.**) and determined the genotype of 69 ethnically matched (Chinese) individuals in the cohort using a SNP array based on fluorescent probe hybridization supplied by Illumina (HumanOmniExpress 24).

We then performed genome-wide linkage eQTL analysis to assess whether Single Nucleotide Polymorphisms (SNPs) affect RNA transcript levels of IL-11 or IL-11RA in unstimulated fibroblasts, in TGFB1 stimulated (5ng/ml, 24h) fibroblasts. We also tested if the increase in IL-11 upon TGFβ1 stimulation (= response) was dependent on the genotype.

At first we quantified the read count for both IL-11 and IL-11RA in all individuals and transformed these counts using the variance stabilization (VST) approach of the DESeq2 method (Love et al., Genome Biology 2014 15:550). We then considered IL-11 and IL-11RA expression in unstimulated (VST_{unstim}) and stimulated (VST_{stim}) cells. To assess the increase in IL-11, we also computed the delta in expression as $VST_{stim} - VST_{unstim}$. We corrected the expression values using covariates such as RNA sequencing library batch, RNA RIN quality score, library concentration, library fragment size, age, gender before analyses. SNP and transcript expression, or delta expression, pairs were analysed using the matrix eQTL approach (Andrey A. Shabalin., Bioinformatics 2012 May 15; 28(10): 1353-1358).

We did not observe variation in *cis* or *trans* that significantly affected IL-11 expression in unstimulated cells. However, we detected distant SNPs that regulated the expression in stimulated = fibrotic fibroblasts. These variants stratify the population between individuals that do express low levels of IL-11 and those that express high amounts of IL-11 in fibrosis. We also detected local and distal variants that predicted the increase in IL-11 expression in response to TGFβ1. These variants can be used to stratify individuals into high and low responders in fibrosis.

The SNPs identified are shown in Figures 33 to 35 and accompanying data is shown in Figures 36 and 37.

Figure 1A

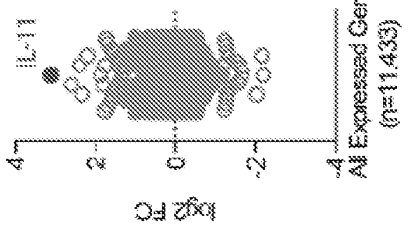


Figure 1B

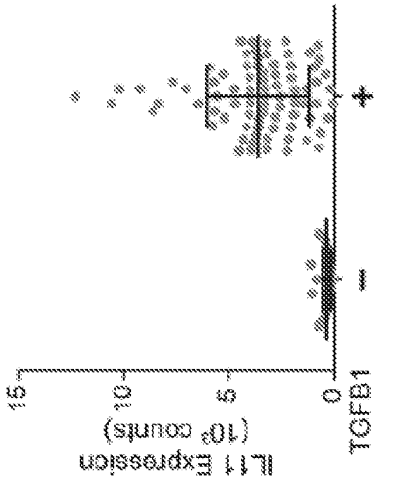


Figure 1C

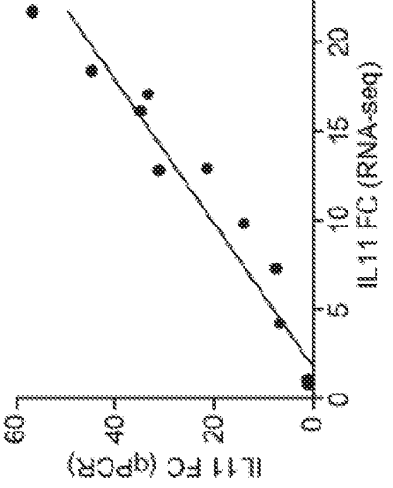


Figure 1D

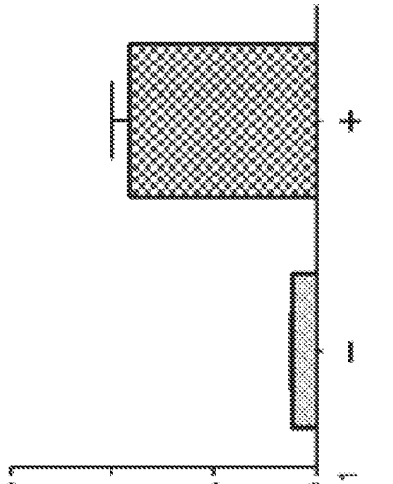


Figure 2A

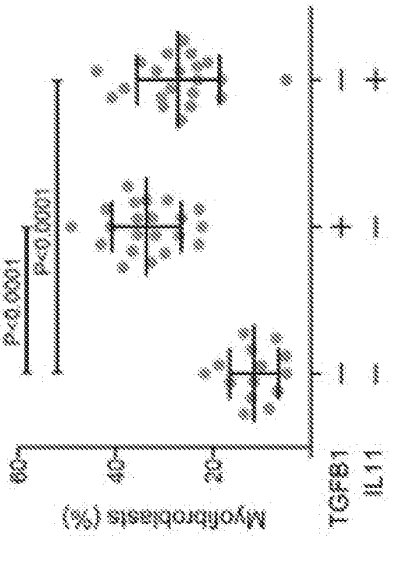


Figure 2B

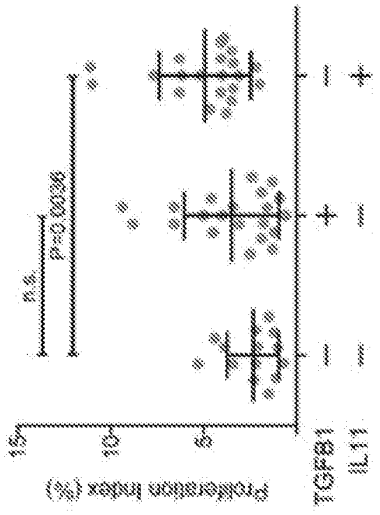


Figure 2C

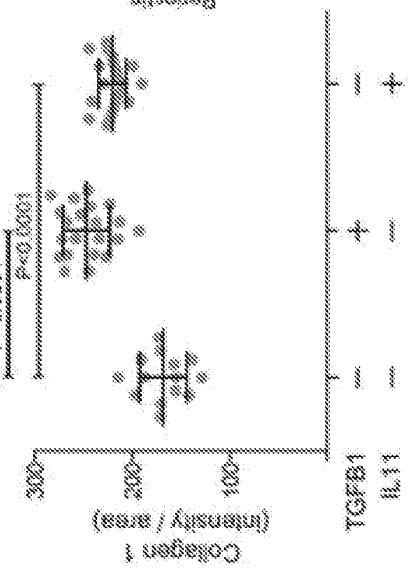
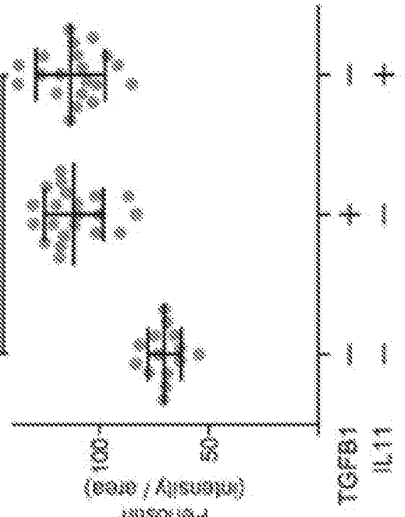


Figure 2D



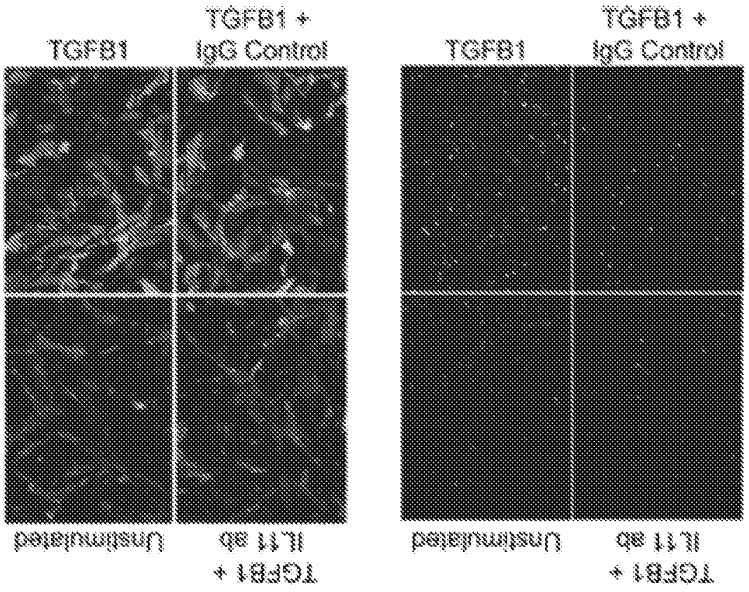


Figure 3A

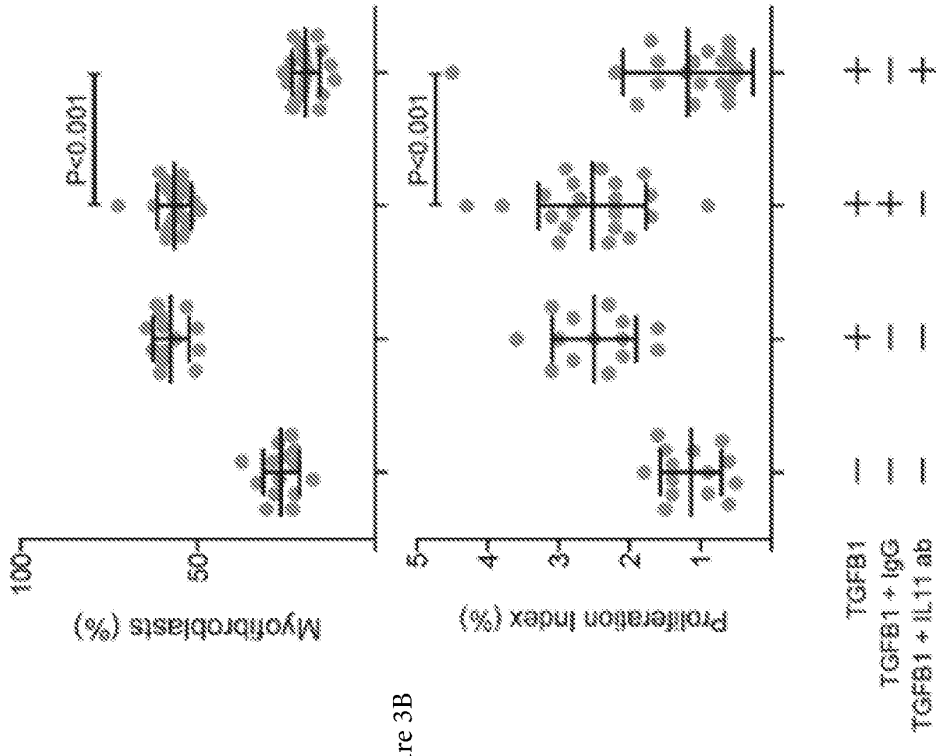
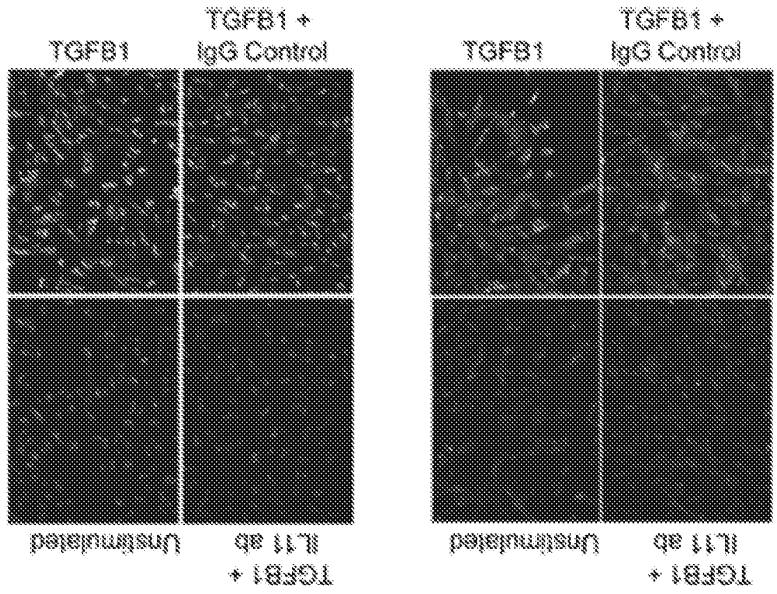


Figure 3B



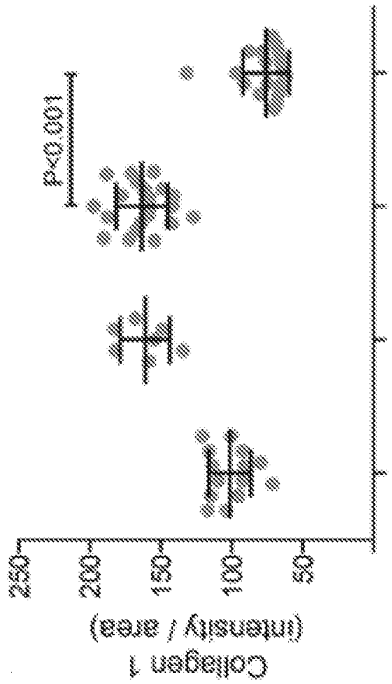


Figure 3C

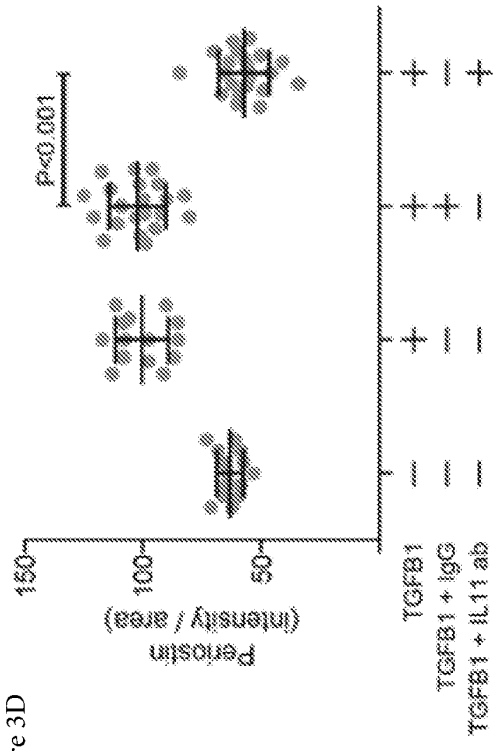


Figure 3D

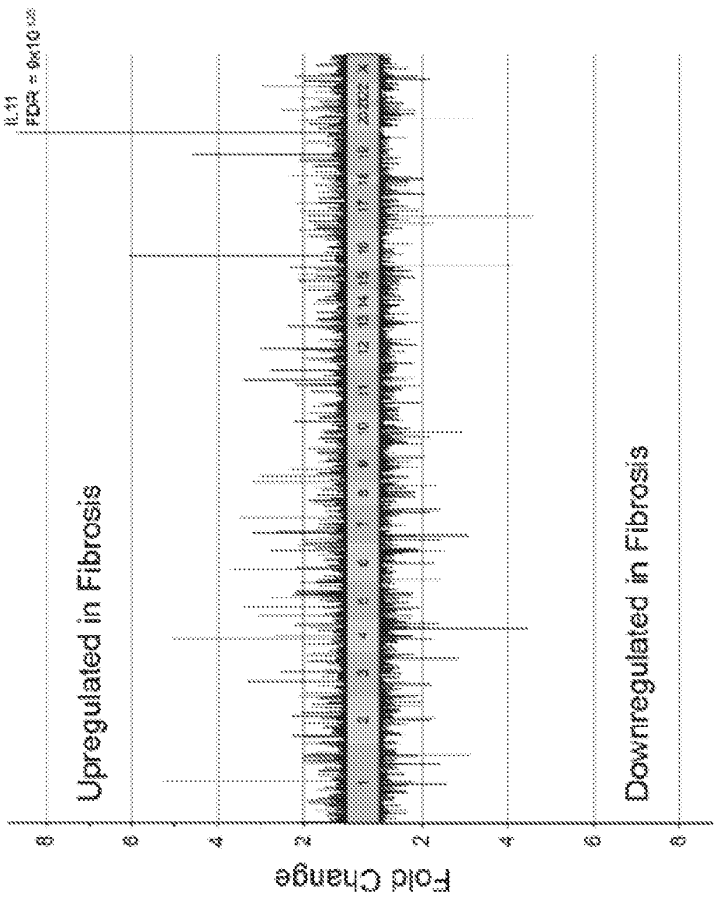

IL11
FDR = 9e10
Upregulated in Fibrosis
Downregulated in Fibrosis
Fold Change
8
6
4
2
2
4
6
8

Figure 4A

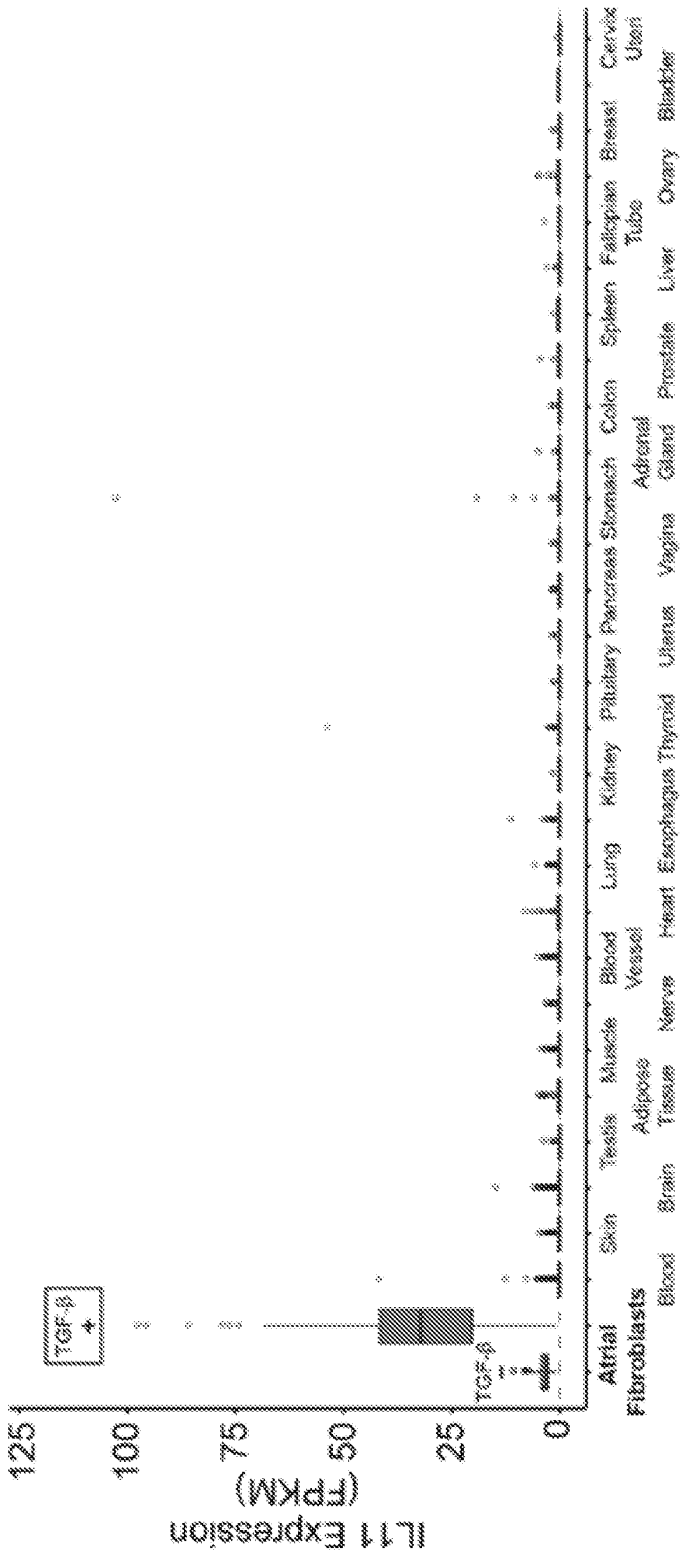

IL11 Expression
(FPKM)
125
100
75
50
25
0
TGF-β
TGF-β
Atrial Fibroblasts
Blood
Skin
Testis
Muscle
Blood Vessel
Lung
Kidney
Pituitary
Pancreas
Stomach
Colon
Spleen
Fallopian Tube
Breast
Cervix Uteri
Brain
Tissue
Adipose
Nerve
Heart
Esophagus
Thyroid
Uterus
Vagina
Adrenal Gland
Prostate
Liver
Ovary
Bladder

Figure 4B

Figure 5A

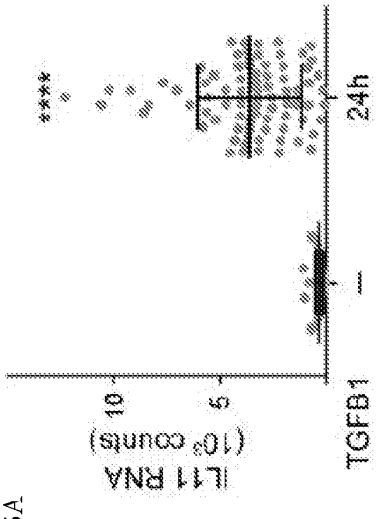


Figure 5B

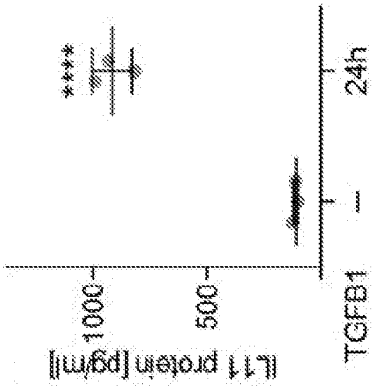
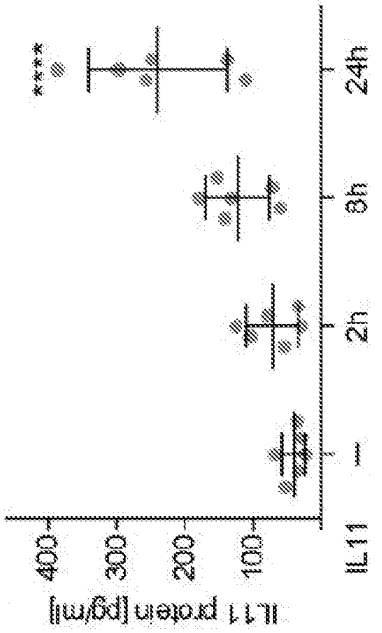


Figure 5C

Figure 5D



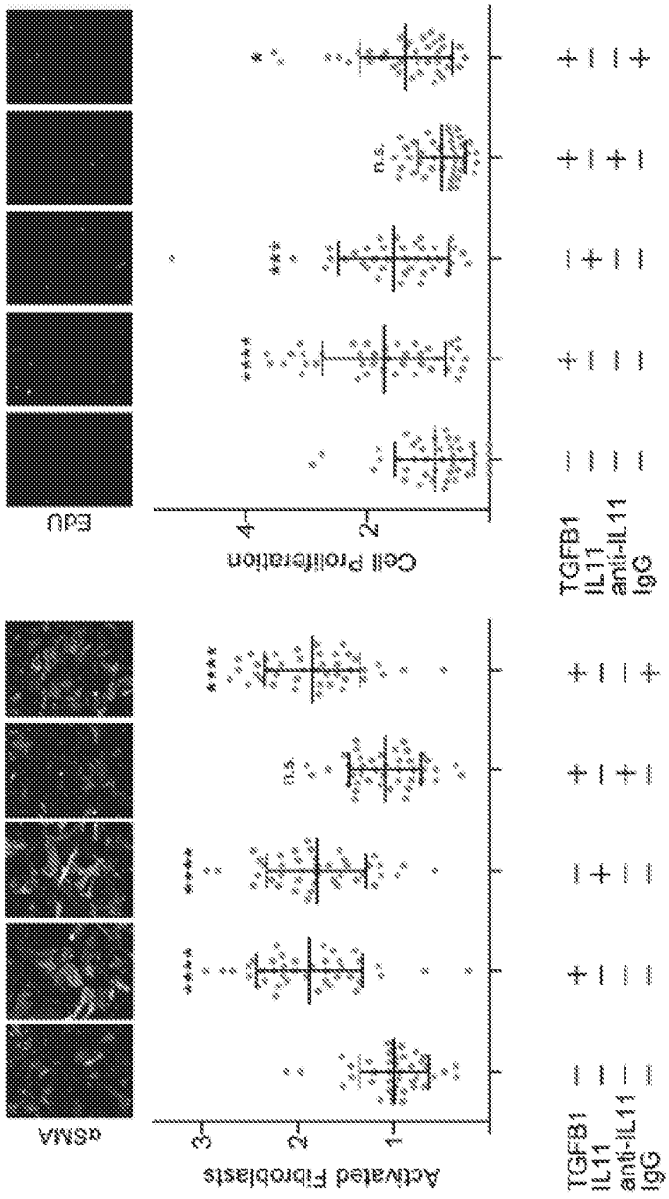


Figure 6A

Figure 6B

Figure 6C

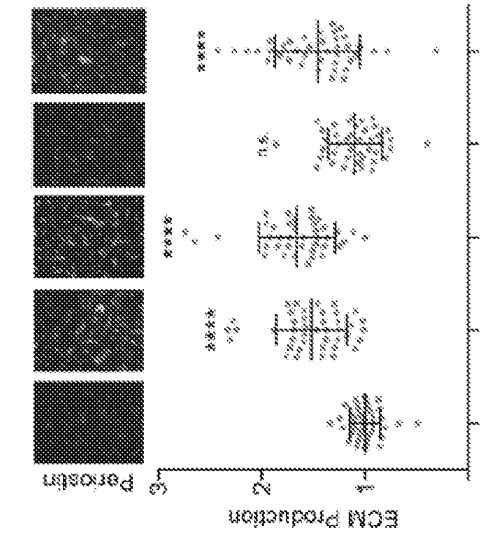


Figure 6D

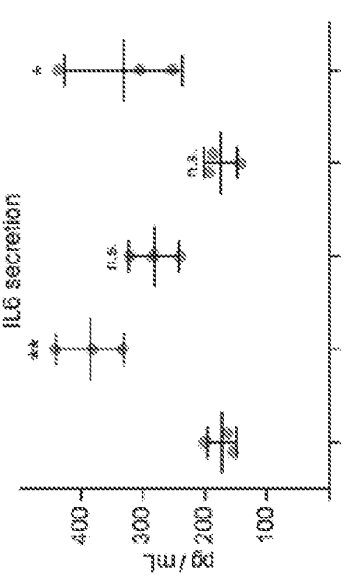


Figure 6E

Figure 6F

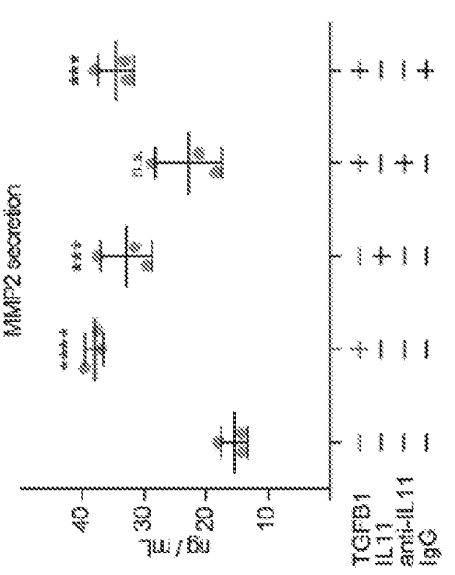


Figure 7A

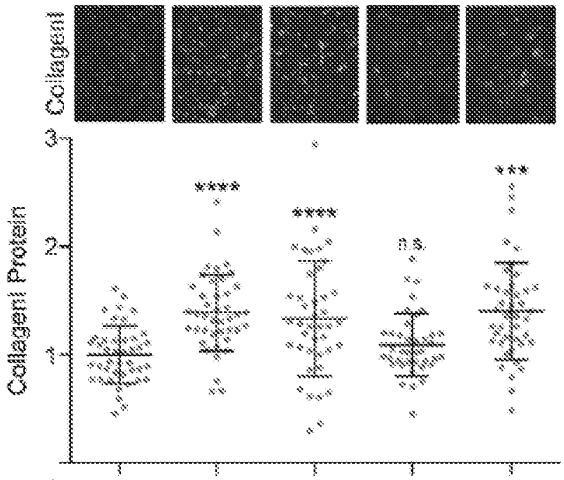


Figure 7B

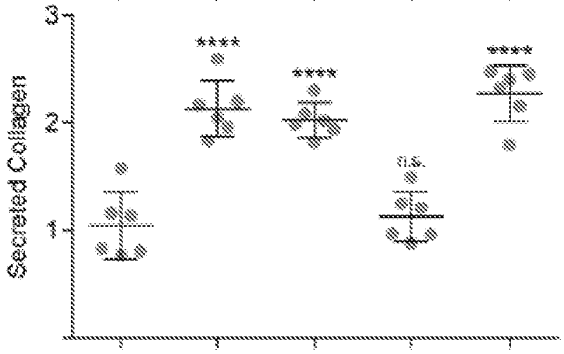


Figure 7C

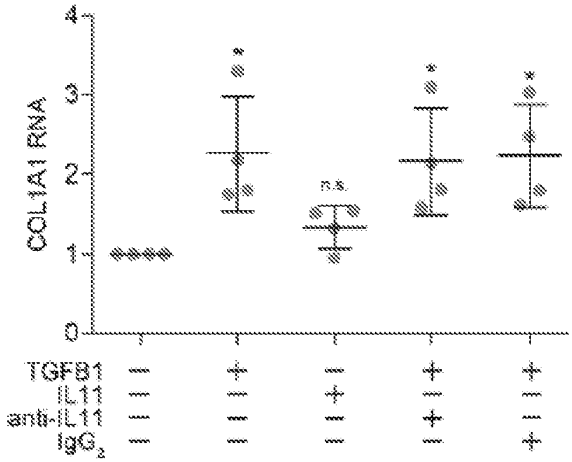


Figure 8A

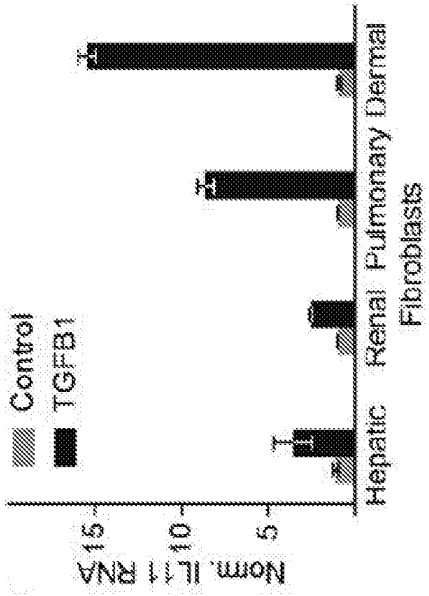


Figure 8B

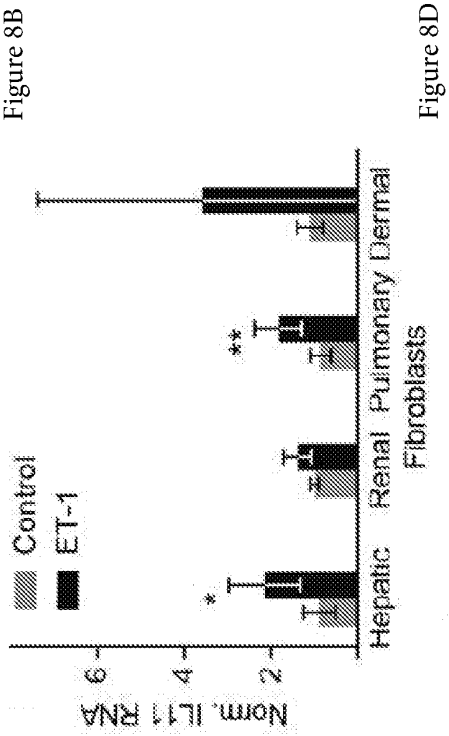
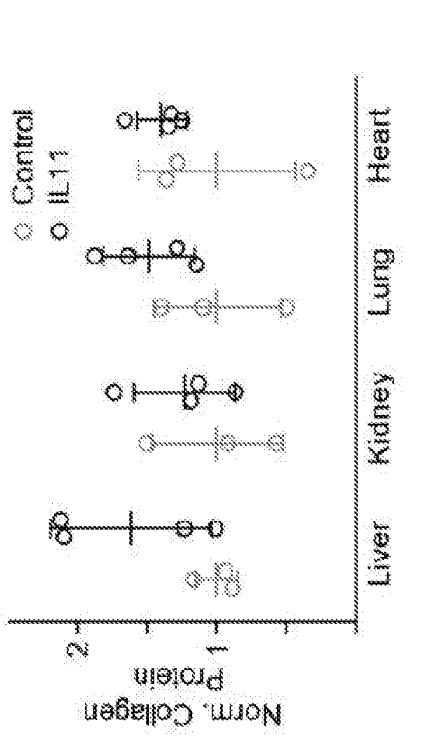


Figure 8C

Figure 8D



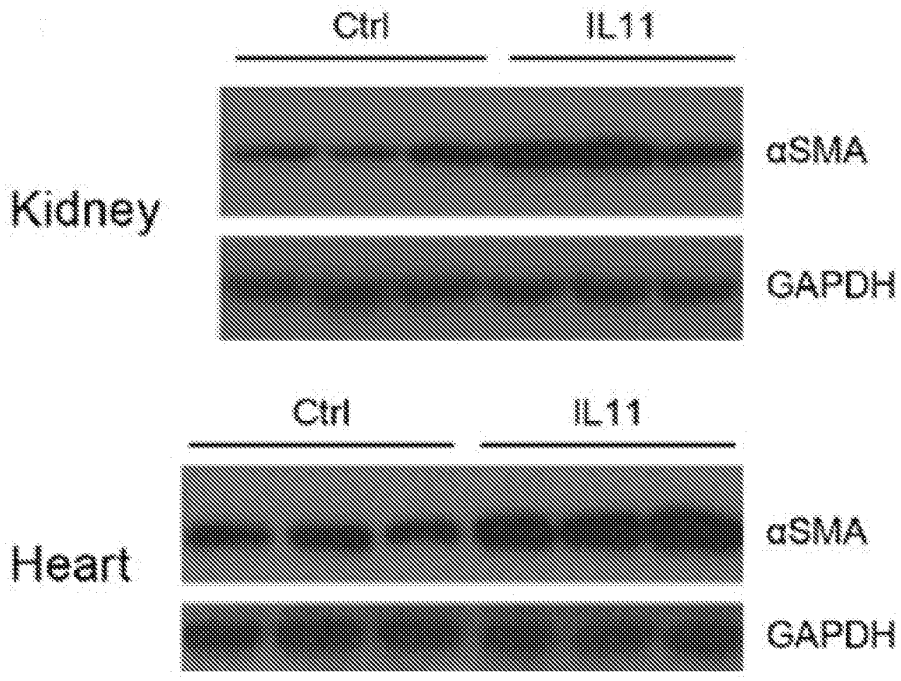


Figure 8E

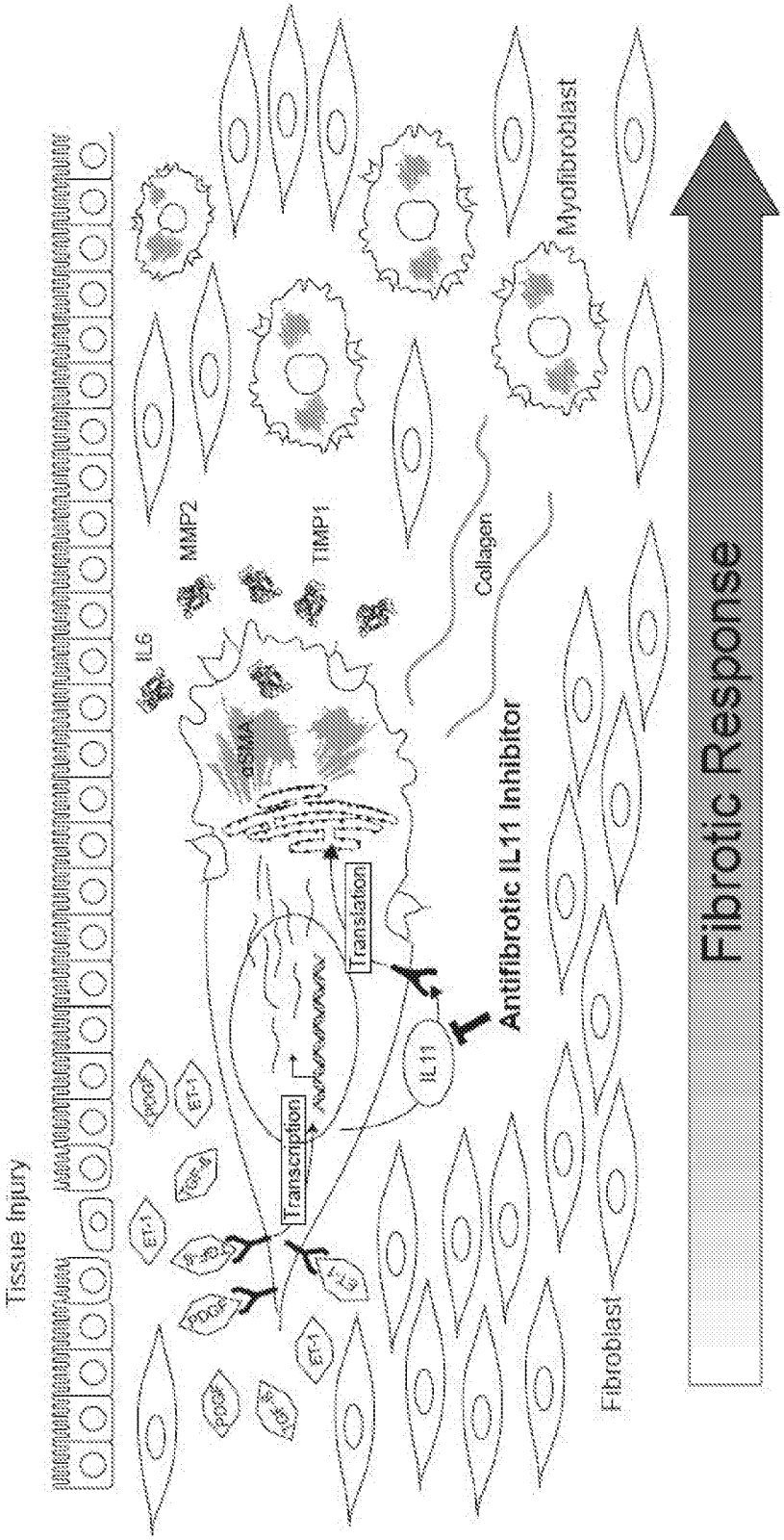

Tissue Injury
IL6
MMP2
TIMP1
Collagen
Myofibroblast
Transcription
Translation
IL11
Antifibrotic IL11 Inhibitor
Fibroblast
Fibrotic Response

Figure 9

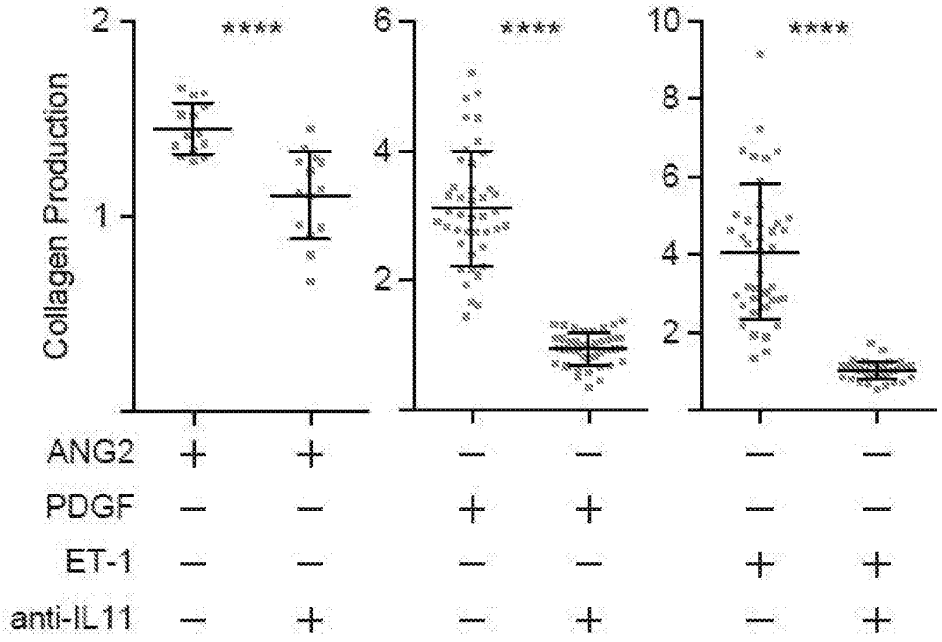


Figure 10

ACTGCCGCGGCCCTGCTGCTCAGGGCACATGCCTCCCCTCCCCAGGCCGCGGCCCAGCTGACCCTCGGGG
CTCCCCCGGCAGCGGACAGGGAAGGGTTAAAGGCCCCCGGCTCCCTGCCCCCTGCCCTGGGGAACCCCTG
GCCCTGTGGGGACATGAACTGTGTTTGCCGCCTGGTCCTGGTCGTGCTGAGCCTGTGGCCAGATACAGCT
GTCGCCCCTGGGCCACCACCTGGCCCCCCTCGAGTTTCCCCAGACCCTCGGGCCGAGCTGGACAGCACCG
TGCTCCTGACCCGCTCTCTCCTGGCGGACACGCGGCAGCTGGCTGCACAGCTGAGGGACAAATTCCCAGC
TGACGGGGACCACAACCTGGATTCCCTGCCCACCCTGGCCATGAGTGCGGGGGCACTGGGAGCTCTACAG
CTCCCAGGTGTGCTGACAAGGCTGCGAGCGGACCTACTGTCCTACCTGCGGCACGTGCAGTGGCTGCGCC
GGGCAGGTGGCTCTTCCCTGAAGACCCTGGAGCCCGAGCTGGGCACCCTGCAGGCCCGACTGGACCGGCT
GCTGCGCCGGCTGCAGCTCCTGATGTCCCGCCTGGCCCTGCCCCAGCCACCCCCGGACCCGCCGGCGCCC
CCGCTGGCGCCCCCCTCCTCAGCCTGGGGGGGCATCAGGGCCGCCCACGCCATCCTGGGGGGGCTGCACC
TGACACTTGACTGGGCCGTGAGGGGACTGCTGCTGCTGAAGACTCGGCTGTGACCCGGGGCCCAAAGCCA
CCACCGTCCTTCCAAAGCCAGATCTTATTTATTTATTTATTTCAGTACTGGGGGCGAAACAGCCAGGTGA
TCCCCCCGCCATTATCTCCCCCTAGTTAGAGACAGTCCTTCCGTGAGGCCTGGGGGGCATCTGTGCCTTA
TTTATACTTATTTATTTCAGGAGCAGGGGTGGGAGGCAGGTGGACTCCTGGGTCCCCGAGGAGGAGGGGA
CTGGGGTCCCGGATTCTTGGGTCTCCAAGAAGTCTGTCCACAGACTTCTGCCCTGGCTCTTCCCCATCTA
GCCCTGGGCAGGAACATATATTATTTATTTAAGCAATTACTTTTCATGTTGGGGTGGGGACGGAGGGGAA
AGGGAAGCCTGGGTTTTTGTACAAAAATGTGAGAAACCTTTGTGAGACAGAGAACAGGGAATTAAATGTG
TCATACATATCCACTTGAGGGCGATTTGTCTGAGAGCTGGGGCTGGATGCTTGGGTAACTGGGGCAGGGC
AGGTGGAGGGGAGACCTCCATTCAGGTGGAGGTCCCGAGTGGGCGGGGCAGCGACTGGGAGATGGGTCGG
TCACCCAGACAGCTCTGTGGAGGCAGGGTCTGAGCCTTGCCTGGGGCCCCGCACTGCATAGGGCCTTTTG
TTTGTTTTTTGAGATGGAGTCTCGCTCTGTTGCCTAGGCTGGAGTGCAGTGAGGCAATCTGAGGTCACTG
CAACCTCCACCTCCCGGGTTCAAGCAATTCTCCTGCCTCAGCCTCCCGATTAGCTGGGATCACAGGTGTG
CACCACCATGCCCAGCTAATTATTTATTTCTTTTGTATTTTTAGTAGAGACAGGGTTTCACCATGTTGGC
CAGGCTGGTTTCGAACTCCTGACCTCAGGTGATCCTCCTGCCTCGGCCTCCCAAAGTGCTGGGATTACAG
GTGTGAGCCACCACACCTGACCCATAGGTCTTCAATAAATATTTAATGGAAGGTTCCACAAGTCACCCTG
TGATCAACAGTACCCGTATGGGACAAAGCTGCAAGGTCAAGATGGTTCATTATGGCTGTGTTCACCATAG
CAAACTGGAAACAATCTAGATATCCAACAGTGAGGGTTAAGCAACATGGTGCATCTGTGGATAGAACGCC
ACCCAGCCGCCCGGAGCAGGGACTGTCATTCAGGGAGGCTAAGGAGAGAGGCTTGCTTGGGATATAGAAA
GATATCCTGACATTGGCCAGGCATGGTGGCTCACGCCTGTAATCCTGGCACTTTGGGAGGACGAAGCGAG
TGGATCACTGAAGTCCAAGAGTTCGAGACCGGCCTGCGAGACATGGCAAAACCCTGTCTCAAAAAAGAAA
GAATGATGTCCTGACATGAAACAGCAGGCTACAAAACCACTGCATGCTGTGATCCCAATTTTGTGTTTTT
CTTTCTATATATGGATTAAAACAAAAATCCTAAAGGGAAATACGCCAAAATGTTGACAATGACTGTCTCC
AGGTCAAAGGAGAGAGGTGGGATTGTGGGTGACTTTTAATGTGTATGATTGTCTGTATTTTACAGAATTT
CTGCCATGACTGTGTATTTTGCATGACACATTTTAAAAATAATAAACACTATTTTTAGAATAACAGAAAA
A [SEQ ID NO:1]

CCTTCCAAAGCCAGATCTT [SEQ ID NO:2]

GCCTGGGCAGGAACATATA [SEQ ID NO:3]

CCTGGGCAGGAACATATAT [SEQ ID NO:4]

GGTTCATTATGGCTGTGTT [SEQ ID NO:5]

Figure 11

GCTGTAGCTGGTGAGAGGAAGTCCTAGAGGCTATGGACACTCTGCTGCTGGGATCACCGAGATGAGCAGC
AGCTGCTCAGGGCTGAGCAGGGTCCTGGTGGCCGTGGCTACAGCCCTGGTGTCTGCCTCCTCCCCCTGCC
CCCAGGCCTGGGGCCCCCCAGGGGTCCAGTATGGGCAGCCAGGGAGGTCCGTGAAGCTGTGTTGTCCTGG
AGTGACTGCCGGGGACCCAGTGTCCTGGTTTCGGGATGGGGAGCCAAAGCTGCTCCAGGGACCTGACTCT
GGGCTAGGGCATGAACTGGTCCTGGCCCAGGCAGACAGCACTGATGAGGGCACCTACATCTGCCAGACCC
TGGATGGTGCACTTGGGGGCACAGTGACCCTGCAGCTGGGCTACCCTCCAGCCCGCCCTGTTGTCTCCTG
CCAAGCAGCCGACTATGAGAACTTCTCTTGCACTTGGAGTCCCAGCCAGATCAGCGGTTTACCCACCCGC
TACCTCACCTCCTACAGGAAGAAGACAGTCCTAGGAGCTGATAGCCAGAGGAGGAGTCCATCCACAGGGC
CCTGGCCATGCCCACAGGATCCCCTAGGGGCTGCCCGCTGTGTTGTCCACGGGGCTGAGTTCTGGAGCCA
GTACCGGATTAATGTGACTGAGGTGAACCCACTGGGTGCCAGCACACGCCTGCTGGATGTGAGCTTGCAG
AGCATCTTGCGCCCTGACCCACCCCAGGGCCTGCGGGTAGAGTCAGTACCAGGTTACCCCCGACGCCTGC
GAGCCAGCTGGACATACCCTGCCTCCTGGCCGTGCCAGCCCCACTTCCTGCTCAAGTTCCGTTTGCAGTA
CCGTCCGGCGCAGCATCCAGCCTGGTCCACGGTGGAGCCAGCTGGACTGGAGGAGGTGATCACAGATGCT
GTGGCTGGGCTGCCCCATGCTGTACGAGTCAGTGCCCGGGACTTTCTAGATGCTGGCACCTGGAGCACCT
GGAGCCCGGAGGCCTGGGGAACTCCGAGCACTGGGACCATACCAAAGGAGATACCAGCATGGGGCCAGCT
ACACACGCAGCCAGAGGTGGAGCCTCAGGTGGACAGCCCTGCTCCTCCAAGGCCCTCCCTCCAACCACAC
CCTCGGCTACTTGATCACAGGGACTCTGTGGAGCAGGTAGCTGTGCTGGCGTCTTTGGGAATCCTTTCTT
TCCTGGGACTGGTGGCTGGGGCCCTGGCACTGGGGCTCTGGCTGAGGCTGAGACGGGGTGGGAAGGATGG
ATCCCCAAAGCCTGGGTTCTTGGCCTCAGTGATTCCAGTGGACAGGCGTCCAGGAGCTCCAAACCTGTAG
AGGACCCAGGAGGGCTTCGGCAGATTCCACCTATAATTCTGTCTTGCTGGTGTGGATAGAAACCAGGCAG
CACAGTAGATCCCTATGGTTGGATCTCAGCTGGAAGTTCTGTTTGGAGCCCATTTCTGTGAGACCCTGTA
TTTCAAATTTGCAGCTGAAAGGTGCTTGTACCTCTGATTTCACCCCAGAGTTGGAGTTCTGCTCAAGGAA
CGTGTGTAATGTGTACATCTGTGTCCATGTGTGACCATGTGTCTGTGAAGGCCAGGGAACATGTATTCCT
CTGCATGCATGTATGTAGGTGCCTGGGAGTGTGTGTGGTCCTTGCTCTGGCCCTTTCCCTTGCAGGGTTG
TGCAGGTGTGAATAAA [SEQ ID NO:6]

GGACCATACCAAAGGAGAT [SEQ ID NO:7]

GCGTCTTTGGGAATCCTTT [SEQ ID NO:8]

GCAGGACAGTAGATCCCT [SEQ ID NO:9]

GCTCAAGGAACGTGTGTAA [SEQ ID NO:10]

Figure 12

Gene Name	*Gene ID/ Accession no.*	*siRNA name*	*siRNA sequence(5'-3')*	*SEQ ID NO:*
IL-11	NM_000641.3	siRNA 1	CCUUCCAAAGCCAGAUCUUdTdT AAGAUCUGGCUUUGGAAGGdTdT	11
IL-11	NM_000641.3	siRNA 2	GCCUGGGCAGGAACAUAUAdTdT UAUAUGUUCCUGCCCAGGCdTdT	12
IL-11	NM_000641.3	siRNA 3	CCUGGGCAGGAACAUAUAUdTdT AUAUAUGUUCCUGCCCAGGdTdT	13
IL-11	NM_000641.3	siRNA 4	GGUUCAUUAUGGCUGUGUUdTdT AACACAGCCAUAAUGAACCdTdT	14

Figure 13

Gene Name	*Gene ID/ Accession no.*	*siRNA name*	*siRNA sequence(5'-3')*	*SEQ ID NO:*
IL-11R	U32324.1	siRNA 5	GGACCAUACCAAAGGAGAUdTdT AUCUCCUUUGGUAUGGUCCdTdT	15
IL-11R	U32324.1	siRNA 6	GCGUCUUUGGGAAUCCUUUdTdT AAAGGAUUCCCAAAGACGCdTdT	16
IL-11R	U32324.1	siRNA 7	GCAGGACAGUAGAUCCCUAdTdT UAGGGAUCUACUGUCCUGCdTdT	17
IL-11R	U32324.1	siRNA 8	GCUCAAGGAACGUGUGUAAdTdT UUACACACGUUCCUUGAGCdTdT	18

Figure 14

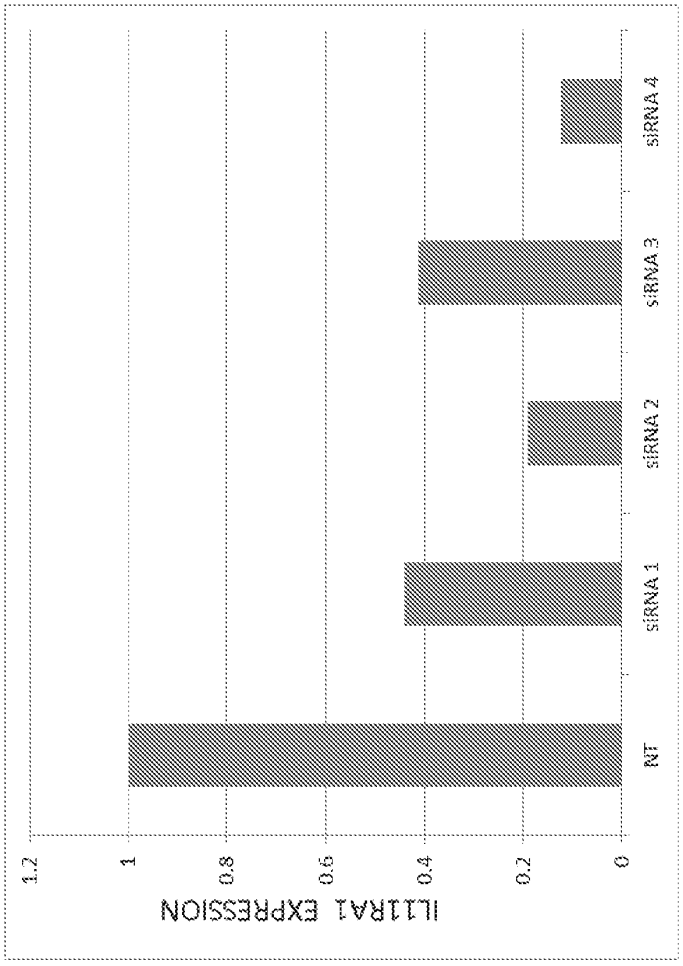

IL1RAP EXPRESSION
1.2
1
0.8
0.6
0.4
0.2
0
NT
siRNA 1
siRNA 2
siRNA 3
siRNA 4

Figure 15

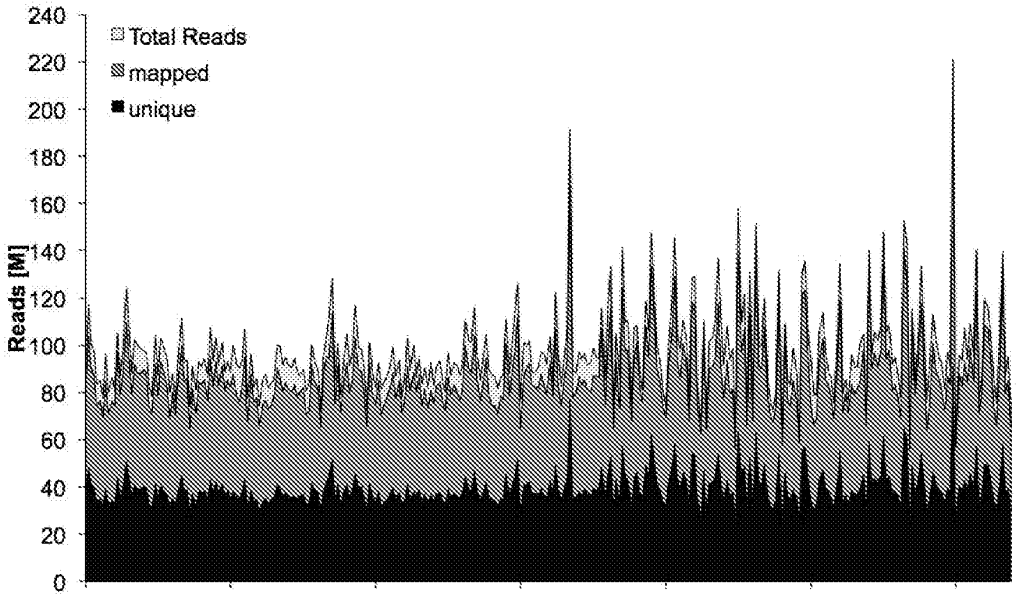


Figure 16

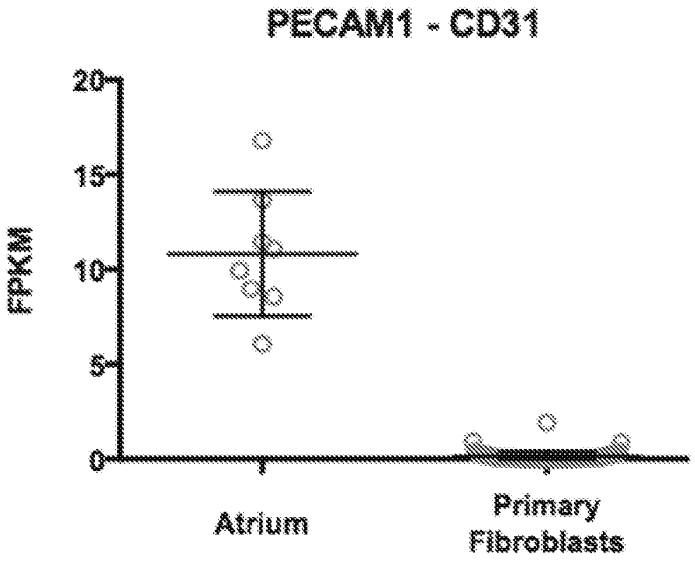


Figure 17A

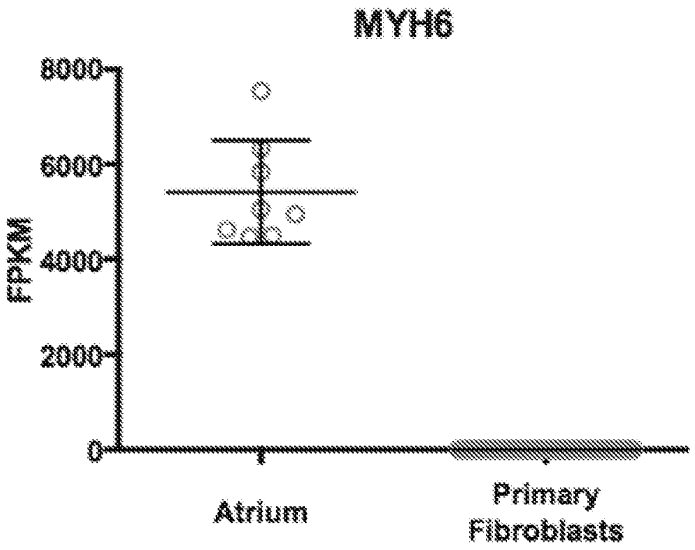


Figure 17B

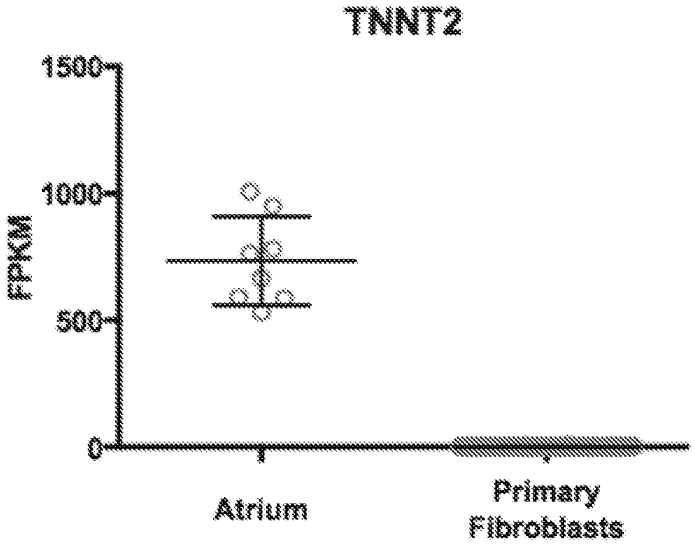


Figure 17C

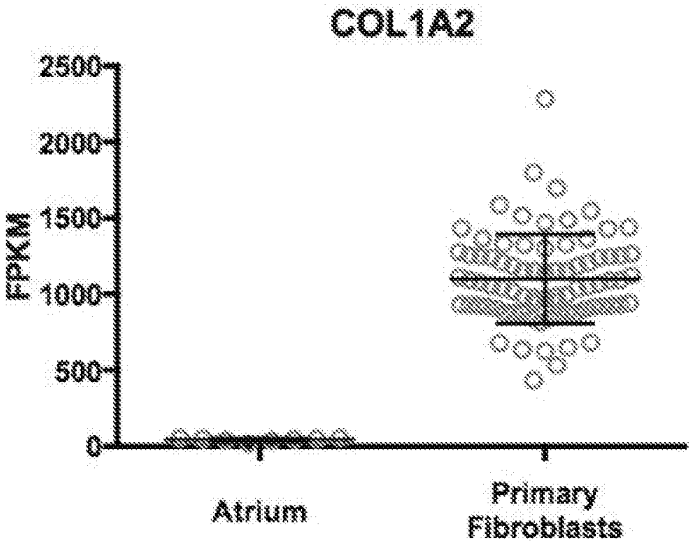


Figure 17D

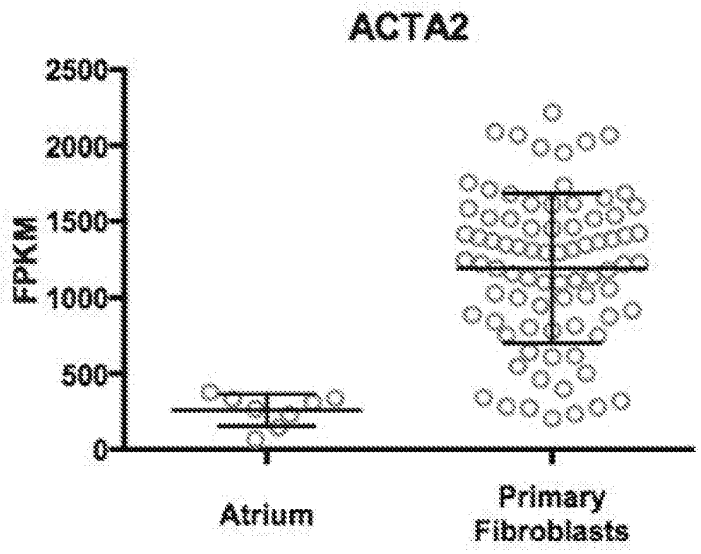


Figure 17E

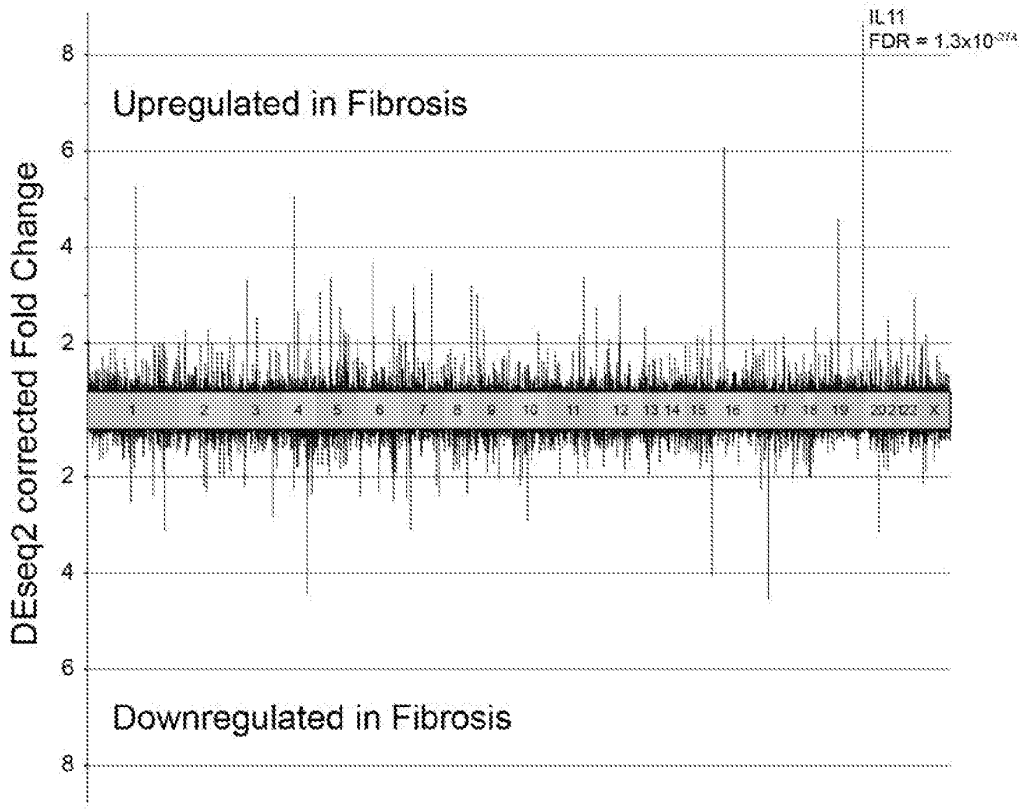


Figure 18A

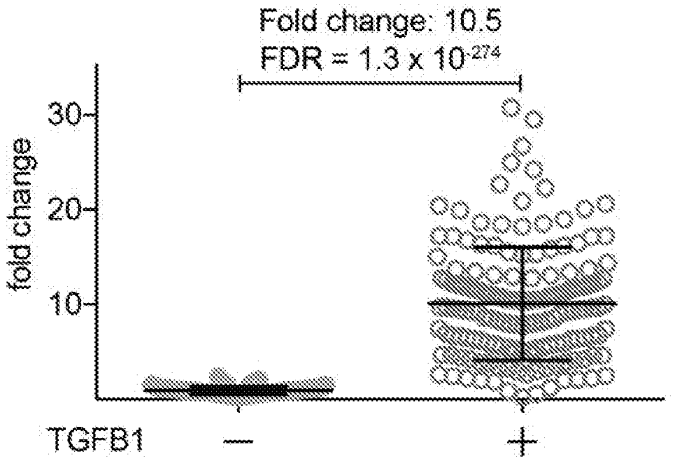


Figure 18B

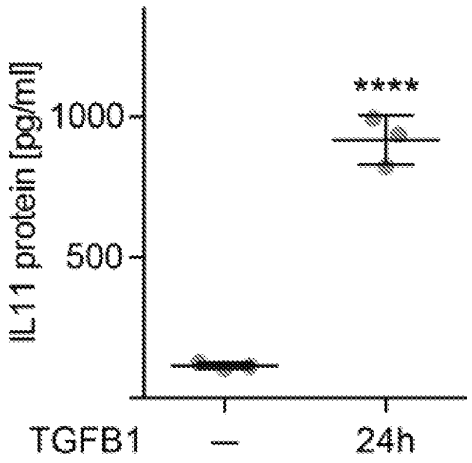


Figure 18C

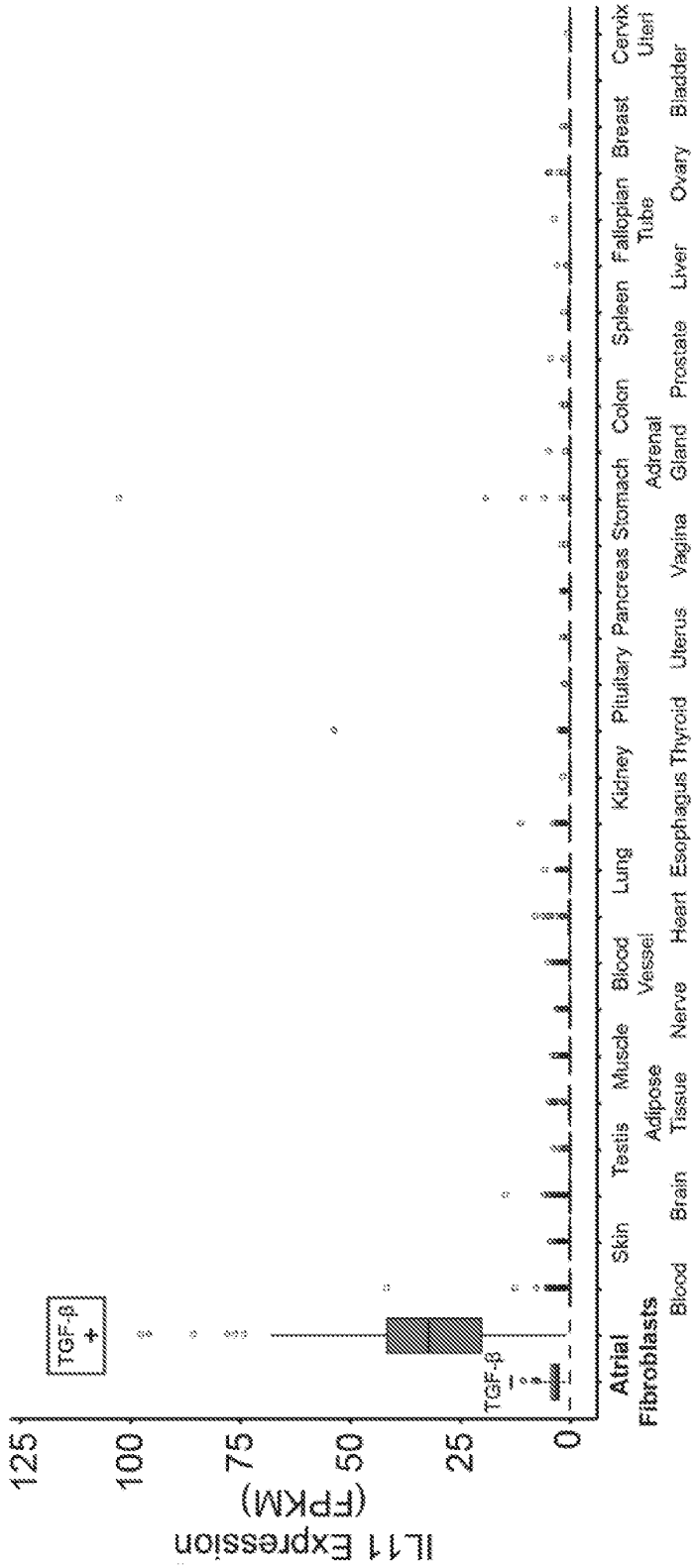

IL11 Expression
(FPKM)
125
100
75
50
25
0
TGF-β
+
TGF-β
-
Atrial Fibroblasts
Blood
Skin
Brain
Testis
Adipose Tissue
Muscle
Nerve
Blood Vessel
Heart
Lung
Esophagus
Kidney
Thyroid
Pituitary
Uterus
Pancreas
Vagina
Stomach
Adrenal Gland
Colon
Prostate
Spleen
Liver
Fallopian Tube
Ovary
Breast
Uteri
Cervix
Bladder

Figure 18D

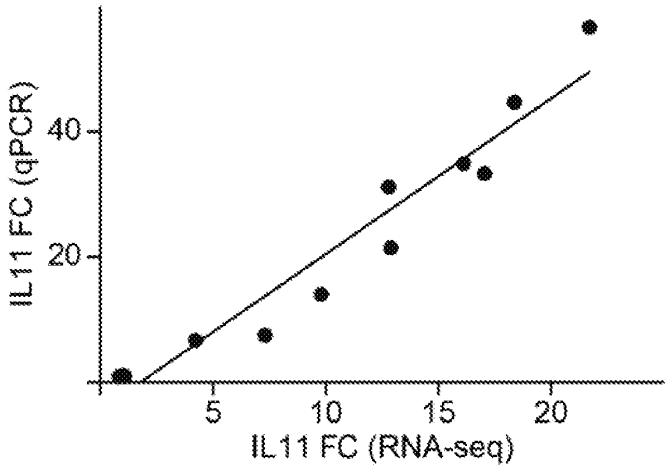


Figure 18E

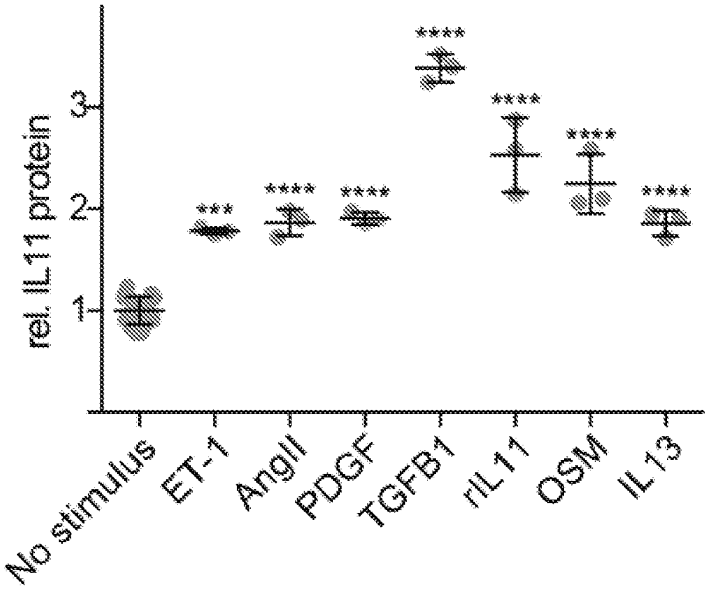


Figure 19A

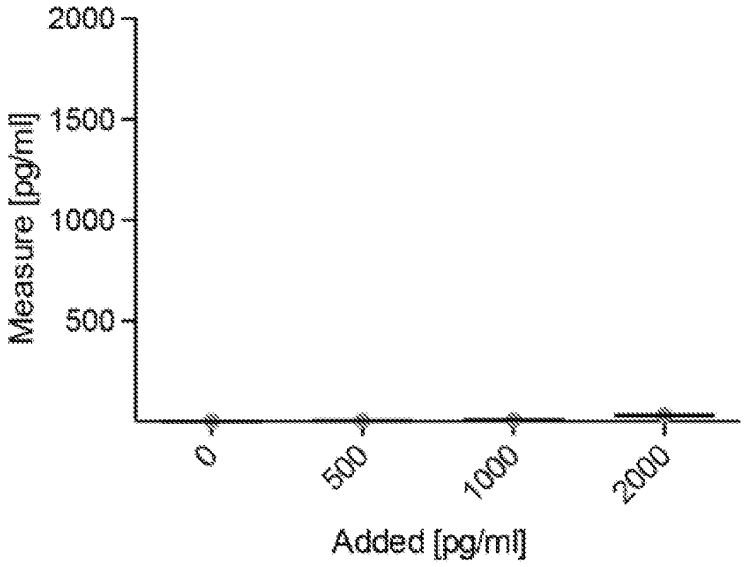


Figure 19B

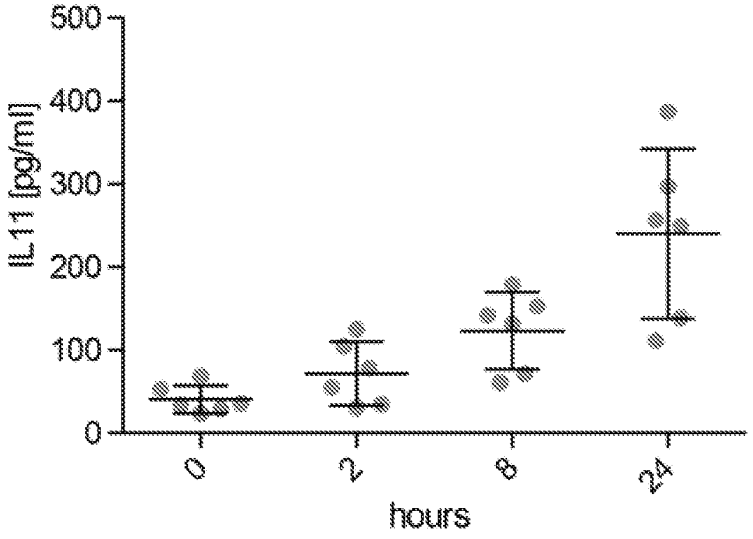


Figure 19C

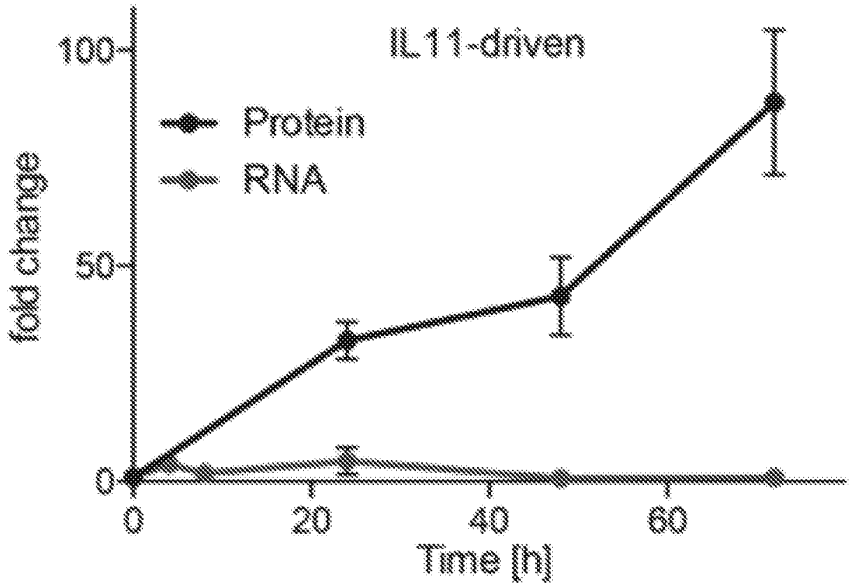


Figure 19D

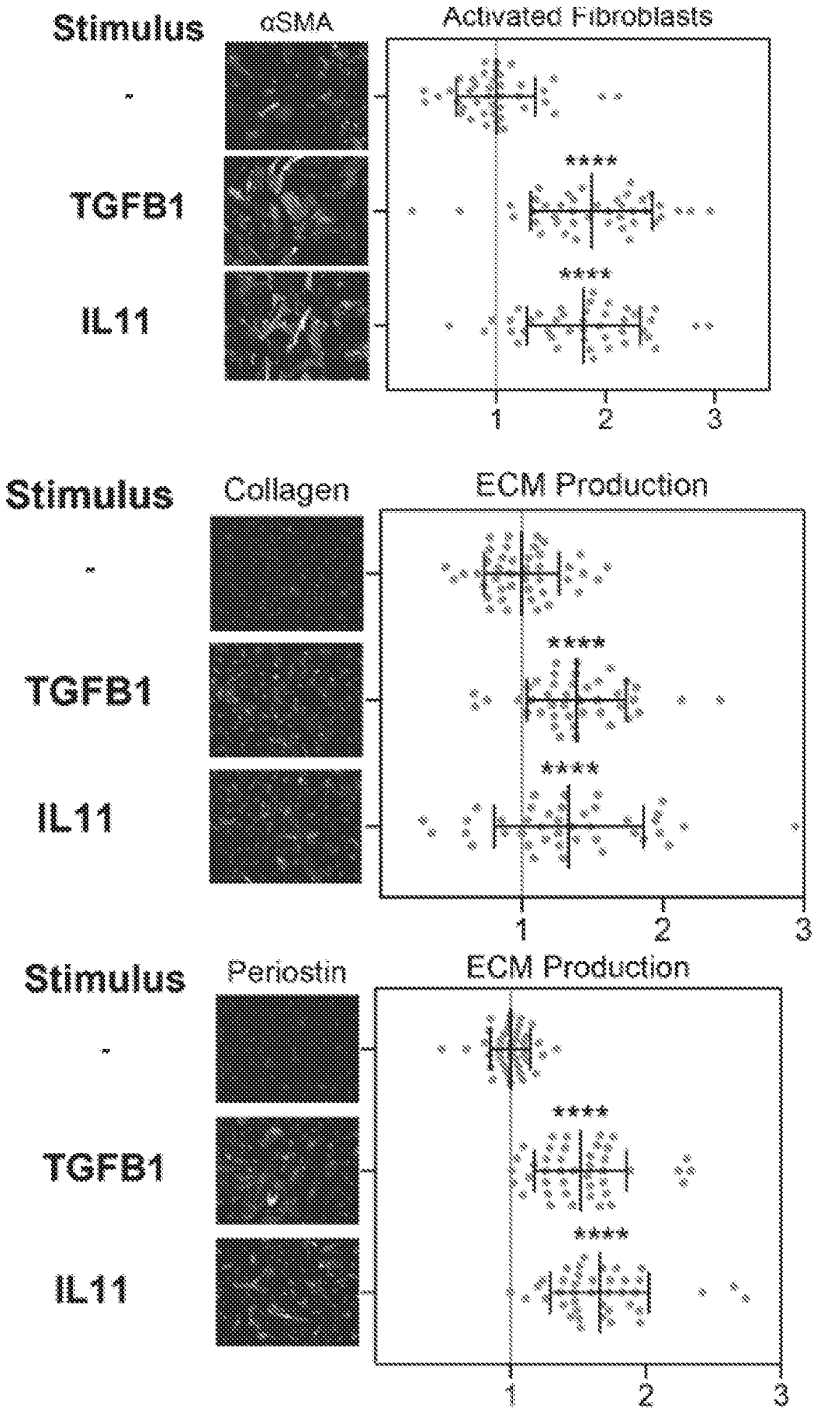

Stimulus
αSMA
Activated Fibroblasts
-
TGFB1
IL11
****
****
1
2
3
Stimulus
Collagen
ECM Production
-
TGFB1
IL11
****
****
1
2
3
Stimulus
Periostin
ECM Production
-
TGFB1
IL11
****
****
1
2
3

Figure 20A

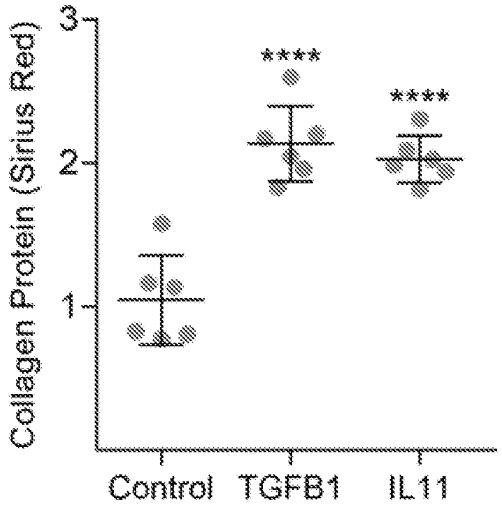


Figure 20B

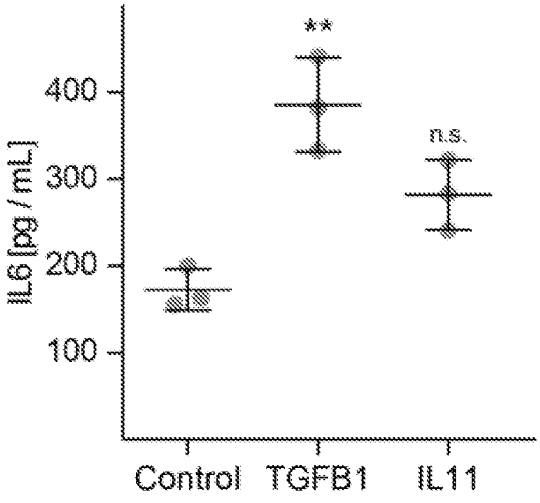


Figure 20C

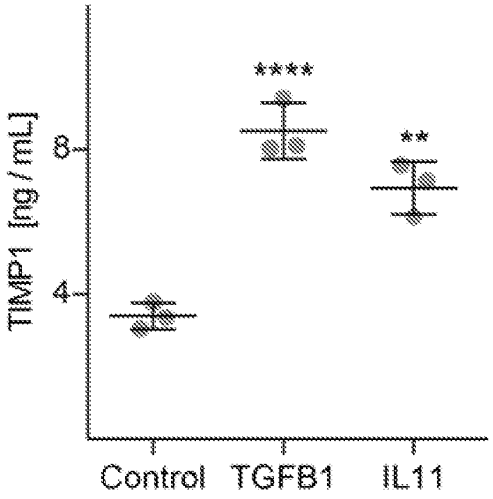


Figure 20D

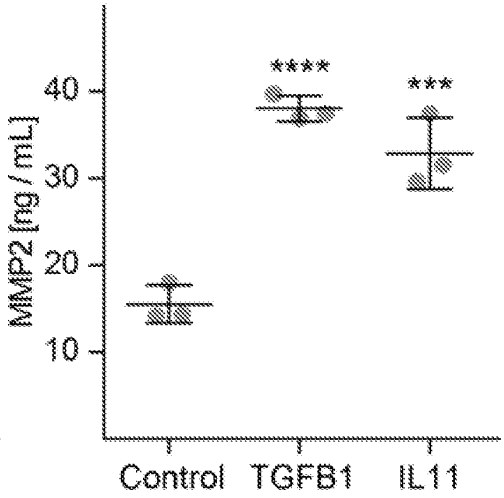


Figure 20E

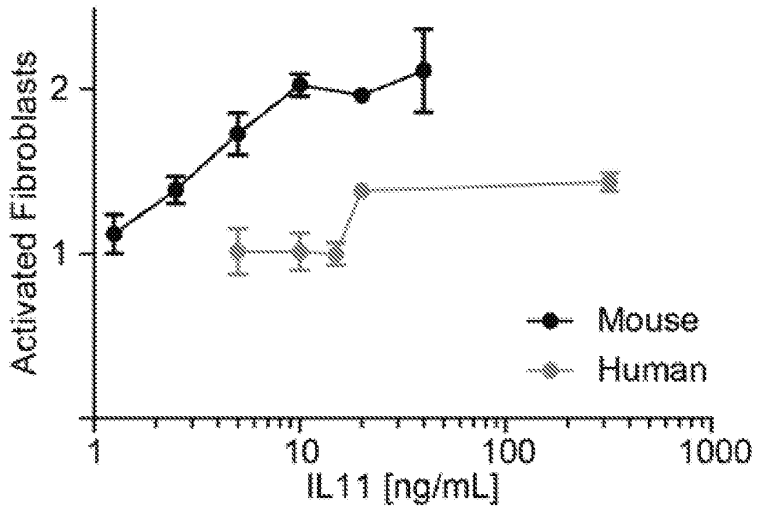

Activated Fibroblasts
2
1
1
10
100
1000
IL11 [ng/mL]
Mouse
Human

Figure 20F

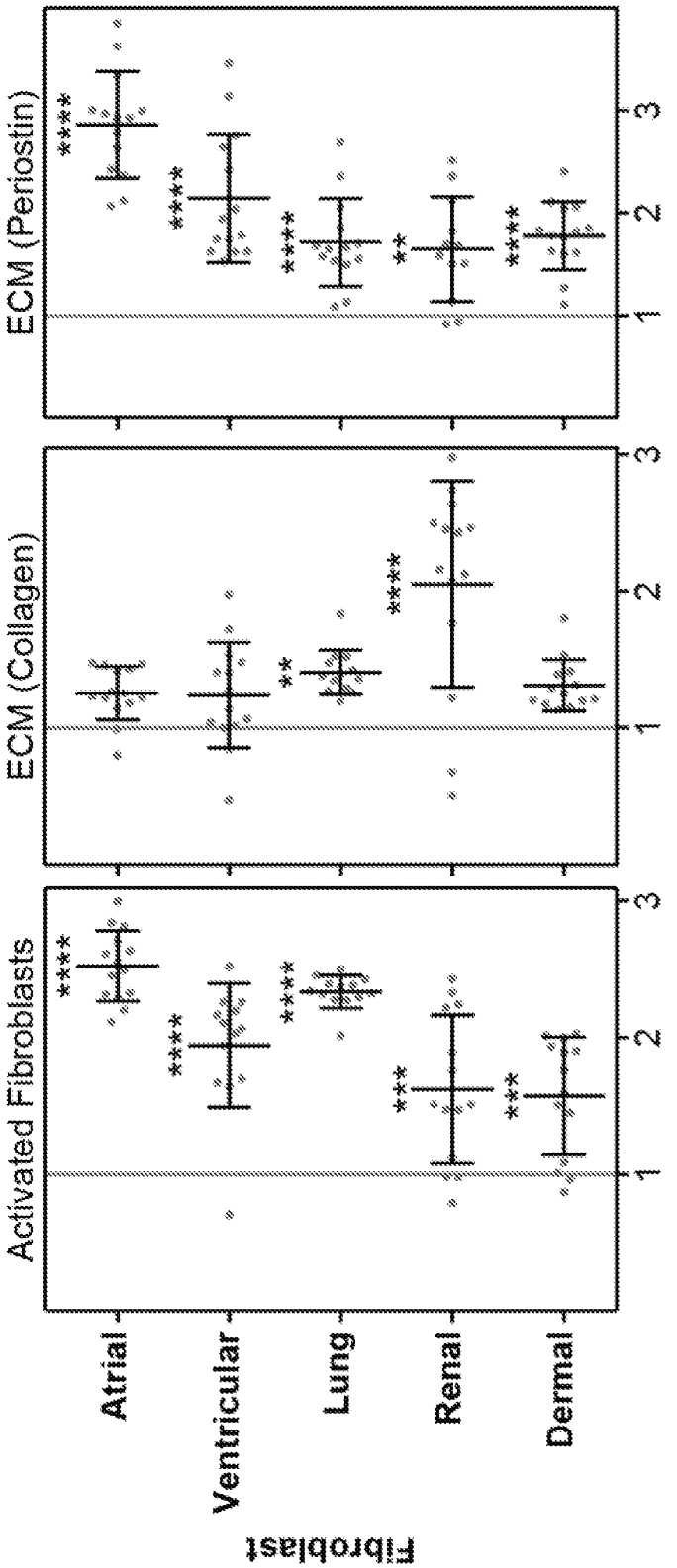

Activated Fibroblasts
ECM (Collagen)
ECM (Periostin)
Fibroblast
Atrial
Ventricular
Lung
Renal
Dermal
1
2
3
****
****
****
***
***
**
****
****
****
**
****

Figure 21A

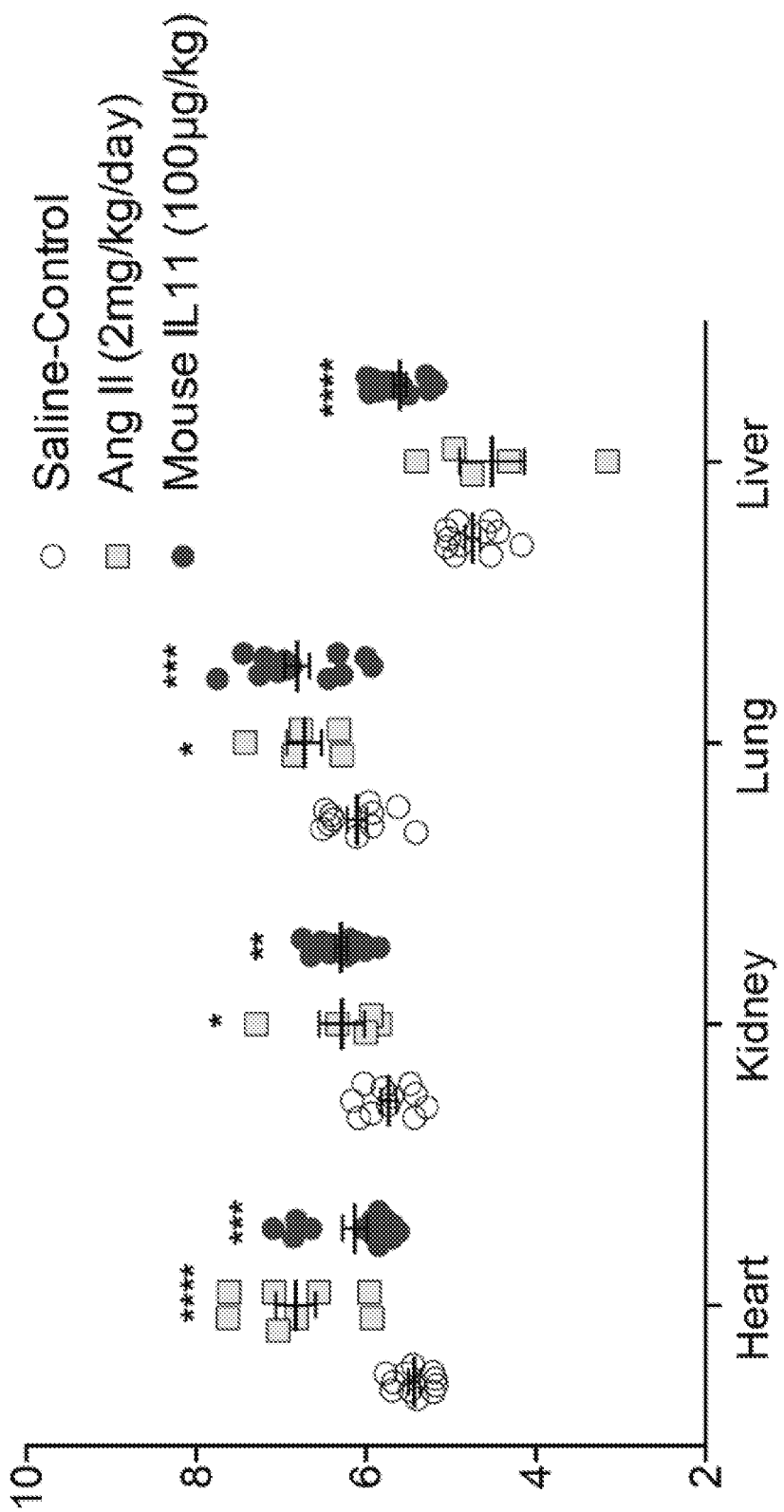

Saline-Control
Ang II (2mg/kg/day)
Mouse IL11 (100µg/kg)
10
8
6
4
2
Heart
Kidney
Lung
Liver
****
***
**
*
*
***
*
****

Figure 21B

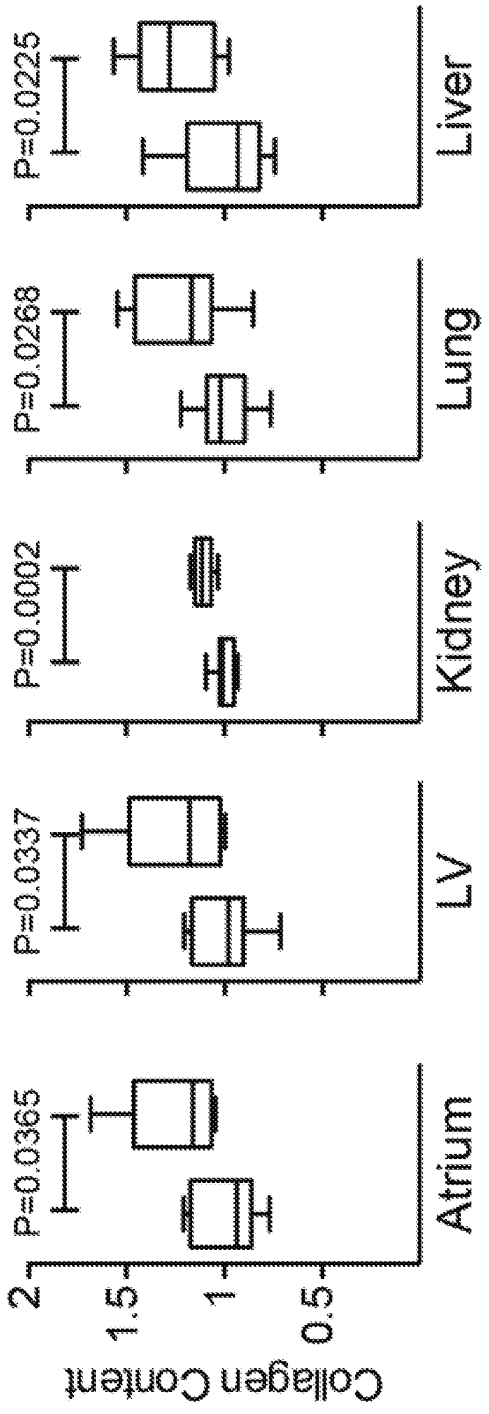

Collagen Content
2
1.5
1
0.5
P=0.0365
P=0.0337
P=0.0002
P=0.0268
P=0.0225
Atrium
LV
Kidney
Lung
Liver

Figure 21C

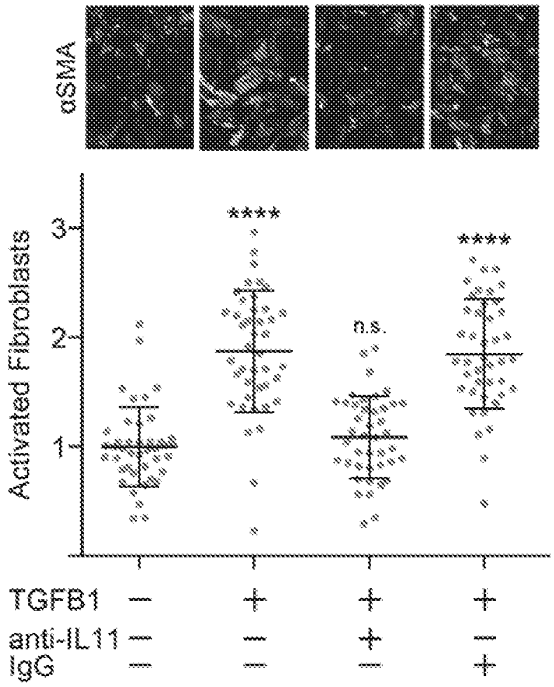


Figure 22A

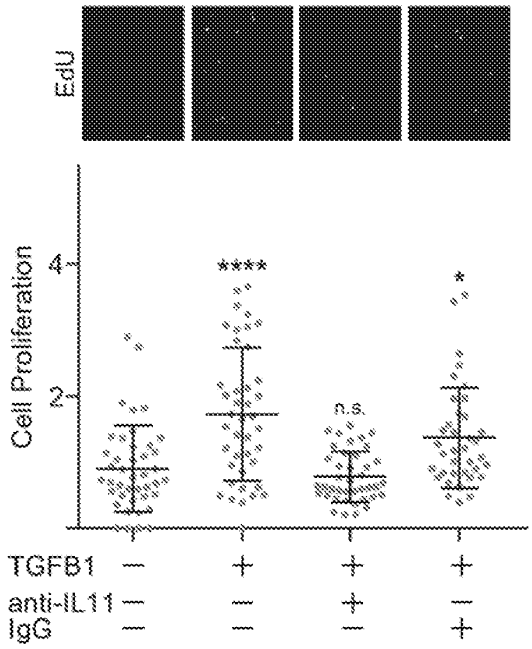


Figure 22B

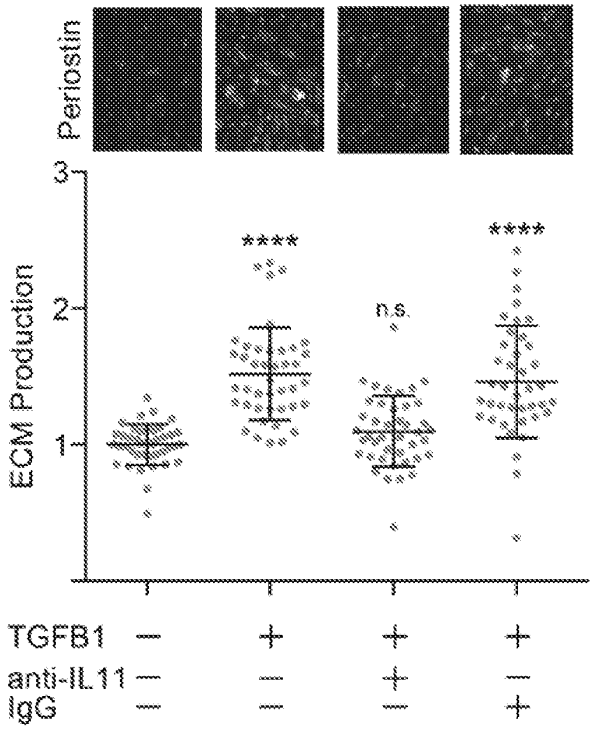


Figure 22C

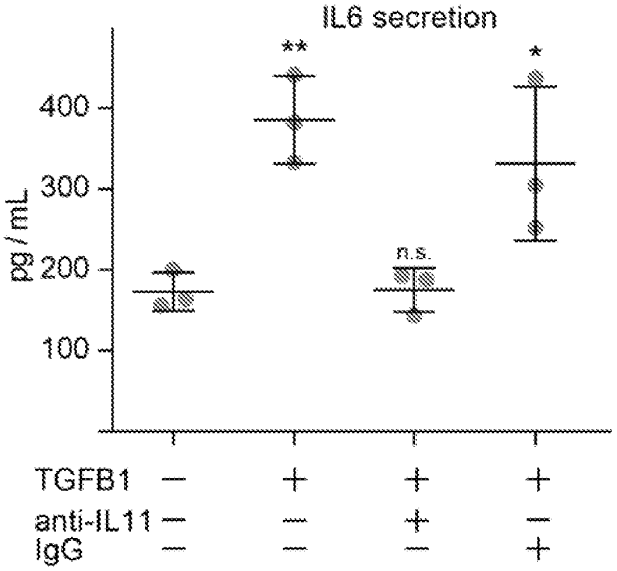


Figure 22D

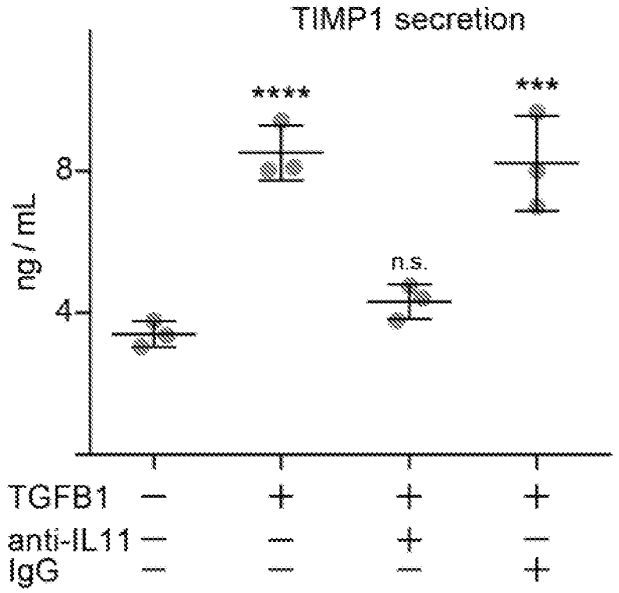


Figure 22E

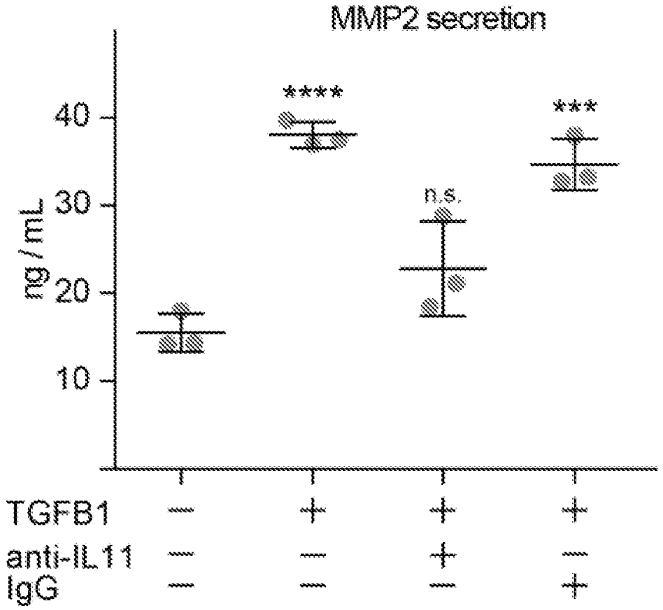


Figure 22F

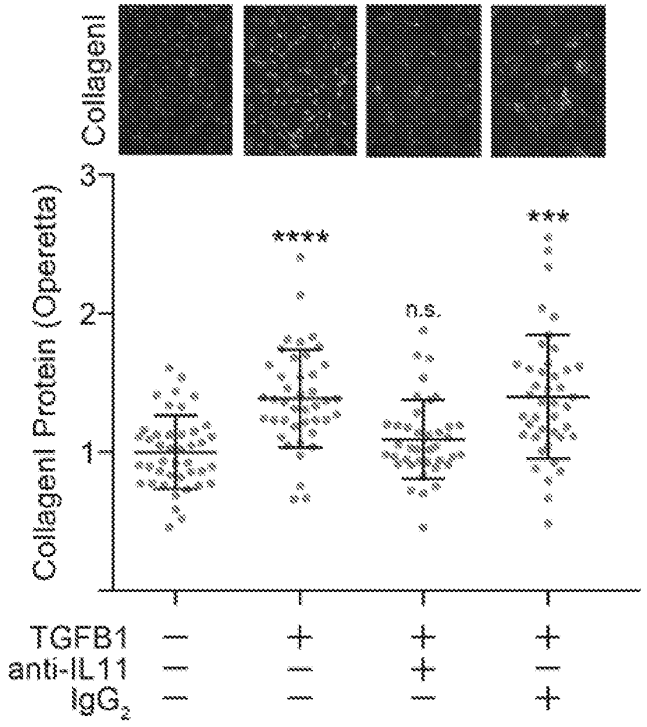


Figure 23A

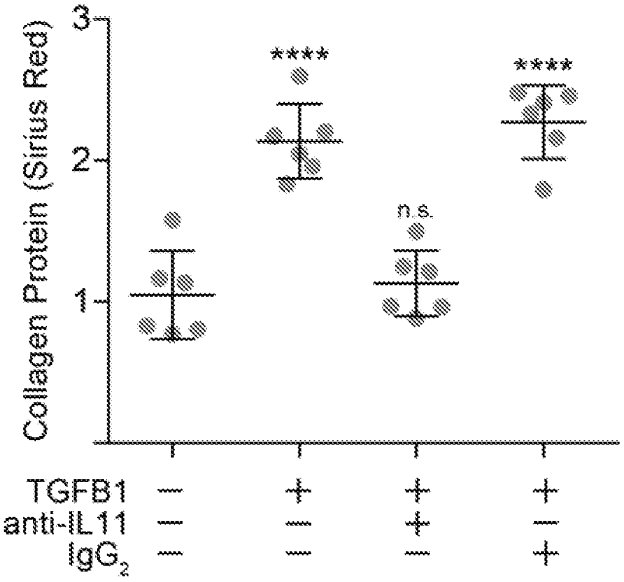


Figure 23B

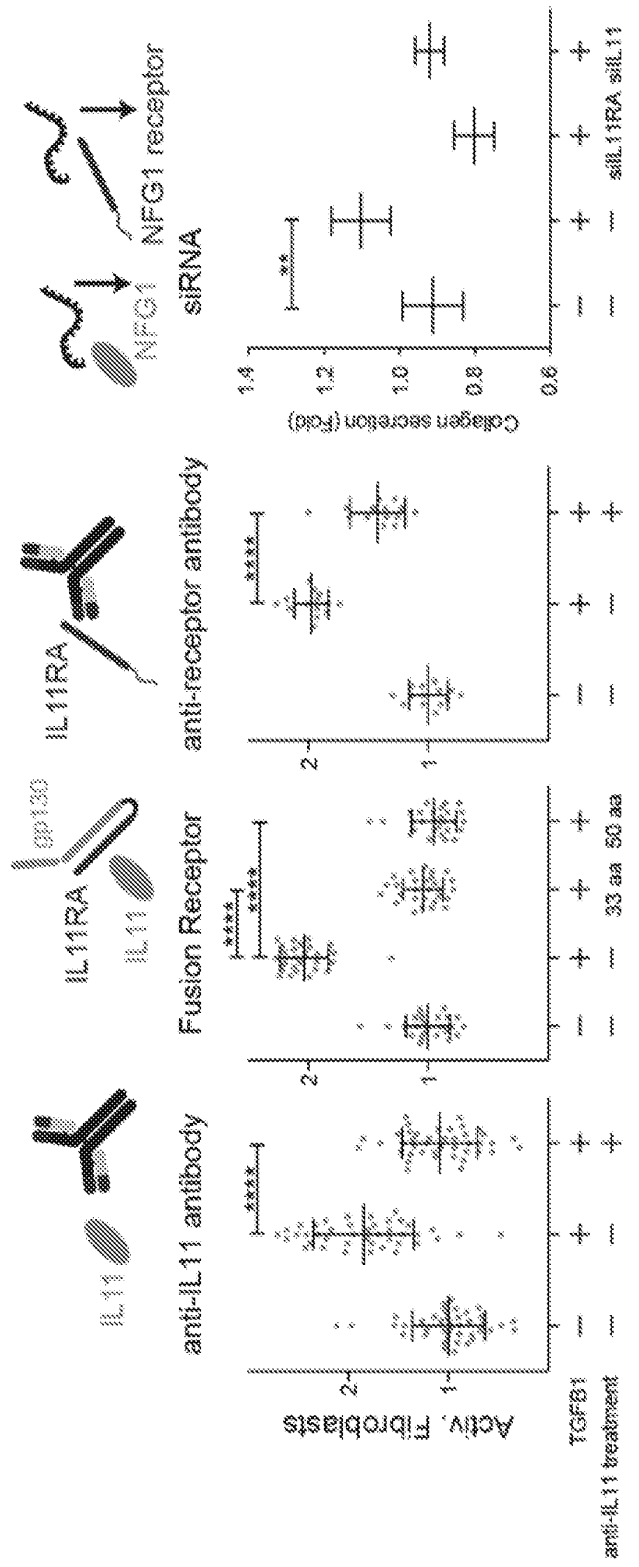

IL11
anti-IL11 antibody
gp130
IL11RA
IL11
Fusion Receptor
IL11RA
anti-receptor antibody
NFG1
NFG1 receptor
siRNA
Activ. Fibroblasts
Collagen secretion (Fold)
TGFB1
anti-IL11 treatment
33 aa
50 aa
linker between gp130 & IL11RA
siIL11RA
siIL11

Figure 24

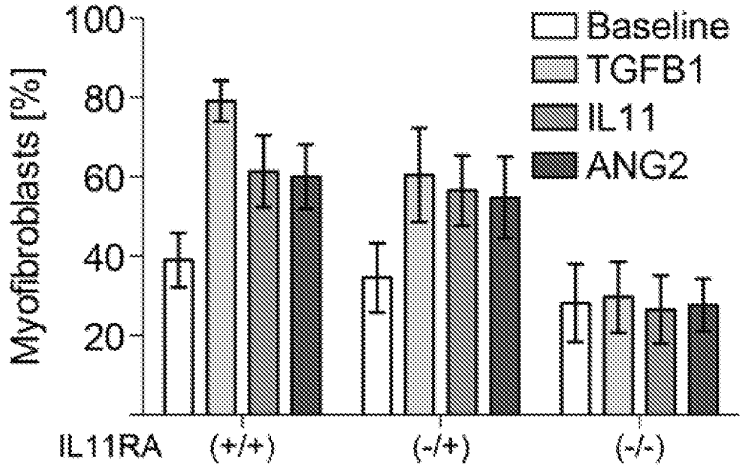


Figure 25A

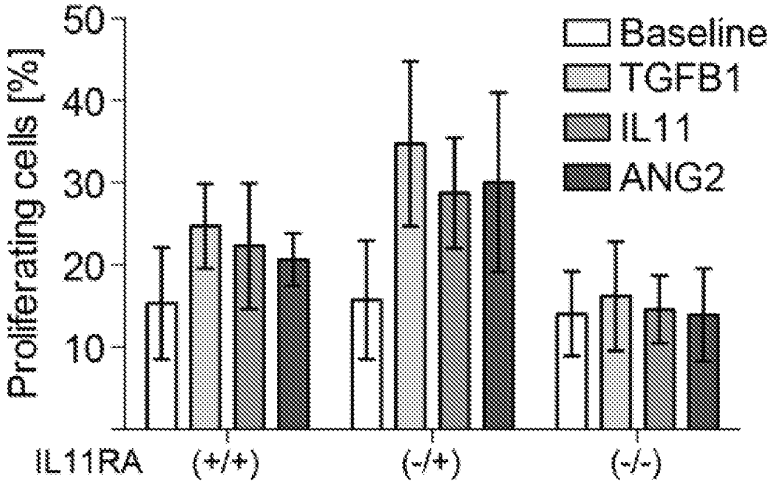


Figure 25B

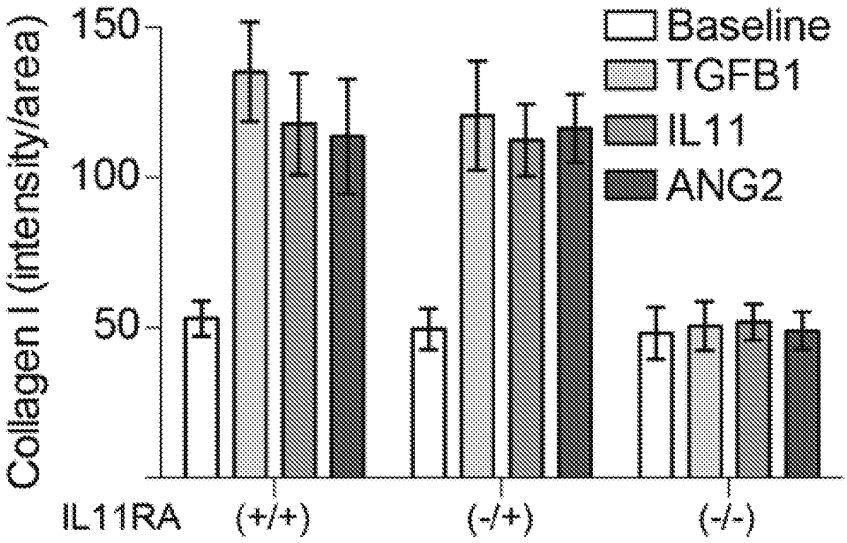


Figure 25C

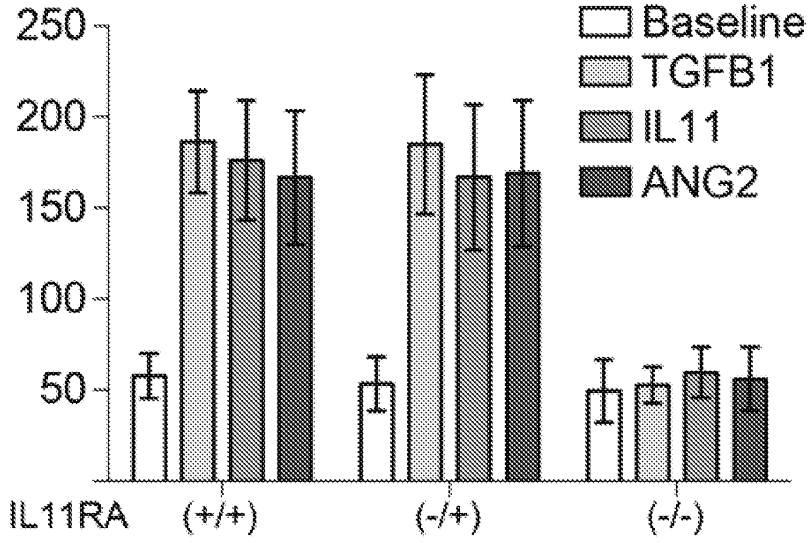


Figure 25D

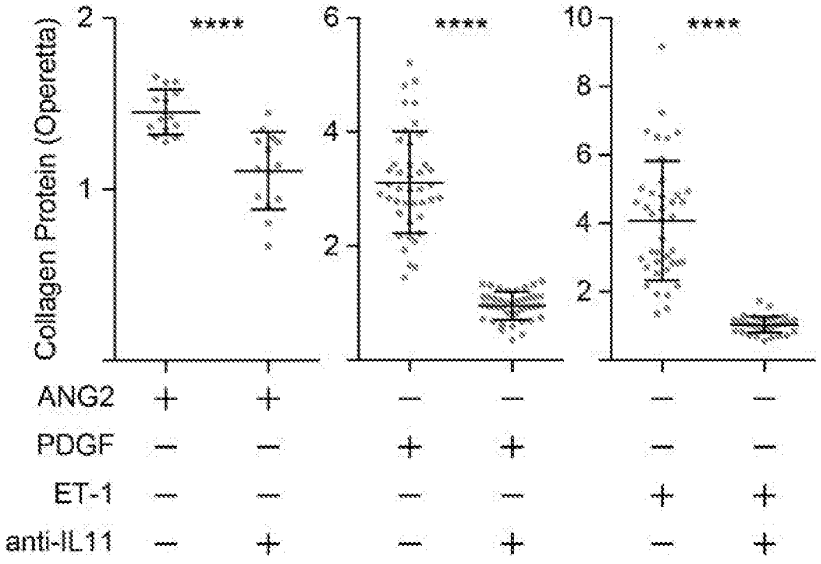


Figure 26A

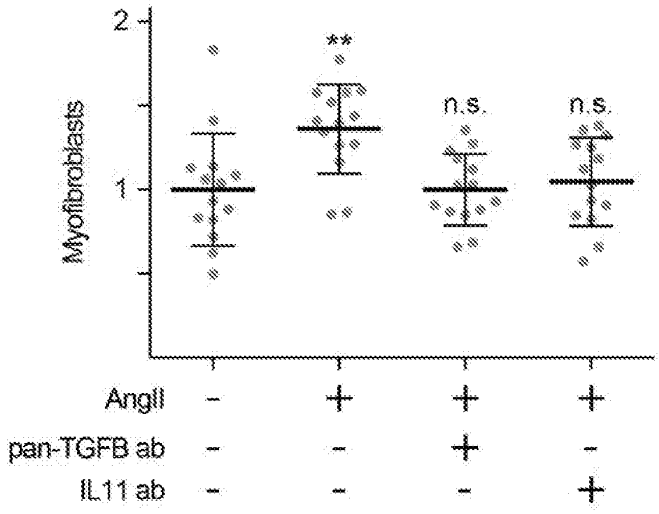


Figure 26B

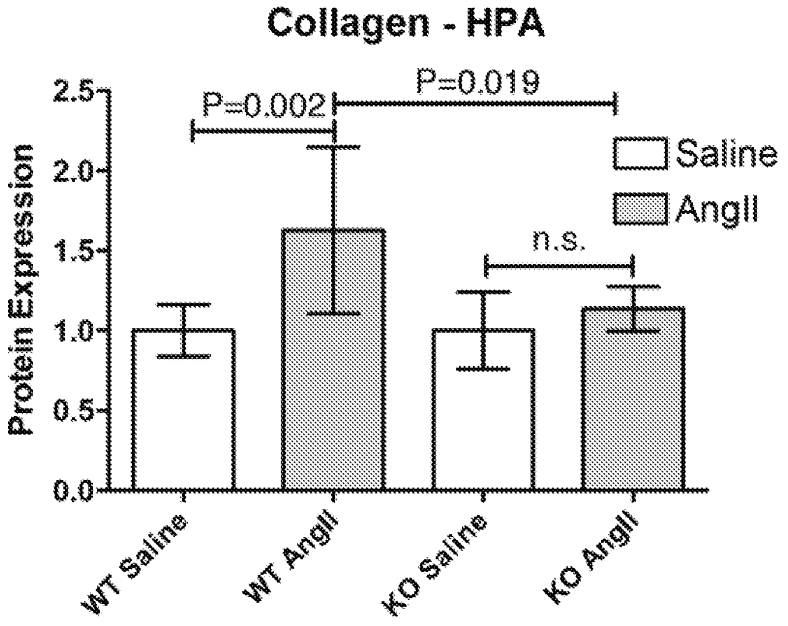


Figure 27A

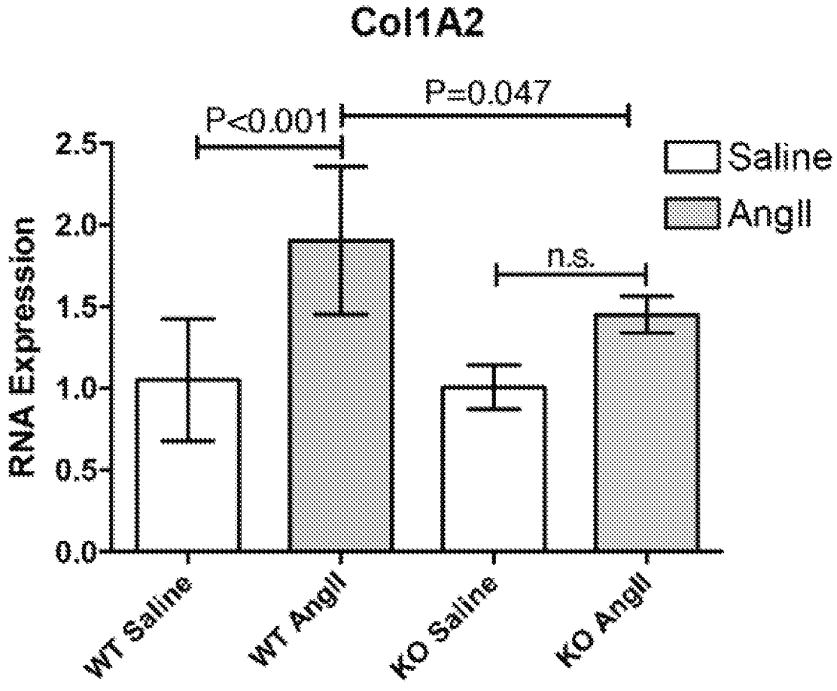


Figure 27B

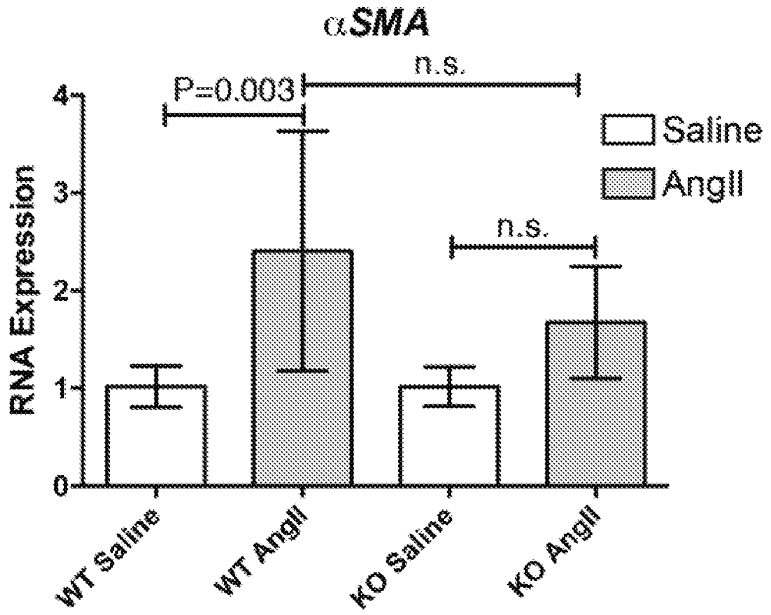


Figure 27C

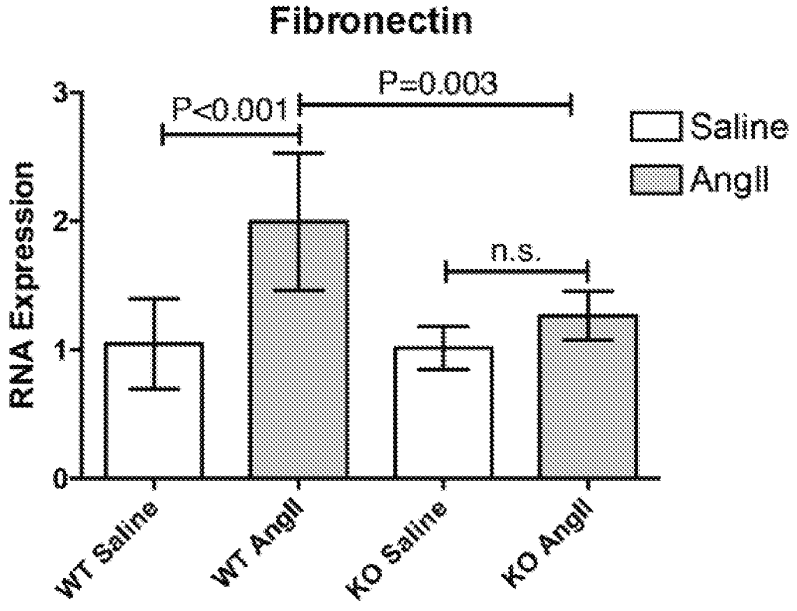


Figure 27D

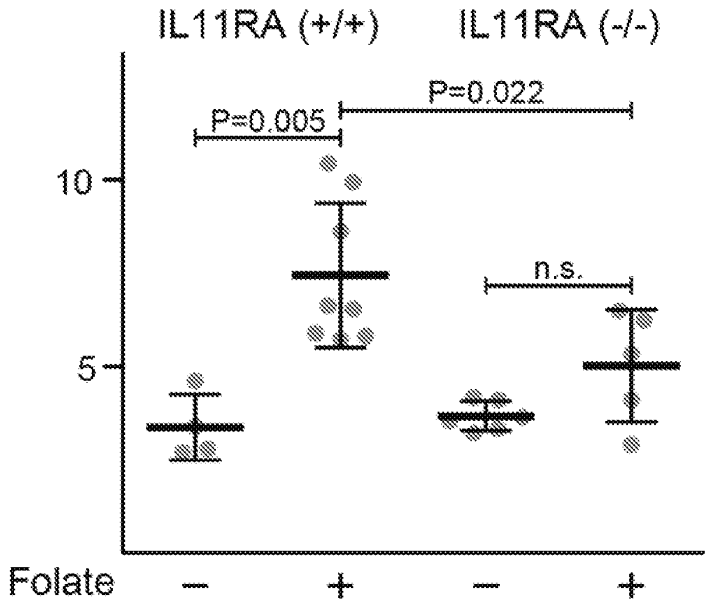


Figure 28

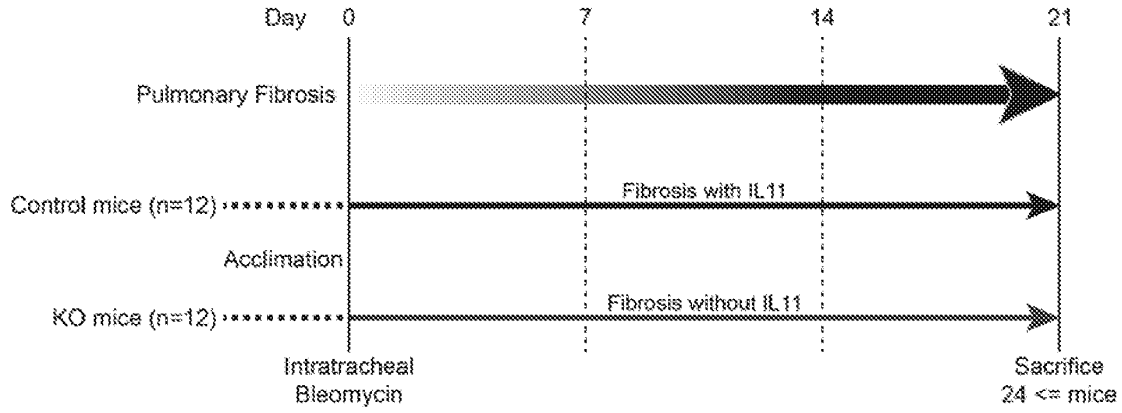


Figure 29A

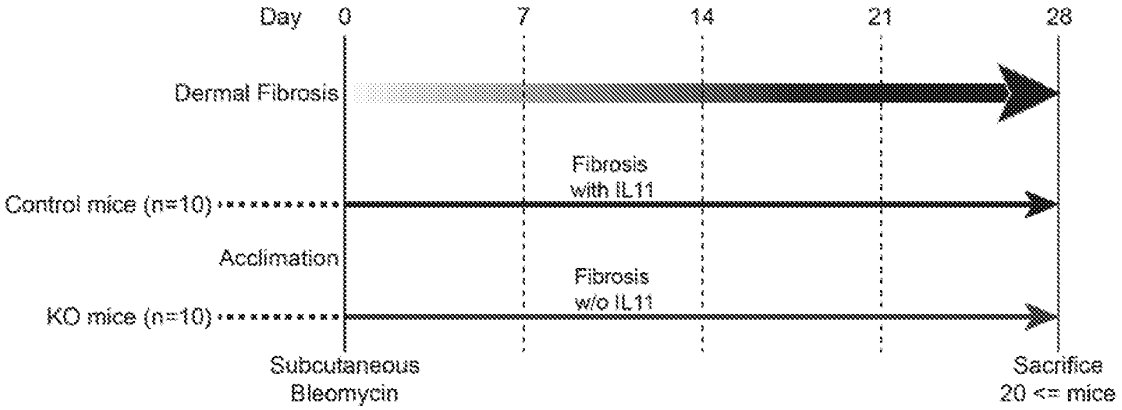


Figure 29B

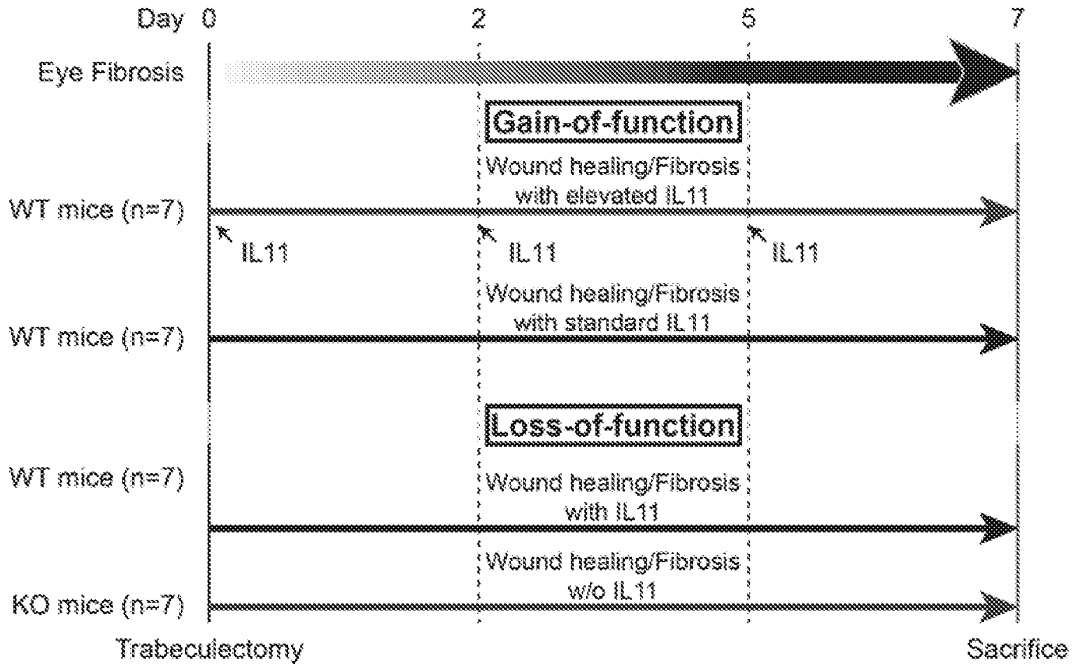

Day 0
2
5
7
Eye Fibrosis
Gain-of-function
Wound healing/Fibrosis with elevated IL11
WT mice (n=7)
IL11
IL11
IL11
Wound healing/Fibrosis with standard IL11
WT mice (n=7)
Loss-of-function
WT mice (n=7)
Wound healing/Fibrosis with IL11
Wound healing/Fibrosis w/o IL11
KO mice (n=7)
Trabeculectomy
Sacrifice

Figure 29C

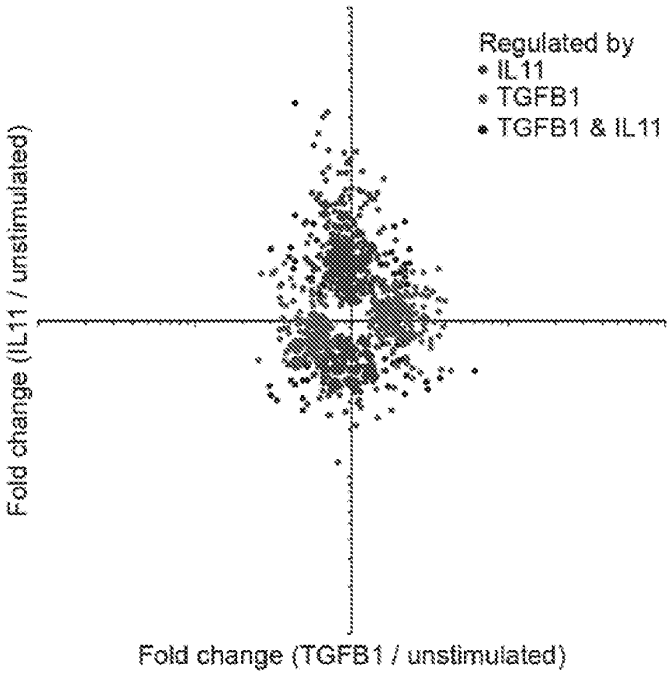


Figure 30A

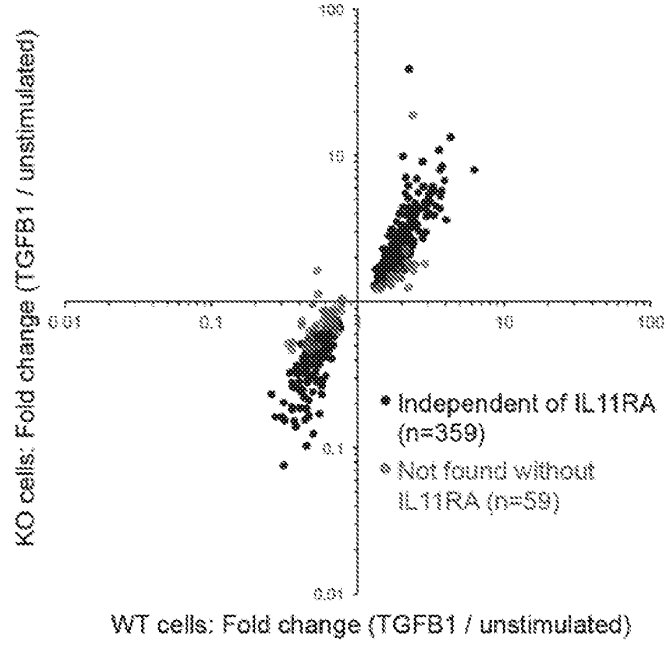


Figure 30B

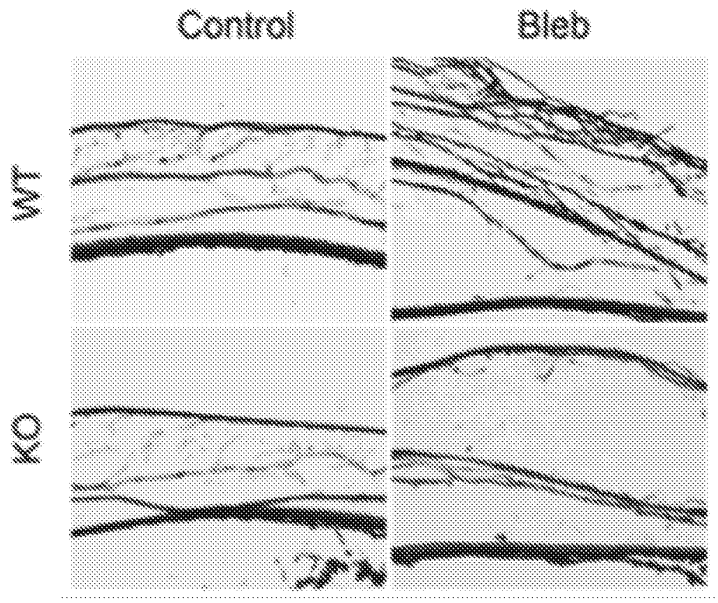


Figure 31A

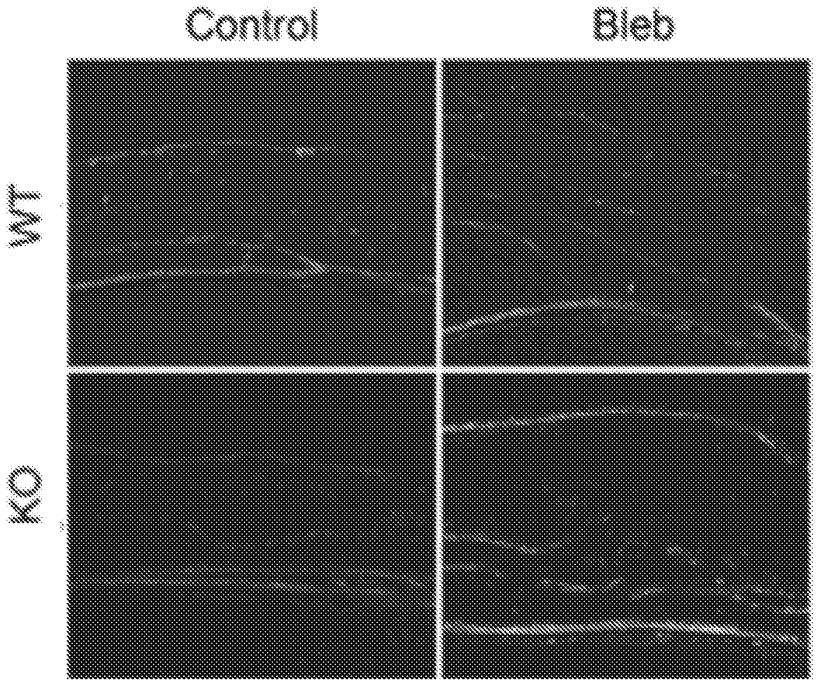


Figure 31B

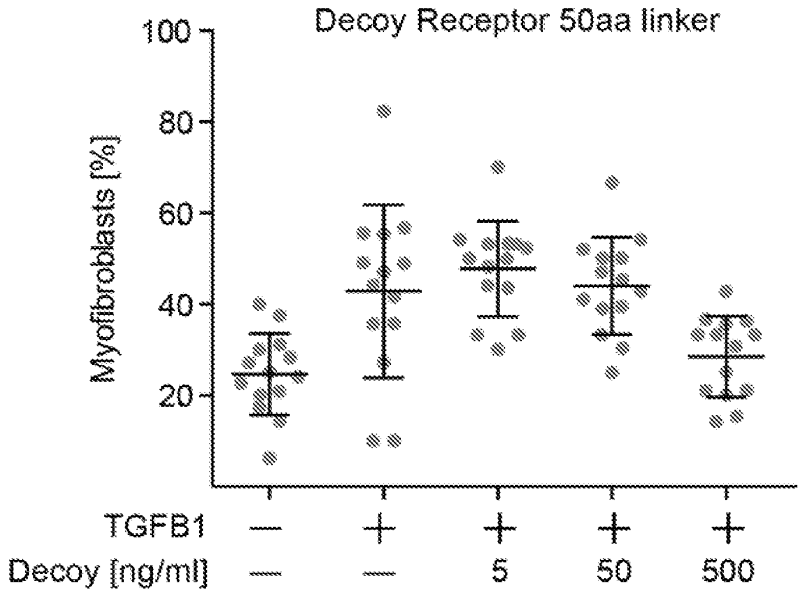


Figure 32A

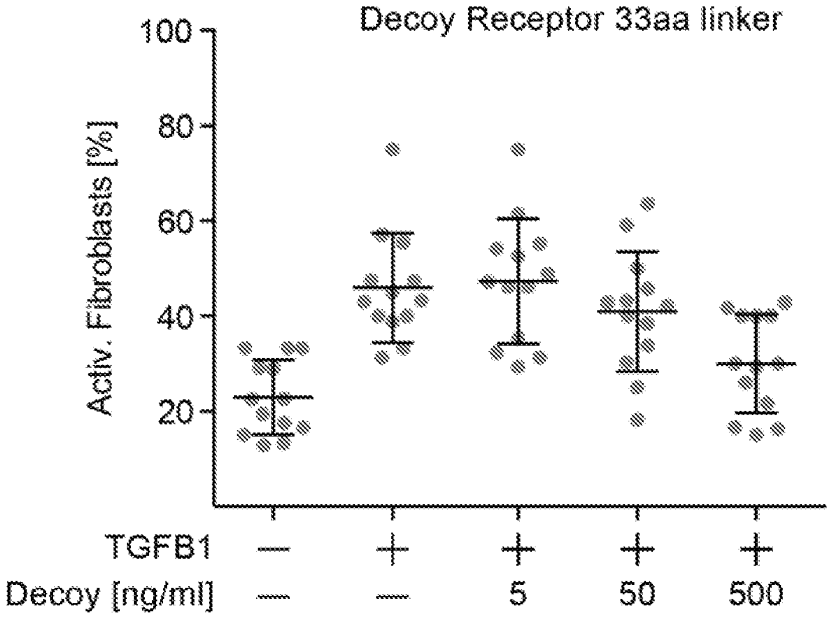


Figure 32B

SNP ID	Position (hg19)	P value	FDR	Genotype	Minor Allele	Sequence	SEQ ID NO
rs10831850	11-12566917	1.76E-07	0.0248	G/A	A	GTAAGGGATGTGAATCGGGTACTGA[A/G]GAAAGAGCCTGGATGCAGAGCCAGC	19
rs4756936	11-12599138	1.76E-07	0.0248	C/T	T	TTGATAACTTCAGCATCTGGATCAC[C/T]GTGGGATTAGCATCTGTTTGTATTT	20
rs6485827	11-12578182	1.76E-07	0.0248	C/T	C	GTGTGATTGCTTAAAAAAAACTACT[C/T]ACATTGTTTTGAATCACACCTCACA	21
rs7120273	11-12581045	1.44E-07	0.0248	T/C	T	GCTCAGCTAATCAATGACCAGTCTC[C/T]TTAATTCTTCTAATGCCTATATGGT	22
rs895468	2-223621273	2.70E-07	0.0306	G/A	G	GCAGTGCTCAGAAGAGCAGCAGCCA[A/G]TGACATTTTGGGGCTATAAGAGGTA	23

Figure 33

SNP ID	Position (hg19)	P value	FDR	Genotype	Minor Allele	Sequence	SEQ ID NO
rs1000574	19-55114775	0.0432	0.6581	C/T	T	TGTCCAGTAAATACTTAACATTTTA[C/T]GTGCA ATGTATGTCATAAATATGGG	24
rs10403345	19-56741808	0.0355	0.6372	G/A	G	GGgtgaagtttggaaacaggtatac[A/G]ttgtgatgcaatcgtca gaaccaag	25
rs10419994	19-55726220	0.0334	0.6372	C/T	T	aaaccataGTATCATCCTTCCCAAA[C/T]AGTCAAC CCAGggaatcacagagat	26
rs10426177	19-56269096	0.0103	0.6372	C/T	T	TAAGACGCTATTCTCTAATTCTGAA[C/T]GGAAG AACTCCTCTCCCAAGACATG	27
rs11084337	19-54916104	0.0370	0.6372	C/T	C	aggtggaacaacacaaagggtgggg[C/T]gaggcgtgcaattta aacattttct	28
rs11671244	19-56502995	0.0242	0.6372	C/A	A	TATTAGATTTTGTGTGGGATTTCAT[A/C]GTTAC ATTTGTTACCAGCCCAATTT	29
rs11882068	19-56227165	0.0005	0.2204	A/G	G	GATTCCAGTTCCAAGTCACATCATC[A/G]CCAG CTGGAAGACCTAGGGCAAAAG	30
rs12104147	19-54935505	0.0150	0.6372	G/A	A	ACCATGACGGTGTCCTCATTGCTTT[A/G]ACCAT TAGTAAtcattcattcattc	31
rs12608558	19-55715090	0.0275	0.6372	A/G	G	ACCTGTCACATTTTGTCAGCTCCCA[A/G]CCAC CCCCTACCACTGTCCTTATAA	32

Figure 33 cont.

rs12610132	19-55032293	0.0408	0.6372	A/G	G	GACACGATGTCTTCAGTCTCCAGCT[A/G]AGCT TGGACTGTGAGGATGGGTCAG	33
rs12610617	19-56443519	0.0030	0.3650	C/T	T	CTCTCAAGTTTGCCCAGGGGATACG[C/T]GGGA AGTGCCCCTGGGGGGCCGACC	34
rs160104	19-56433203	0.0012	0.2352	G/T	T	ctggtctcttccagctctggtggct[G/T]ccagtatttcttgggttgtggc ctc	35
rs16986899	19-56549510	0.0265	0.6372	T/C	C	AACAAGGTGACAGACCAGGGAGTAA[C/T]GCCT CTCAGTGATGCCTTGAGAGTC	36
rs17815373	19-56608900	0.0483	0.7007	G/A	A	CGGCAGGCAGTAGGATGGACTGCGT[A/G]GAC GGCGGCCAGCATGTAAATGAAA	37
rs1895375	19-55035001	0.0329	0.6372	T/C	C	AAGTAAGGTGTCAAGGAGGCCATGC[C/T]CACT CTGTAGGTTCTAGGAAAGAAT	38
rs2043690	19-56383651	0.0305	0.6372	T/C	T	ATGCCTGAAAGAAACAAGAGCAAAT[C/T]GTCT CAGGAGGTAGGTAATAGGATG	39
rs2194	19-56257259	0.0064	0.5195	G/A	A	AGCATATTCATTGATTTCCTTACAT[A/G]CAAAT GCTCCTTTTTAAGTGCTCAA	40
rs2288419	19-55693244	0.0140	0.6372	A/G	G	TCAGTACGTATTCCTGCATCAGTGC[A/G]TCCT GCGGTTCCTCCAACAGTCAGC	41
rs2288521	19-55708557	0.0262	0.6372	T/C	T	AGTGGAGGCCCTGGAACCCGGGACG[C/T]TGT ACAATTTCACCGTGTGGGCAGA	42
rs2288527	19-55699077	0.0454	0.6738	A/G	G	TGCCATATAATCTCAGGGTGCAACG[A/G]ATAA ACAAGGGGTGATGCCGAAGAA	43

Figure 33 cont.

rs2637107	19-56566741	0.0317	0.6372	G/A	A	CTCTGTCCCCTCAACTTCTTTCTAC[A/G]TGGTC ATGTCCCTTCTTTAGTTCCT	44
rs299164	19-56319796	0.0273	0.6372	A/C	C	GCAACAAAAATCTTATACATCACCA[A/C]ATGTC TGCTTAGCGGCAGAATTGCC	45
rs299169	19-56316796	0.0324	0.6372	G/A	G	GCTAGGTAAAGGACTCTGAAAATAC[A/G]GCAA CATGGAAAACATCCAGTCTCC	46
rs303997	19-56424443	0.0007	0.2204	T/C	T	TCCATTTGCCCAGTGCAGCATAGCC[C/T]GCAT TGCCAAGGTGGTCTTCCCAAC	47
rs304001	19-56423668	0.0015	0.2352	C/T	C	CGTTTAACAAAGAAGACACTGAGAT[C/T]GAGG GCCTGGAAGTGCCTTTCATTG	48
rs304002	19-56423254	0.0141	0.6372	C/T	C	TTCTACGACTTTTTCACTGCCTACA[C/T]GAGTC CCAGGAGGAAGACTTCACAA	49
rs306463	19-56497077	0.0068	0.5195	T/G	T	gccaaatgtgtttgaaaattccatt[G/T]gaagaatttatggtgaatgc atttt	50
rs310445	19-56173551	0.0396	0.6372	G/A	G	GAAGATTGTTCAAGAAAAGGCAGAG[A/G]GCAT GATGACAACACAAAATGAAGA	51
rs3745429	19-54937593	0.0233	0.6372	A/G	G	CCTGGGACTATCCCCTGGCCGGGCC[A/G]CAC ACATGTGCCCTGTGACCAGGGA	52
rs3786867	19-55684200	0.0394	0.6372	C/T	T	GCTGGCTGTGAGGAGTCCGCGAGAA[C/T]TCC CTTGCTTGTCCATGAATTTATC	53
rs4629084	19-56420157	0.0243	0.6372	G/T	G	AAGGTGAAGAGTGGGAAAAGGCAGA[G/T]GATC AGGAAAAATAACTAATGGGTA	54

Figure 33 cont.

rs464765	19-56430224	0.0208	0.6372	A/C	A	ggcttaataaaaggaattagagagc[A/C]ctccctcctctccaacatctttttc	55
rs4801278	19-56246520	0.0268	0.6372	G/A	A	TGGTCTGTGCTCTCCTTGCACACTC[A/G]TCTGTGGACATCACAGGAGGGAACA	56
rs4801635	19-56372705	0.0352	0.6372	C/T	C	GTGCGTATATGTGTTACAAGTGGCT[C/T]GTGTTGACCGCCTGCCTGTGGAAAG	57
rs516022	19-56609371	0.0408	0.6372	C/A	C	GCCTCGGGTGCTGACCGGGGGTGCC[A/C]TTGCTGGGCTTAGCAGGCCGGGCTT	58
rs6509882	19-55030183	0.0400	0.6372	A/G	G	TGATGTGCCACATCCTGTATAGGAA[A/G]CAGGTGATGTGGAAATGAGTCAGAC	59
rs6509883	19-55030376	0.0378	0.6372	A/G	G	CATGAAAACCCCTCAGATGGTACCA[A/G]AAATATAGACAATTGATCCAGAGAG	60
rs6509933	19-55683104	0.0188	0.6372	T/C	T	gtcccagctactcacacaggaggat[C/T]gcaggagtttgaaaccagcctgtgc	61
rs6509939	19-55851708	0.0337	0.6372	T/C	C	CACCCTCAGGGCCTCTCCCTGACCC[C/T]TCTCTCACCCCCGGGACCTCCCTGC	62
rs8110255	19-56568978	0.0045	0.4527	T/C	C	GCAGGTCTGTGTTCCTTGTTATGTT[C/T]CCTTGCTACAGGCACTCTCAGCCTT	63
rs8112791	19-56723492	0.0236	0.6372	C/A	C	gtcagtttgtgaacaggtaaaatca[A/C]aatgctcatgttctctacagggaaa	64
rs873732	19-56745520	0.0194	0.6372	G/A	A	AGCCAATTCGTCAGTGAATGAGGCA[A/G]AGAAATTGGTAAAAAGAAGGAAAGT	65

Figure 34

SNP ID	Position (hg19)	P value	FDR	Genotype	Minor Allele	Sequence	SEQ ID NO
rs2823699	21-17628689	1.62E-08	0.0033	T/C	T	TACATAGTAGGCTTAAGAGCAAATG[C/T]CTACC TTTTCCTCTGTTTTCAACTC	66
rs4973978	3-41667545	1.69E-08	0.0033	T/G	G	GTTGGTTGGTTTGTTTCCCCTTTAA[G/T]GGTGC CATTTAATGACAGATTTCAT	67
rs11744285	5-124622296	1.75E-08	0.0033	A/G	G	GCCCCGGTGACAAGAATGGCAAAAC[A/G]TTTA TTCGGCATTAACAATGTGTAA	68
rs7120273	11-12581045	3.32E-08	0.0041	T/C	T	GCTCAGCTAATCAATGACCAGTCTC[C/T]TTAAT TCTTCTAATGCCTATATGGT	69
rs10831850	11-12566917	5.12E-08	0.0041	G/A	A	GTAAGGGATGTGAATCGGGTACTGA[A/G]GAAA GAGCCTGGATGCAGAGCCAGC	70
rs4756936	11-12599138	5.12E-08	0.0041	C/T	T	TTGATAACTTCAGCATCTGGATCAC[C/T]GTGG GATTAGCATCTGTTTGTATTT	71
rs6485827	11-12578182	5.12E-08	0.0041	C/T	C	GTGTGATTGCTTAAAAAAAACTACT[C/T]ACATT GTTTTGAATCACACCTCACA	72

Figure 34 cont.

rs7079768	10-131271870	9.92E-08	0.0070	G/A	A	ACTTGTGCCAGGCTGGCTTTGCAAC[A/G]ATGAGCCTGAGAAGCTGTTAGAAGT	73
rs1293764	12-113425679	1.27E-07	0.0080	A/G	A	GAGACACAAGAGGTGGGCAGGTCTT[A/G]GGGATTTAGGAGTTGGGTTCAAGGC	74
rs1862945	7-50936184	6.84E-07	0.0387	G/A	A	TGAGTCTGTGAGGAGAAATGAACAA[A/G]TCTACCACAGTCATCCAGAATGAGA	75
rs10496038	2-55340963	9.41E-07	0.0484	C/T	T	ATCAGAAGGCTAAGGAACCACCTGT[C/T]TAATAGTCTGGTGCCAAACACAGGC	76
rs12669489	7-50961697	1.05E-06	0.0493	G/A	A	catcaataagaaaaacaaaaataat[A/G]taatagaaaaatgcataagagactt	77

Figure 35

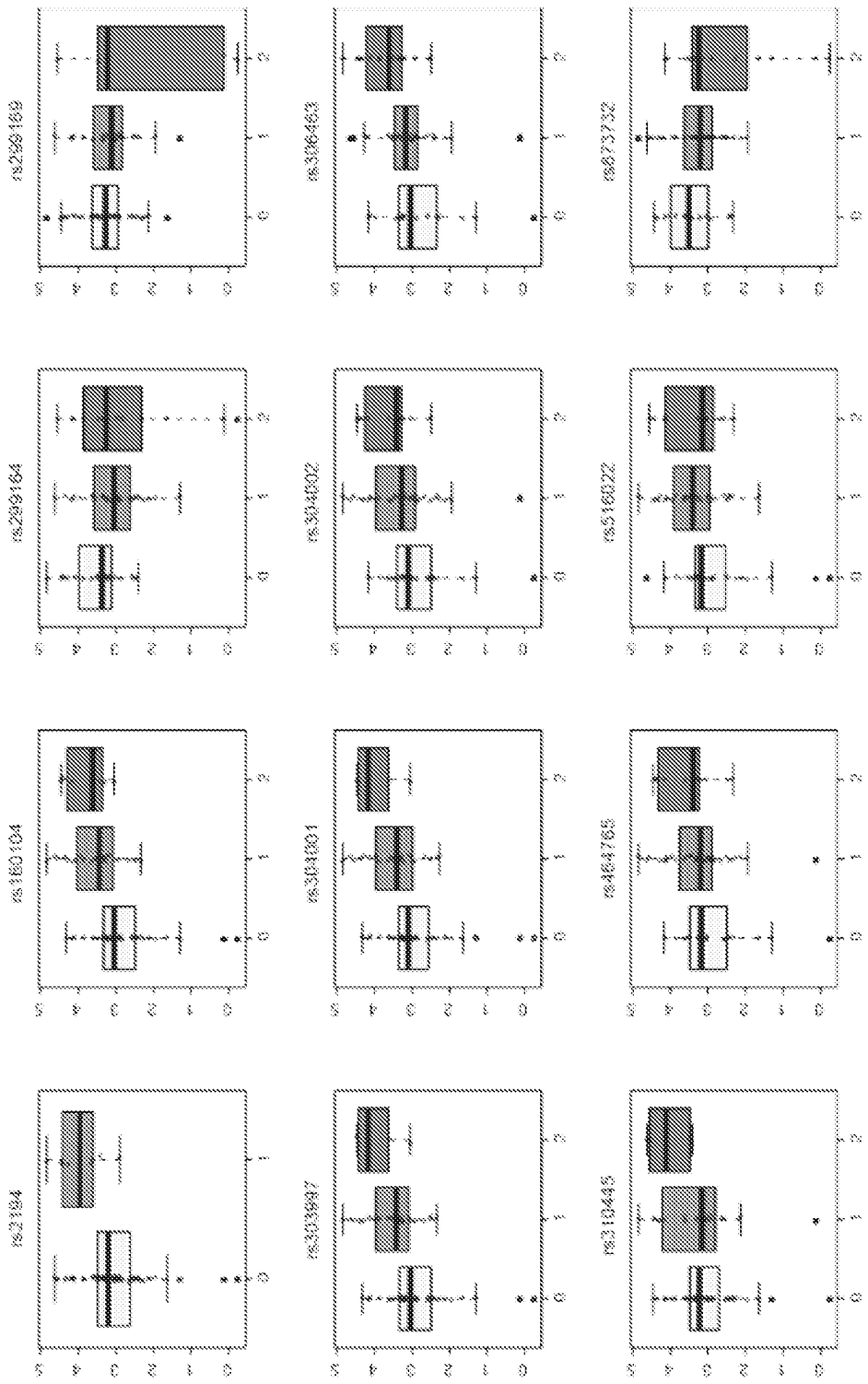

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Figure 36A

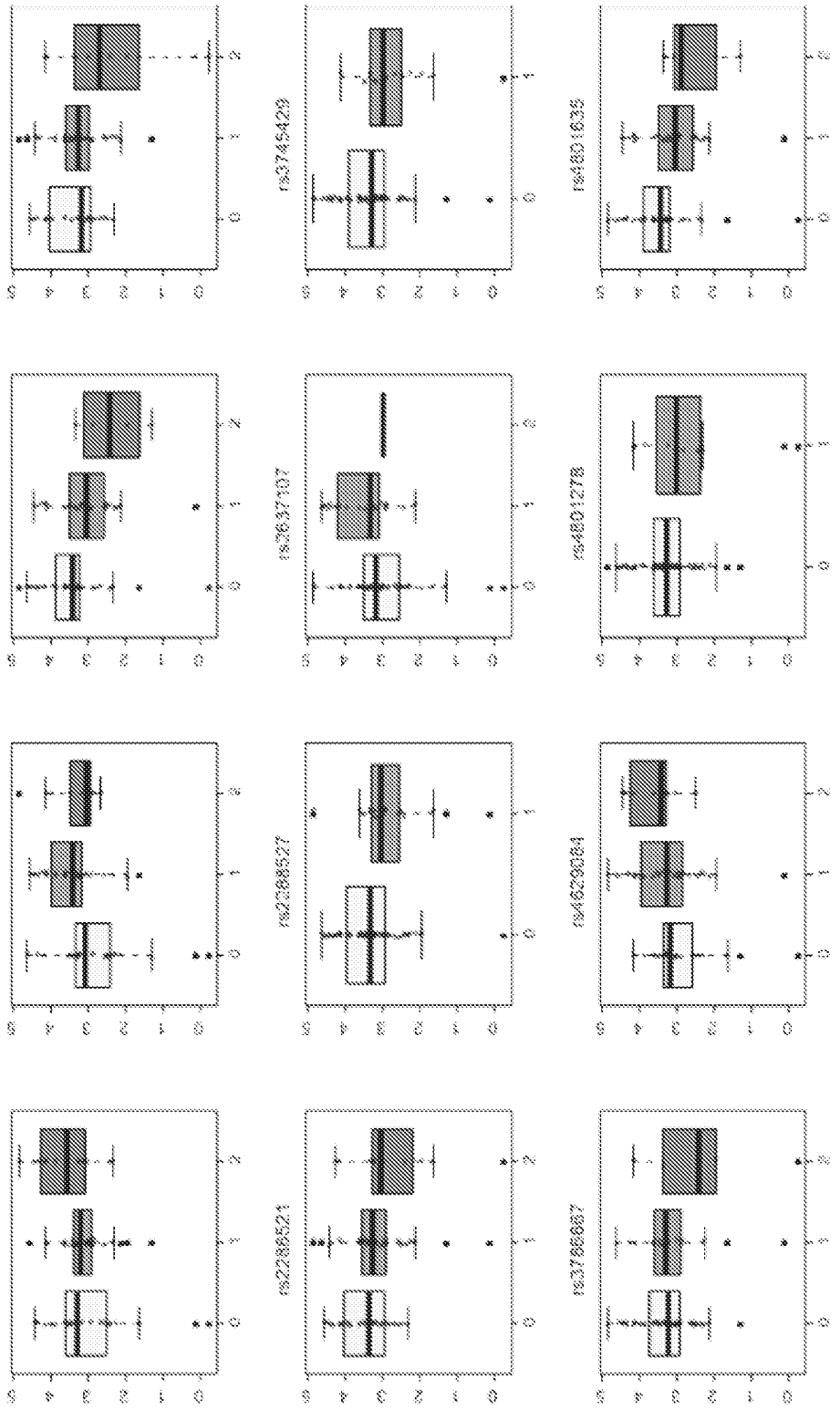
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Figure 36B

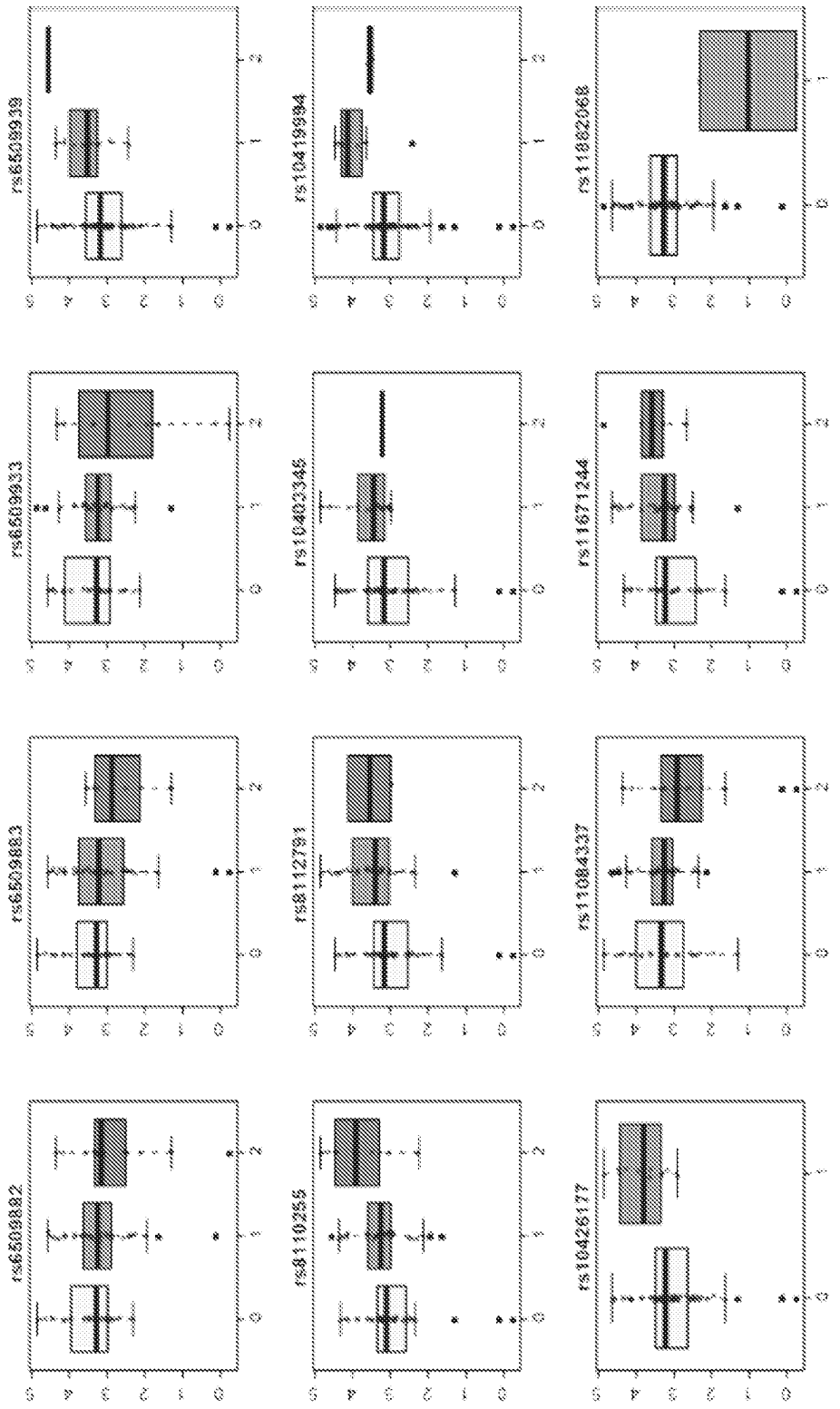
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Figure 36C

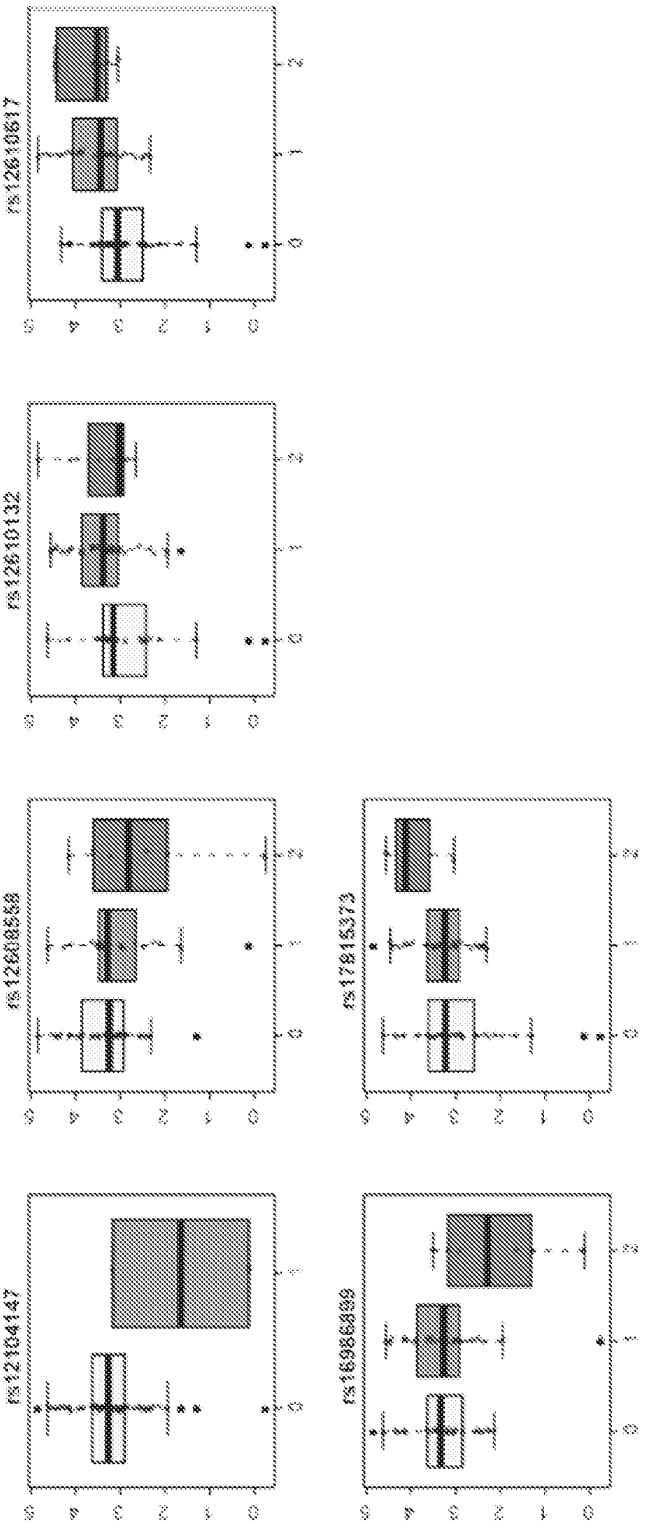
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Figure 36D

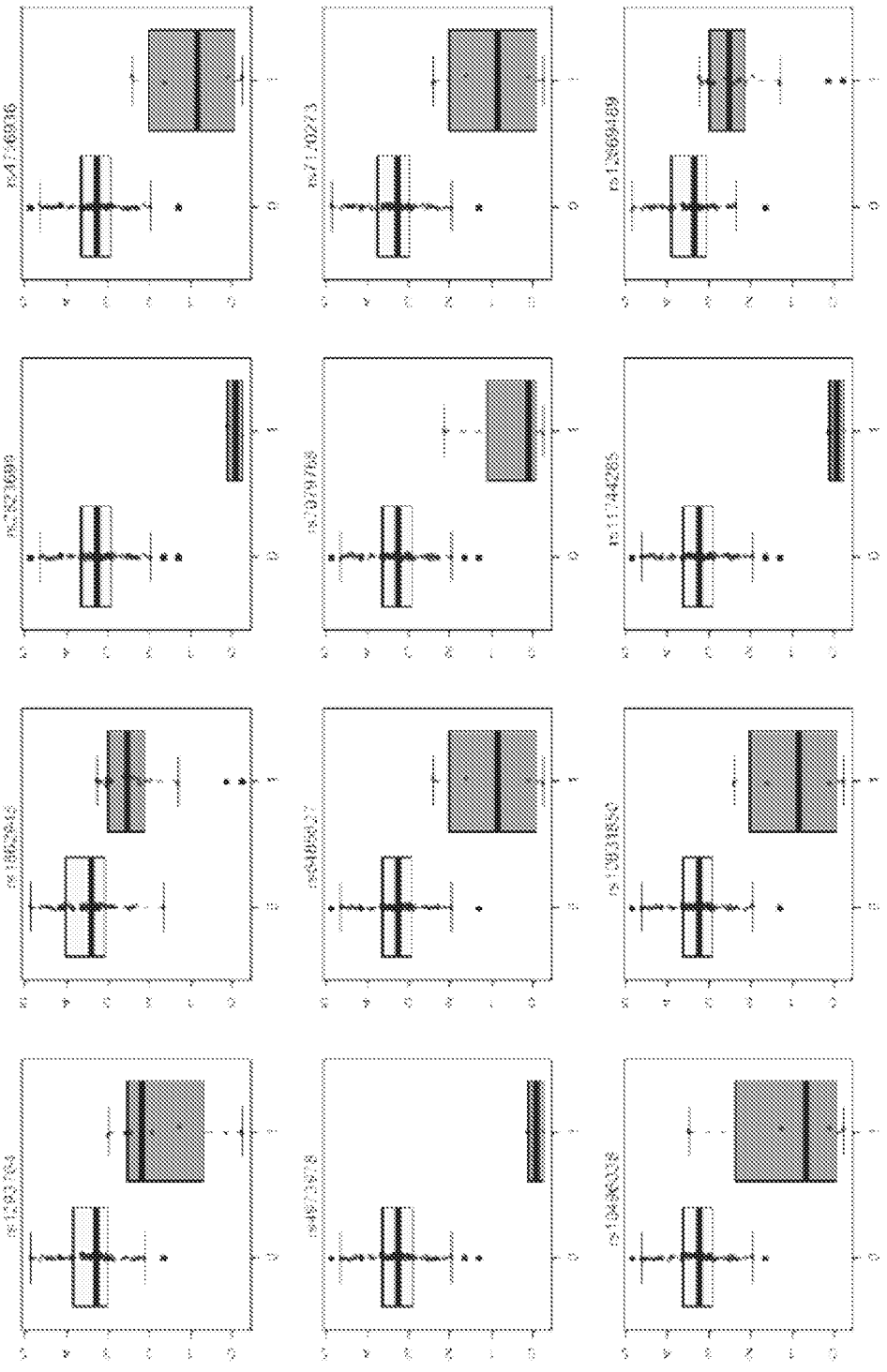

Figure 37

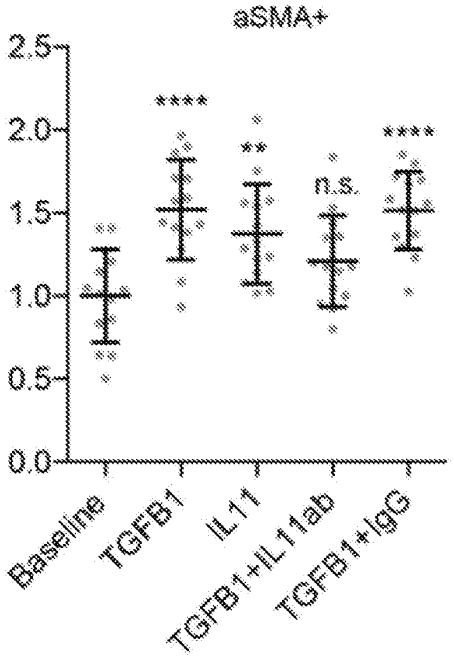

Figure 38A

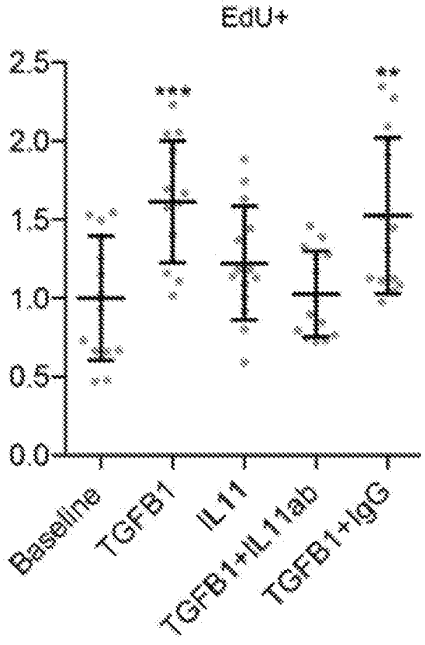

Figure 38B

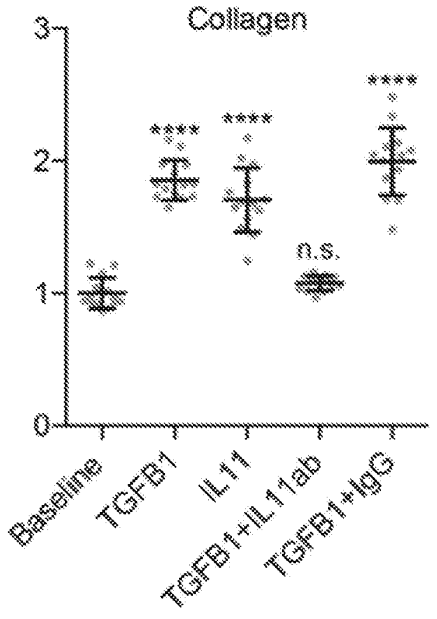

Figure 38C

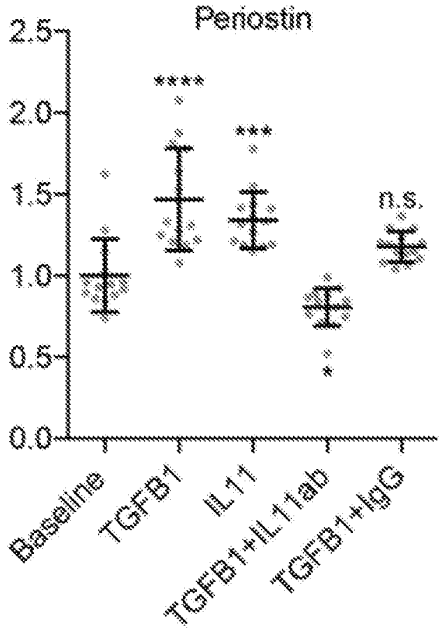

Figure 38D

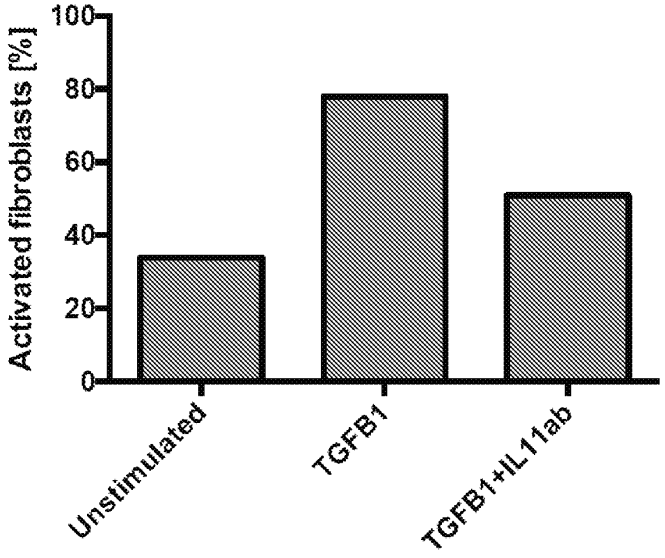

Figure 39

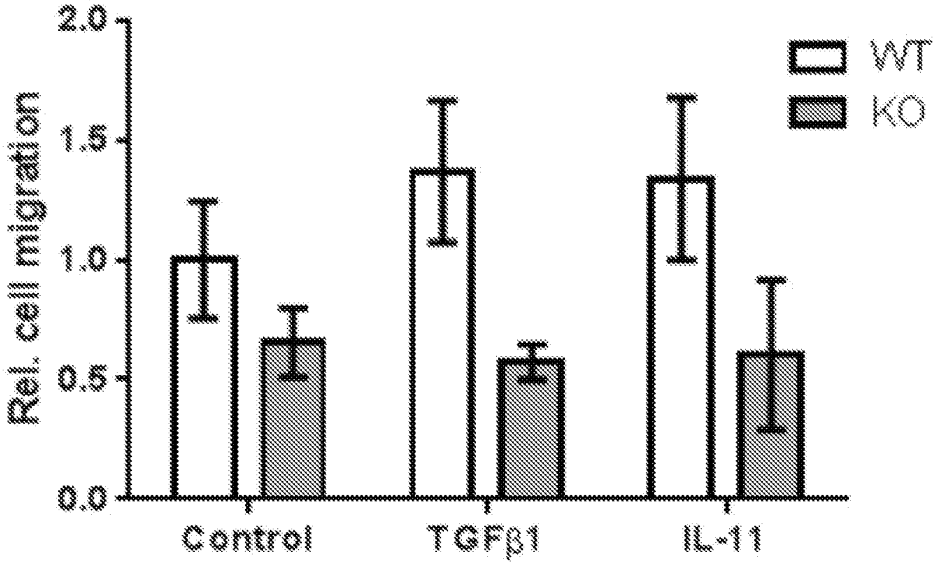

Figure 40

DECLARATION (37 C.F.R. § 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 C.F.R. § 1.76)

Title of Invention	TREATMENT OF FIBROSIS

As the below named inventor, I declare that:

This declaration is directed to:

☐ The attached application, or

☒ United States application or PCT international application number 15/381,822 filed on December 16, 2016

The above-identified application was made or authorized to be made by me.

I believe I am the original inventor or an original joint inventor of a claimed invention in the application.

I have reviewed and understand the contents of the above-identified application, including the claims.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in 37 C.F.R. § 1.56.

All statements made herein of my own knowledge are true, and all statements made herein on information and belief are believed to be true.

I hereby acknowledge that any willful false statement and the like made in this declaration is punishable by fine or imprisonment of not more than five (5) years, or both, under 18 U.S.C. § 1001, and may jeopardize the validity of the application or any patent issuing thereon.

LEGAL NAME OF SOLE OR FIRST INVENTOR

Inventor one: Stuart Alexander Cook Date: 26/1/17

Signature: Stuart Cook

LEGAL NAME OF ADDITIONAL JOINT INVENTOR, if any

Inventor two: Sebastian Schaefer Date: 25/1/17

Signature: S. Schaefer

LEGAL NAME OF ADDITIONAL JOINT INVENTOR, if any

Inventor three: _____ Date: _____

Signature: _____

LEGAL NAME OF ADDITIONAL JOINT INVENTOR, if any

Inventor four: _____ Date: _____

Signature: _____

☐ Additional inventors are being named on _____ Additional form(s) attached hereto.

Application Data Sheet

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Correspondence Information

Correspondence Customer Number::	23628

Application Information

Application Type::	Regular
Subject Matter::	Utility
CD-ROM or CD-R?::	None
Sequence submission?::	Yes
Computer Readable Form (CRF)?::	Text Format
Title::	TREATMENT OF FIBROSIS
Attorney Docket Number::	M0546.70012US01
Request for Early Publication?::	No
Request for Non-Publication?::	No
Total Drawing Sheets::	66
Small Entity?::	No
Petition included?::	No
Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2::	No

Representative Information

Representative Customer Number::	23628

Domestic Priority Information

Application::	Continuity Type::	Parent Application::	Parent Filing Date::	Prior Appl Status::
This Application	Division of	15/381622	12/16/16	Pending

Foreign Priority Information

Country::	Application number::	Filing Date::	Priority Claimed::	DAS Access::
United Kingdom	1522186.4	12/16/15	Yes	

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Country of mailing address::	Republic of Singapore
Postal or Zip Code of mailing address::	168753

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City of mailing address::	Singapore

Country of mailing address::	Republic of Singapore
Postal or Zip Code of mailing address::	119077

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Country of mailing address::	Republic of Singapore
Postal or Zip Code of mailing address::	168753

Assignee Number::	2
Organization Name::	National University of Singapore
Street of mailing address::	21 Lower Kent Ridge Road
City of mailing address::	Singapore
Country of mailing address::	Republic of Singapore
Postal or Zip Code of mailing address::	119077

Authorization or Opt-Out of Authorization to Permit Access

When this Application Data Sheet is properly signed and filed with the application, applicant has provided written authority to permit a participating foreign intellectual property (IP) office access to the instant application-as-filed (see paragraph A in subsection 1 below) and the European Patent Office (EPO) access to any search results from the instant application (see paragraph B in subsection 1 below).

Should applicant choose not to provide an authorization identified in subsection 1 below, applicant **must opt-out** of the authorization by checking the corresponding box A or B or both in subsection 2 below.

NOTE: This section of the Application Data Sheet is **ONLY** reviewed and processed with the **INITIAL** filing of an application. After the initial filing of an application, an Application Data Sheet cannot be used to provide or rescind authorization for access by a foreign IP office(s). Instead, Form PTO/SB/39 or PTO/SB/69 must be used as appropriate.

1. Authorization to Permit Access by a Foreign Intellectual Property Office(s)

A. Priority Document Exchange (PDX) - Unless box A in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the State Intellectual Property Office of the People's Republic of China (SIPO), the World Intellectual Property Organization (WIPO), and any other foreign intellectual property office participating with the USPTO in a bilateral or multilateral priority document exchange agreement in which a foreign application claiming priority to the instant patent application is filed, access to: (1) the instant patent application-as-filed and its related bibliographic data, (2) any foreign or domestic application to which priority or benefit is claimed by the instant application and its related bibliographic data, and (3) the date of filing of this Authorization. See 37 CFR 1.14(h)(1).

B. Search Results from U.S. Application to EPO - Unless box B in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the EPO access to the bibliographic data and search results from the instant patent application when a European patent application claiming priority to the instant patent application is filed. See 37 CFR 1.14(h)(2).

The applicant is reminded that the EPO's Rule 141(1) EPC (European Patent Convention) requires applicants to submit a copy of search results from the instant application without delay in a European patent application that claims priority to the instant application.

2. Opt-Out of Authorizations to Permit Access by a Foreign Intellectual Property Office(s)

☐ A. Applicant **DOES NOT** authorize the USPTO to permit a participating foreign IP office access to the instant application-as-filed. If this box is checked, the USPTO will not be providing a participating foreign IP office with any documents and information identified in subsection 1A above.

☐ B. Applicant **DOES NOT** authorize the USPTO to transmit to the EPO any search results from the instant patent application. If this box is checked, the USPTO will not be providing the EPO with search results from the instant application.

NOTE: Once the application has published or is otherwise publicly available, the USPTO may provide access to the application in accordance with 37 CFR 1.14.

Signature:

NOTE: This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b). **However, if this Application Data Sheet is submitted with the INITIAL filing of the application and either box A or B is not checked in subsection 2 of the "Authorization or Opt-Out of Authorization to Permit Access" section, then this form must also be signed in accordance with 37 CFR 1.14(c).** This Application Data Sheet **must** be signed by a patent practitioner if one or more of the applicants is a **juristic entity** (e.g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, **all** joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of **all** joint inventor-applicants. See 37 CFR 1.4(d) for the manner of making signatures and certifications.			
Signature	/Amy J. McMahon/	Date (YYYY-MM-DD)	2018-05-24
Name	Amy J. McMahon, PhD	Registration Number	73,073

REMARKS

Entry of this amendment prior to examination and calculation of the claim fees is respectfully requested.

The application has been amended to update the Related Applications section of the specification. No new matter has been added.

Claims 1-20 were previously pending in this application. By this amendment, Applicant is canceling claims 2-20 without prejudice or disclaimer. Claim 1 is amended. New claims 21-29 are added. Support for the claims as amended can be found in the application as filed, e.g., as shown in the below table. As a result, claims 1 and 21-29 are pending for examination with claim 1 being an independent claim. No new matter has been added.

Table of Example Claim Support

Claim	Example Support from Application as Filed
1	Page 3, lines 27-29; page 6, lines 27-29; page 7, lines 12-21; page 8, lines 1-3; page 24, lines 20-24; page 25, lines 17-18
21	Page 2, lines 32-36; page 3, lines 31-32; page 25, lines 28-30
22	Page 4, lines 4-7; page 7, lines 20-21
23	Page 8, lines 6-9
24	Page 8, lines 11-13
25	Page 8, lines 15-17
26	Page 8, lines 19-21
27	Page 8, lines 23-26
28	Page 8, lines 28-31
29	Page 8, lines 33-36

The pending application is believed to be in condition for substantive examination.

Dated: May 24, 2018

Respectfully submitted,

By /Amy J. McMahon/
Amy J. McMahon, PhD
Registration No.: 73,073
WOLF, GREENFIELD & SACKS, P.C.
600 Atlantic Avenue
Boston, Massachusetts 02210-2206
617.646.8000

AMENDMENTS TO THE CLAIMS

Please replace all prior versions, and listings, of claims in the application with the following list of claims:

1. (Currently Amended) A method of treating fibrosis in a human subject, the method comprising administering to a human subject in need of treatment a therapeutically effective amount of an Interleukin 11 receptor α (IL-11Rα)~~anti interleukin 11 (IL-11)~~ antibody which is capable of~~, wherein the anti-IL-11 antibody binds to IL-11 and inhibits~~ inhibiting Interleukin 11 (IL-11) mediated signalling, wherein the fibrosis is fibrosis of the heart, liver, kidney or eye.

2-20. (Canceled)

21. (New) The method of claim 1, wherein the antibody is capable of inhibiting or reducing the binding of IL-11 to an IL-11 receptor.

22. (New) The method of claim 1, wherein the antibody is an IL-11Rα binding antibody.

23. (New) The method of claim 1, wherein the fibrosis is in the heart and is associated with dysfunction of the musculature or electrical properties of the heart, or thickening of the walls or valves of the heart.

24. (New) The method of claim 1, wherein the fibrosis is in the liver and is associated with chronic liver disease or liver cirrhosis.

25. (New) The method of claim 1, wherein the fibrosis is in the kidney and is associated with chronic kidney disease.

26. (New) The method of claim 1, wherein the fibrosis is in the eye and is retinal fibrosis, epiretinal fibrosis, or subretinal fibrosis.

27. (New) The method of claim 1, wherein the method of treating fibrosis comprises administering said antibody to a subject in which IL-11Rα expression is upregulated.

28. (New) The method of claim 1, wherein the method of treating fibrosis comprises administering said antibody to a subject in which IL-11Rα expression has been determined to be upregulated.

29. (New) The method of claim 1, wherein the method of treating fibrosis comprises determining whether IL-11Rα expression is upregulated in the subject and administering said antibody to a subject in which IL-11Rα expression is upregulated.

AMENDMENTS TO THE SPECIFICATION

Please amend the paragraph beginning on page 1, line 5 entitled "RELATED APPLICATIONS" with the following amended paragraph:

RELATED APPLICATIONS

The present application is a divisional application of U.S. Application No. 15/381,622, filed December 16, 2016, which claims priority under 35 USC § 119(a)-(d) to United Kingdom Application No. 1522186.4, filed December 16, 2015. The entire contents of the aforementioned applications are~~this application is~~ hereby incorporated by reference herein.

Docket No.: M0546.70012US01
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Stuart Alexander Cook
Application No.:	Not Yet Assigned
Confirmation No.:	Not Yet Assigned
Filed:	Concurrently Herewith
For:	TREATMENT OF FIBROSIS
Examiner:	Not Yet Assigned
Art Unit:	Not Yet Assigned

FIRST PRELIMINARY AMENDMENT UNDER 37 C.F.R. § 1.115

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

Dear Sir:

INTRODUCTORY COMMENTS

Prior to examination on the merits, please amend the above-identified U.S. patent application as follows:

Amendments to the Specification begin on page 2 of this paper.

Amendments to the Claims are reflected in the listing of claims which begins on page 3 of this paper.

Remarks/Arguments begin on page 5 of this paper.

Doc Code: PA..
Document Description: Power of Attorney

PTO/AIA/82B (07-13)
Approved for use through 11/30/2014. OMB 0651-0051
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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POWER OF ATTORNEY BY APPLICANT

I hereby revoke all previous powers of attorney given in the application identified in either the attached transmittal letter or the boxes below.

Application Number	Filing Date
15/381,622	December 16, 2016

(Note: The boxes above may be left blank if information is provided on form PTO/AIA/82A.)

[X] I hereby appoint the Patent Practitioner(s) associated with the following Customer Number as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above: 23628

OR

[ ] I hereby appoint Practitioner(s) named in the attached list (form PTO/AIA/82C) as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the patent application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above. (Note: Complete form PTO/AIA/82C.)

Please recognize or change the correspondence address for the application identified in the attached transmittal letter or the boxes above to:

[ ] The address associated with the above-mentioned Customer Number

OR

[ ] The address associated with Customer Number:

OR

Firm or Individual Name					
Address					
City		State		Zip	
Country					
Telephone		Email			

I am the Applicant (if the Applicant is a juristic entity, list the Applicant name in the box):

National University of Singapore

[ ] Inventor or Joint Inventor (title not required below)

[ ] Legal Representative of a Deceased or Legally Incapacitated Inventor (title not required below)

[X] Assignee or Person to Whom the Inventor is Under an Obligation to Assign (provide signer's title if applicant is a juristic entity)

[ ] Person Who Otherwise Shows Sufficient Proprietary Interest (e.g., a petition under 37 CFR 1.46(b)(2) was granted in the application or is concurrently being filed with this document) (provide signer's title if applicant is a juristic entity)

SIGNATURE of Applicant for Patent

The undersigned (whose title is supplied below) is authorized to act on behalf of the applicant (e.g., where the applicant is a juristic entity).

Signature		Date (Optional)	
Name	Sean P. Flanigan		
Title	Director		

NOTE: Signature - This form must be signed by the applicant in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. If more than one applicant, use multiple forms.

[ ] Total of 1 forms are submitted.

5184560.1

Doc Code: PA..
Document Description: Power of Attorney

PTO/AIA/82B (07-13)
Approved for use through 11/30/2014. OMB 0651-0051
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POWER OF ATTORNEY BY APPLICANT

I hereby revoke all previous powers of attorney given in the application identified in either the attached transmittal letter or the boxes below.

Application Number	Filing Date
15/381,622	December 16, 2016

(Note: The boxes above may be left blank if information is provided on form PTO/AIA/82A.)

[X] I hereby appoint the Patent Practitioner(s) associated with the following Customer Number as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above: 23628

OR

[ ] I hereby appoint Practitioner(s) named in the attached list (form PTO/AIA/82C) as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the patent application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above. (Note: Complete form PTO/AIA/82C.)

Please recognize or change the correspondence address for the application identified in the attached transmittal letter or the boxes above to:

[ ] The address associated with the above-mentioned Customer Number

OR

[ ] The address associated with Customer Number:

OR

Firm or Individual Name					
Address					
City		State		Zip	
Country					
Telephone		Email			

I am the Applicant (if the Applicant is a juristic entity, list the Applicant name in the box):

Singapore Health Services PTE LTD.

[ ] Inventor or Joint Inventor (title not required below)

[ ] Legal Representative of a Deceased or Legally Incapacitated Inventor (title not required below)

[X] Assignee or Person to Whom the Inventor is Under an Obligation to Assign (provide signer's title if applicant is a juristic entity)

[ ] Person Who Otherwise Shows Sufficient Proprietary Interest (e.g., a petition under 37 CFR 1.46(b)(2) was granted in the application or is concurrently being filed with this document) (provide signer's title if applicant is a juristic entity)

SIGNATURE of Applicant for Patent

The undersigned (whose title is supplied below) is authorized to act on behalf of the applicant (e.g., where the applicant is a juristic entity).

Signature	ENNY KIESWORO Chief Operating Officer, Assignee	Date (Optional)	Jan 26, 2017
Name	SINGAPORE HEALTH SERVICES PTE LTD		
Title			

NOTE: Signature - This form must be signed by the applicant in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. If more than one applicant, use multiple forms.

[ ] Total of 1 forms are submitted.

5184559.1

Electronic Patent Application Fee Transmittal

Application Number:	
Filing Date:	
Title of Invention:	TREATMENT OF FIBROSIS
First Named Inventor/Applicant Name:	Stuart Alexander Cook
Filer:	Amy Jeanette McMahon/Nancy Arsenault
Attorney Docket Number:	M0546.70012US01

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
UTILITY APPLICATION FILING	1011	1	300	300
UTILITY SEARCH FEE	1111	1	660	660
UTILITY EXAMINATION FEE	1311	1	760	760
Pages:				
UTILITY APPL SIZE FEE PER 50 SHEETS >100	1081	1	400	400
Claims:				
Miscellaneous-Filing:				
Petition:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	2120

Electronic Acknowledgement Receipt

EFS ID:	32717634
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:	24-MAY-2018
Filing Date:	
Time Stamp:	15:09:17
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	CARD
Payment was successfully received in RAM	$2120
RAM confirmation Number	052518INTEFSW15102800
Deposit Account	232825
Authorized User	Wolf Greenfield
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: 37 CFR 1.16 (National application filing, search, and examination fees)	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal of New Application	M054670012US01-TRN-AM.pdf	32217 89697bb34985057b0ab25a92edf5ef505350c6f5	no	1
Warnings:					
Information:					
2	Fee Worksheet (SB06)	M054670012US01-FEE-AM.pdf	29862 dce2f3c72b1fc21b86782e561faac9b4fcbfaf9d	no	1
Warnings:					
Information:					
3		M054670012US01-UTLAPL-AM.pdf	467461 d35ecd873c3055f83003e9f4c5dae42769e497ae	yes	96

Multipart Description/PDF files in .zip description

Document Description	Start	End
Abstract	96	96
Claims	93	95
Specification	1	92

Warnings:					
Information:					
4	Drawings-other than black and white line drawings	M054670012US01-DRW-AM.pdf	5131255 3f4853caadc9a9a9156463 17eced9ede4bba3e28	no	66
Warnings:					
Information:					
5	Oath or Declaration filed	M054670012US01-INVDEC-AM.pdf	134507 8ab6b23c41464e0353761d3a38a5f2f5eaeeb475	no	1

Warnings:					
Information:					
6	Application Data Sheet	M054670012US01-ADS-AM.pdf	29039 dc991bc5f64c26bf7ee6b32f8bd52a1e2a3b0164	no	6
Warnings:					
Information:					
This is not an USPTO supplied ADS fillable form					
7		M054670012US01-PAMN-AM.pdf	41734 a9b38598f3e2f79ba2eebabab1966fb00dc7865c	yes	6

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Applicant Arguments/Remarks Made in an Amendment	5	6
	Claims	3	4
	Specification	2	2
	Preliminary Amendment	1	1

Warnings:					
Information:					
8	Power of Attorney	M054670012US01-POA-NUS-AM.pdf	85252 db2c7ea489ad147aba15f666ae5f92aad1f1ffe1	no	1
Warnings:					
Information:					
9	Power of Attorney	M054670012US01-POA-SHS-AM.pdf	98087 108991988b735aeb22c23863a26224fe635e6570	no	1
Warnings:					
Information:					
10	Sequence Listing (Text File)	M054670012US01-SEQ-AM.txt	21738	no	-
Warnings:					
Information:					

11	Fee Worksheet (SB06)	fee-info.pdf	36814 2781966d3ecd84e396528af73d83f1e916e36406	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	6107966	

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

New Applications Under 35 U.S.C. 111

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

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

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

New International Application Filed with the USPTO as a Receiving Office

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