Activation of Key Profibrotic Mechanisms in Transgenic Fibroblasts Expressing Kinase-deficient Type II Transforming Growth Factor-β Receptor (TβRIIΔk)*

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We have generated transgenic mice expressing a kinase-deficient type II transforming growth factor-β (TGFβ) receptor selectively on fibroblasts (TβRIIΔk-fib). These mice develop dermal and pulmonary fibrosis. In the present study we explore activation of TGFβ signaling pathways in this strain and examine the profibrotic properties of explanted transgenic fibroblasts including myofibroblast differentiation and abnormal metalloproteinase production. Gene expression profiles of littermate wild type or transgenic fibroblasts were compared using high-density gene arrays and validated by Taqman reverse transcriptase-PCR, Northern and Western blotting. Using a specific inhibitor (SD-208) we demonstrate that the abnormal phenotype of these cells is dependent upon TβRII kinase (ALK5) activity, and that transgenic fibroblasts show enhanced expression and activation of TGFβ together with increased levels of wild type TβRII. Moreover, we confirm that transgene expression is itself regulated by TGFβ and that expression at low levels facilitates signaling, whereas high level expression is inhibitory. For a subset of TGFβ responsive genes basal up-regulation is normalized or suppressed by exogenous recombinant TGFβ1 at time points coincident with increased transgene expression. These findings explain the profound refractoriness of TβRIIΔk-fib fibroblasts to exogenous TGFβ1, despite their activated phenotype. Thus, transgenic fibroblasts recapitulate many hallmark biochemical properties of fibrotic cells, including high level CTGF (CCN2) expression and type I collagen overproduction, altered MMP production, and myofibroblast differentiation. These cells also show an enhanced ability to contract collagen gel matrices. Our study demonstrates that altered high affinity TGFβ receptor function may lead to ligand-dependent activation of downstream signaling, and provides further evidence of a pivotal role for sustained TGFβ overactivity in fibrosis.

A growing body of evidence implicates overactivity of transforming growth factor β (TGFβ) in fibrosis (1, 2) leading to sustained extracellular matrix overproduction and promoting myofibroblast differentiation (3–5). For example, the prototypic multisystem fibrotic disease systemic sclerosis (SSc) has been associated with increased activity of TGFβ signaling pathways (6, 7), altered expression of high and low affinity TGFβ receptors (8–11), and autocrine overproduction of several TGFβ-regulated genes (12, 13). In addition, several recent studies suggest altered expression of TGFβ pathway signaling intermediates in SSc including Smad3 and Smad7 (14, 15), although the precise nature of altered Smad expression is unclear (reviewed in Ref. 6). Gene profiling or PCR-based differential display experiments comparing SSc with healthy control dermal fibroblasts point toward a gene expression pattern for SSc similar to that of TGFβ-activated normal fibroblasts (16–18). Finally, myofibroblasts are more frequent in SSc skin and lung samples and the myofibroblast phenotype is maintained in explanted cultures from SSc skin or lung biopsies (19).

We have sought to directly test in vivo the hypothesis that sustained alteration in TGFβ signaling or responsiveness in fibroblasts may recapitulate the SSc phenotype. Transgenic mice have been generated for these experiments using a potent fibroblast-specific promoter subcloned from the pro-α(2)I collagen gene (20) to drive expression of TGFβ receptors. Surprisingly, we achieved our goal of sustained activation of TGFβ signaling by expressing a kinase-deficient type II TGFβ receptor (TβRIIΔk) in fibroblasts. Initial characterization of a novel transgenic mouse strain in which TβRIIΔk is expressed selectively on fibroblasts by linking it to a lineage-specific promoter demonstrated development of skin and lung fibrosis (21). Our findings were unexpected because kinase-deficient TGFβ receptors have previously been shown to be dominant negative inhibitors of signaling in several experimental systems, albeit at high expression levels compared with wild type receptors (22). In the present study the fibrotic phenotype of explanted dermal fibroblasts from TβRIIΔk-fib mice is delineated. We determine that expression of the mutant receptor leads to multilevel activation of the TGFβ ligand-receptor axis and that

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1 The abbreviations used are: TGFβ, transforming growth factor β; α-SMA, α-smooth muscle actin; MMP, matrix metalloproteinase; FPCd, fibroblast-populated collagen gel lattice; SSc, systemic sclerosis; PAI-1, plasminogen activator inhibitor; PBS, phosphate-buffered saline; PIPES, 1,4-piperazine-N,N'-diethanesulfonic acid; RT, reverse transcriptase; TIMP, tissue inhibitor of metalloproteinase.
activation is dependent upon endogenous TβRII receptor kinase activity. Two cardinal features of this mouse strain are identified: altered metalloproteinase expression and function, and myofibroblast differentiation. In addition, we confirm that the fibroblast-specific expression cassette is itself regulated by TGFβ and that low level expression of the kinase-deficient TβRII activates TGFβ signaling, whereas higher expression levels are inhibitory. We hypothesize that the profound refractoriness of explanted transgenic dermal fibroblasts to recombinant TGFβ1 is because of transgene up-regulation in ligand-activated cells. It is possible that analogous signaling perturbations may lead to balanced up-regulation of profibrotic pathways and sustain abnormal fibroblast properties in fibrotic disease. If so, then consequences of blocking TGFβ ligand or receptor activity are unpredictable, and animal models such as the TβRIIΔk-fib strain are likely to be valuable tools in the development and evaluation of targeted anti-fibrotic therapies.

**EXPERIMENTAL PROCEDURES**

**Transgenic Mice**—The generation and initial characterization of the transgenic mouse strain used in this report have been described (23). In brief, a fibroblast-specific expression cassette was subdivided from the upstream region of the pro-α2II collagen gene (Col1a2). This incorporates a fragment between −19.5 and −13.5 kb upstream of the transcription start site that, when linked to an endogenous minimal promoter drives gene expression at high levels in fibroblasts, but not in other type I collagen producing cells. Reporter genes linked to this promoter-enhancer show high level fibroblast-specific expression in embryonic development from 8-day post-conception and postnatally, as previously described. The mouse strain TβRIIΔk-fib was generated by subcloning the cDNA encoding the extracellular and transmembrane portion of the human type II TGFβ receptor into the Sall site of the pCD3 expression vector. A bacterial β-galactosidase gene (LacZ) is coexpressed from a dicistronic transgene mRNA product via an encephalomyocarditis virus internal ribosome entry site (IRE) (full details of this construction are provided in Ref. 21. Fibroblasts cultured from 6-kb LacZ transgenic mice, in which a bacterial β-galactosidase reporter gene is regulated by the same fibroblast-specific reporter transgene as the TβRIIΔk-fib strain were used in control experiments (23).

**Fibroblast Culture**—Fibroblast cultures were derived from skin biopsies from the lower back of neonatal transgenic or control littermate mice. Cells were cultured in the presence of antibiotics and passaged at confluence. Fibroblasts were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen), 100 units/ml penicillin, 100 μg/ml streptomycin, and cultured in a humidified atmosphere of 5% CO2 in air. Fibroblasts were subcultured at confluence and used between passages 2 and 5. Fibroblast monolayers were serum and reconstituted human serum-free medium for testing sera with high expression of α-smooth muscle actin. Fibroblasts were cultured in a low serum medium overnight, and then stimulated with recombinant TGFβ1 (4 ng/ml) for between 30 min and 24 h, in the presence or absence of the novel inhibitor. For studies examining regulation of fibroblast-specific expression cassette by TGFβ1 signaling, 10 ng/ml TGFβ1 was used to inhibit SPl (27) and curcumin was used as an inhibitor of AP-1 (28). In some experiments the broad spectrum metalloproteinase inhibitor GM6001 was used to block protease activity in monolayer or gel lattice culture (29). To assess the effect of neutralizing antibody against TGFβ1 (1D11) on transgene expression in TβRIIΔk-fib mice, neonatal animals were injected with 1D11 or the isotype-matched control monoclonal 13C4 (2.5 mg/kg intraperitoneal) and β-galactosidase activity assayed in tail biopsy lysate 48 h later.

**Affymetrix GeneChips**—Total RNA was harvested from fibroblasts using the isothiocyanate/cesium chloride method (26) or by TRIzol (Invitrogen) according to the manufacturer's instructions. Levels of transcription of total RNA. Northern blot analysis—RNA isolated from fibroblasts cultures and compare responsiveness to recombinant TGFβ1 ligand Western blot analysis was performed. Cell layer lysates and tissue culture supernatants were examined from independent strains derived from transgenic or non-transgenic littermates (n = 5). Supernatants were concentrated by ammonium sulfate precipitation to selectively enrich samples for secreted matrix proteins. After SDS-PAGE, proteins were electroblotted onto nylon membranes and probed with specific antibodies. These were localized by chemiluminescence using a specific secondary antibody. For supernatants, specific antibodies to collagen type I (Southern Biotechnology Inc., Birmingham, AL) were used. Other studies examined CTGF (R&D Systems) and MMP13, TGFβ, TGFα1, using the Megakaryocytes (SG224) or 5 mg/ml anti-TGFβ1 monoclonal antibodies (Santa Cruz, CA) using specific primary antibodies. Mouse-specific primary antibodies were used to ensure that endogenous TβRII rather than truncated TβRIIΔk was detected.

**Northern Blot Analysis**—Total RNA was isolated from fibroblasts using the isothiocyanate/cesium chloride method (26) or by TRIzol (Invitrogen) according to the manufacturer’s instructions. Levels of the TGFβ ligand or ligand-receptor axis in determining the abnormal properties of transgenic fibroblasts. A novel, highly specific, inhibitor of the TGFβRII rather than TGFβRI kinase (ALK5) activity with a specificity of >100-fold against TGFβRII and at least 17-fold over members of a panel of related protein kinases including p38, p42/p44, JNK1, ERK1, MAPKAPK2, MKK6, ERK2, PKC, PKA, PKD, CDC2, and CaMKII. Preliminary experiments defined the effective concentration producing specific ALK5 inhibition. For these fibroblast experiments were cultured to confluence, incubated in a low serum medium overnight, and then stimulated with recombinant TGFβ1 (4 ng/ml) for between 30 min and 24 h, in the presence or absence of the novel inhibitor. For studies examining regulation of fibroblast-specific expression cassette by TGFβ1 signaling, 10 ng/ml TGFβ1 was used to inhibit SPl (27) and curcumin was used as an inhibitor of AP-1 (28). In some experiments the broad spectrum metalloproteinase inhibitor GM6001 was used to block protease activity in monolayer or gel lattice culture (29). To assess the effect of neutralizing antibody against TGFβ (1D11) on transgene expression in TβRIIΔk-fib mice, neonatal animals were injected with 1D11 or the isotype-matched control monoclonal 13C4 (2.5 mg/kg intraperitoneal) and β-galactosidase activity assayed in tail biopsy lysate 48 h later.

**Quantitative RT-PCR Methods**—Fluorescent real-time PCR (Taqman) was used to confirm basal and TGFβ1-induced differences in gene expression. Total RNA was extracted and treated with RNa-free DNase (Roche, Lewes, UK) for 10 min at 37 °C and heat inactivation at 70 °C for 15 min. 18 S rRNA band intensity was used to confirm equal gel loading. Probes were labeled with [32P]dCTP to a specific activity of 106 cpm/μg using the Megaprobe kit. Probes directed to β-actin, GAPDH, and ribosomal protein L32 were used. Other studies examined CTGF (R&D Systems) and MMP13, TGFβ1, TGFβ3, using the Megakaryocytes (SG224) or 5 mg/ml anti-TGFβ1 monoclonal antibodies (Santa Cruz, CA) using specific primary antibodies. Mouse-specific primary antibodies were used to ensure that endogenous TβRII rather than truncated TβRIIΔk was detected.

**Affymetrix GeneChips**—Total RNA was harvested from confluent fibroblast monolayers (TRIzol, Invitrogen) and quantified, and integrity was verified by denaturing gel electrophoresis. Equal amounts of identically treated RNA were pooled and reverse transcribed (Invitrogen) into cDNA that was then in vitro transcribed into biotinylated cRNA. The target cRNA was then fragmented and hybrid-
ized to the mouse U74A2v2 array (Affymetrix, Santa Clara, CA), covering 12,000 mouse genes, as described by the manufacturer. Hybridization of cRNA, signal amplification, and data collection were performed using a fluids station and chip reader following the Affymetrix protocol. Arrays were scaled to an average intensity of 100 per gene and analyzed using the Affymetrix Microarray Analysis Suite version 5.0.

Briefly, transcripts were defined as up-regulated or down-regulated only when identified as “present” and as significantly different (p < 0.001) in two independently probed genechip pairs. The fold-change between treated and untreated samples had to be at least 2.5-fold to designate a transcript as being differentially expressed.

**Contraction of Free-floating or Fixed Fibroblast Populated Collagen Lattices**—To study collagen gel contraction, fibroblasts were cultured within three-dimensional collagen lattices (fibroblast populated collagen lattices; FPCL). These were prepared as previously described (30). In brief, 24-well tissue culture plates (Costar) were pre-coated with sterile 2% bovine serum albumin in phosphate-buffered saline (PBS) (2 ml/well) by incubation at 37 °C overnight, and were then washed three times with sterile PBS. For FPCL, neutral collagen solution (containing one part of 0.2% HEPES, pH 8.0; four parts collagen (Vitrogen-100, 3 mg/ml, Celltrix, Santa Clara, CA) and five parts of MCDB 104 medium (Sigma, 2 times concentrate) was prepared and mixed with fibroblasts that were resuspended in 2 times MCDB 104 medium, to bring the final concentration of collagen 1 mg/ml and 0.1% tri- nulin in PBS (7.5 g/ml, BA-9500; Vector Laboratories) for 30 min, luted in PBS (7.5 g/ml, BA-9500; Vector Laboratories) for 30 min, and mounted with Gelmount (Biomed, Foster City, CA). Specificity of staining was confirmed in control sections incubated with an isotype-matched irrelevant control antibody (13C4) in place of 1D11.

**TGFB Bioassy Using Mink Lung Epithelial Cells**—To examine TGFB bioactivity in fibroblast culture supernatants we used a validated bioassay incorporating mink lung epithelial cells stably transfected with a PAIL-1 luciferase reporter construct. The assay was performed as described previously (24). For each supernatant, aliquot basal activity (active TGFB) and activity after heating the supernatant at 80 °C for 10 min (total TGFB) was compared. Samples were analyzed in triplicate. Briefly, 1.6 × 10⁵ per well were plated in 96-well tissue culture dishes. After attachment for 3 h at 37 °C in 5% CO2, medium was replaced by test sample and after 14 h at 37 °C luciferase activity was determined.

Recombinant TGFβ1 and neutralizing anti-TGFβ antibody (1D11) or isotype matched irrelevant antibody (13C4) were used to verify specificity of the assay. Equal numbers of transgenic or wild type cells were seeded at baseline and luciferase expression data were expressed per unit supernatant volume. Equivalent transgenic or wild type cell numbers at the time of harvesting the supernatant was confirmed by direct counting.

**Statistical Analysis**—For quantitative variables, mean (±S.E.) in replicate samples or combined independent experiments, where inter-experiment variation allowed reliable combination of raw data, were compared. Means were compared by Student’s paired or unpaired t test as appropriate and p < 0.05 taken as statistically significant.

**RESULTS**

**Profibrotic Phenotype of TβRIIΔ-k-fib Transgenic Fibroblasts**—We have generated transgenic mice that develop dermal and sporadic pulmonary fibrosis using a fibroblast-specific expression cassette to direct expression of a kinase-deficient type II TGFβ receptor (21). The basis for paradoxical activation of fibrotic mechanisms by a non-signaling receptor is the focus of the present study. Our initial experiments confirmed basal overproduction of pro(I) collagen compared with littermate wild type cells and also demonstrated sustained refractoriness of neonatal transgenic fibroblasts to recombinant TGFβ1 (Fig. 1A). We also observed constitutive expression of CTGF (CCN2, FSP12) protein by transgenic fibroblasts compared with littermate wild type cells. To confirm that activation of the TGFB signaling pathway determined constitutive overexpression of pro(I) collagen or CTGF, SD-208, a novel TβRI-kinase (ALK5) inhibitor was used. Inhibition of ALK5 reduced pro(I) collagen and CTGF expression in transgenic fibroblasts to levels seen in littermate wild type cells (Fig. 1B). In parallel experiments, mRNA levels of pro-collagen(I) collagen and CTGF were reduced to wild type levels in transgenic fibroblasts following treatment with the inhibitor SD-208 (Fig. 1C). We also examined expression of the major type I collagen degrading enzyme MMP13 in mice. Levels of both precursor and active enzyme were substantially increased in transgenic fibroblast compared with littermate wild type cells, suggesting that protein overproduction rather than reduced degradation is more likely the cause of increased matrix collagen in the TβRIIΔ-k-fib transgenic mice (Fig. 1D).

We hypothesized that expression of TβRIIΔk on fibroblasts may alter basal levels of endogenus wild type TGFβ receptors. A series of Western blot experiments using mouse-specific primary antibodies confirmed substantially increased levels of wild type TβRI in transgenic fibroblast lysates. This provides a plausible explanation for increased TGFβ signaling in these cells. Levels of wild type TβRI were not altered in transgenic fibroblasts (Fig. 2A). Later, we confirmed that transgenic fibroblasts but not wild type cells demonstrated nuclear staining for phosphorylated Smad2/3 (Fig. 2, B and C). Consistent with previous observations concerning the refractoriness of TβRIIΔ-k-fib cells to recombinant TGFβ1, while there was a marked nuclear
localization of pSmad2/3 in wild type fibroblasts 10 min after TGFβ1 activation, no change in staining occurred in littermate transgenic fibroblasts (Fig. 2, D and E). Western blot analysis of wild type or transgenic fibroblast lysates confirmed increased Smad2/3 phosphorylation in TpRII-transgenic fibroblasts (Fig. 2, F), and its dependence upon ALK5 activation because it is abrogated by SD-208 (Fig. 2, F). Data are representative of a series of three independent experiments.

**Fig. 1.** Biochemical phenotype of TpRIIΔk-fib dermal fibroblasts. A, pro(I) collagen overexpression and refractoriness to TGFβ1 demonstrated by Western blot analysis of dermal fibroblast supernatants after stimulation by recombinant murine TGFβ1 (4 ng/ml). B, collagen and CTGF protein overexpression is abrogated by TpRII kinase (ALK5) inhibition (SD-208). C, Northern blot analysis shows Col1a2 and CTGF mRNA overexpression is abrogated by TpRII kinase inhibition (SD-208). D, transgenic (tg) fibroblasts overexpress the major fibrillar collagen-degrading protease MMP-13. Results shown are representative of a series of four independent experiments. wt, wild type.

**Global Expression Profiling of TpRIIΔk Transgenic Fibroblasts**—Based upon the similarity in phenotype between the TpRIIΔk-fib mouse strain and human scleroderma (SSc) we were interested in exploring the similarity between the gene expression profile of transgenic fibroblasts and that reported in a number of earlier studies of dermal fibroblasts from SSc (12, 13, 17). In these reports genes that are overexpressed or down-regulated have generally supported a role for TGFβ in SSc pathogenesis (33). In the present study we have used high density oligonucleotide genechips to compare gene expression for transgenic or littermate wild type fibroblasts in a series of independent experiments. Our data confirm that the gene expression profile of transgenic fibroblasts replicates many reported features observed in chronic fibrotic fibroblasts. Using the MAS5.0 gene expression profiling software (Affymetrix) independent analysis of two pairs of transgenic fibroblasts and non-transgenic littermates identified 5846 expressed genes. Of these, 297 were up-regulated at least 2-fold and 420 genes were down-regulated 2-fold. These genes included a range of matrix components, cytokines, growth factors, and signaling intermediates consistent with the complex profibrotic phenotype previously reported for these transgenic fibroblasts. In our analysis of these profiles we have concentrated on genes that are more than 2.5-fold differentially expressed. Overexpressed genes (n = 41) are listed in Table I and down-regulated genes are listed in Table II (n = 21). Transcript levels of a number of growth factors increased, including several that promote fibrosis through activation of fibroblasts (interleukin-6, platelet-derived growth factor-β, and Cyr61, and perhaps most significantly TGFβ1). Two of the most overexpressed transcripts, PAI-1 (14.7-fold) and CCN2/CTGF (11.5-fold), are prototypic markers of TGFβ activation, consistent with our hypothesis that these transgenic fibroblasts have sustained activation of TGFβ signaling pathways. Other TGFβ up-regulated genes were thrombomodulin, osteoprotegerin, and OSF1. A number of potentially important genes were down-regulated in transgenic fibroblasts (see Table II), including growth factors such as insulin-like growth factor 1, insulin-like growth factor 2, CCL11, and SDF1.

**Fig. 2.** TpRIIΔk-fib dermal fibroblasts have increased levels of endogenous TpRII and altered phosphorylation of Smad2/3. A, Western blot analysis of littermate fibroblast pairs confirms increased levels of endogenous TpRII in transgenic (tg) cells using a mouse-specific primary antibody. Levels of TpRI were not significantly different. Coomassie-stained polyacrylamide gel confirms equal protein loading. Nuclear staining of phosphorylated Smad2/3 (arrow) is absent in wild type (wt) fibroblasts (B) but present in transgenic TpRIIΔk-fib cells (C). There is strong staining of wild type nuclei 10 min after incubation with recombinant TGFβ1 (4 ng/ml) (D) but transgenic fibroblasts show no change (E). Western blot analysis of wild type or transgenic fibroblast lysates confirmed increased Smad2/3 phosphorylation in TpRIIΔk-fib, and its dependence upon ALK5 phosphorylation in TpRIIΔk-fib, and its dependence upon ALK5 activation because it is abrogated by SD-208 (F). Data are representative of a series of three independent experiments.
**Fibrotic Mechanisms in TpRIIΔk-fib Transgenic Mice**

### TABLE I

| Affymetrix ID | Accession No. | Wild type | TpRIIΔk | -Fold change | Descriptions                                                                 |
|--------------|---------------|-----------|---------|--------------|-----------------------------------------------------------------------------|
| 94147_at     | M39960        | 147.9     | 2181.6  | 14.7         | PAI-1                                                                        |
| 93294_at     | M70642        | 207.8     | 2394    | 11.5         | CTGF (CCN-2, FISP12), fibroblast-inducible secreted protein                  |
| 104467_at    | M88242        | 85.5      | 906.65  | 10.6         | Mouse glucocorticoid-regulated inflammatory prostaglandin G/H synthase     |
| 100277_at    | X69619        | 148.6     | 1405.6  | 9.5          | Inhibin β-A                                                                 |
| 98885_at     | U52554        | 64.7      | 512.9   | 7.9          | Hyaluronan synthase 2                                                       |
| 94378_at     | U94828        | 113.6     | 878.2   | 7.7          | *Mus musculus* retinally abundant regulator of G-protein signaling mRGS-r  |
| 96055_at     | X59520        | 88.4      | 517.5   | 5.9          | Cholecytokinin                                                              |
| 93550_at     | d88792        | 102.95    | 554.2   | 5.4          | Cysteine and glycine-rich protein 2                                         |
| 102218_at    | X54542        | 67.75     | 351.2   | 5.2          | Interleukin 6                                                               |
| 103065_at    | M73696        | 111.75    | 533.6   | 4.8          | Solute carrier family 20, member 1,murine Glvr-1                            |
| 102663_at    | X62700        | 31.05     | 144.4   | 4.7          | Urokinase oplasminogen activator receptor M. *musculus* muPAR1              |

### TABLE II

| Affymetrix tag | Accession No. | TpRIIΔk | -Fold change | Wild type | Gene                                                                 |
|----------------|---------------|---------|--------------|-----------|----------------------------------------------------------------------|
| 95545_at       | U22399        | 1218.1  | 22.6         | 53.8      | Cyclin-dependent kinase inhibitor 1C                                  |
| 94813_at       | X65128        | 1299.9  | 5.7          | 226.2     | Growth arrest specific 1                                             |
| 101918_at      | M17298        | 62.95   | 2.9          | 182.2     | Transforming growth factor-β1                                         |
| 102298_at      | M17298        | 58.55   | 2.9          | 161.0     | Nerve growth factor, β                                               |
| 96701_at       | U53823        | 230.45  | 2.9          | 660.9     | Ubiquitin-like 1 (sentrin) activating enzyme E1B                      |
| 96957_at       | L10244        | 353.5   | 2.9          | 101.1     | Spredmin/spermine N3-actetyltransferase                                |
| 160102_at      | Z37164        | 132.95  | 2.8          | 375.2     | *Mus musculus* chaperonin subunit 8 (theta) Ccq                       |
| 94932_at       | M29464        | 180.75  | 2.7          | 496.4     | Platelet-derived growth factor α                                      |
| 101973_at      | y15163        | 132.15  | 2.7          | 357.9     | Cbp/p300-interacting transactivator                                   |
| 102887_at      | U94331        | 91.8    | 2.7          | 245.9     | Osteoprotegerin (OPG)                                                |
| 94288_at       | J03482        | 241.3   | 2.7          | 645.3     | Histone H1 gene                                                      |
| 95580_at       | U39473        | 83.85   | 2.7          | 223.6     | Histidyl-tRNA synthetase                                             |
| 104601_at      | X14432        | 161.4   | 2.7          | 423.3     | Thrombomodulin                                                       |
| 99158_at       | U58888        | 159.4   | 2.6          | 416.3     | Osteoclast stimulating factor 1                                       |
| 94826_at       | Y11460        | 96.2    | 2.6          | 250.55    | β3 Integrin interactor                                               |
| 101966_at      | AF029482      | 80.55   | 2.6          | 209.15    | Nidogen 2                                                            |

*Mean expression determined from two independently performed experiments using the Affymetrix U74v2 genechips. Genes were determined as present in all samples and designated significantly up-regulated (p < 0.001) in both transgenic samples compared with wild type littermates.*
Fibrotic Mechanisms in TβRIIΔk-fib Transgenic Mice

Myofibroblast Differentiation in Transgenic Dermal Fibroblast Cultures—Gene expression profiling identified consistent up-regulation of a several transcripts characteristic of a myofibroblast phenotype including calponin (3.2-fold) and myosin heavy chain (1.7-fold). This led us to explore differences in expression of α-smooth muscle actin (α-SMA) in transgenic fibroblasts. Gene and protein expression levels in cell lysates did not significantly differ between wild type and transgenic fibroblasts but immunostaining of cultured cells was strikingly different. There was basal expression of α-SMA in transgenic fibroblasts (Fig. 3, A and B) with formation of a myofibril-rich cytoskeleton. Wild type littermate fibroblasts responded to recombinant TGFβ1 by forming α-SMA-rich fibers, whereas there was no change in α-SMA expression by transgenic fibroblasts in response to TGFβ1 (Fig. 3, C and D). The novel TβRII kinase (ALK5) inhibitor SD-208 inhibited α-SMA filament formation in both transgenic or wild type fibroblasts treated with TGFβ1 (Fig. 3, E and F). In transgenic fibroblasts SD-208 altered the constitutive pattern of α-SMA staining leading to fragmentation or condensation of myofibrils, whereas basal expression in wild type cells was not altered (Fig. 3, G and H).

Metalloproteinase Expression and Regulation by TGF-β1 Is Altered in Transgenic Fibroblasts—The marked differences in MMP13 protein expression between transgenic and wild type fibroblasts and potential contribution of altered metalloproteinase expression to the development of fibrosis prompted a systematic analysis of MMP and TIMP gene expression. We first asked whether expression of collagen-degrading enzymes was reduced or if levels of the inhibitors of MMP activity TIMP1, -2, and -3 increased. Expression levels for the MMP and TIMP genes included on the U74v2 genechip are summarized in Table III. Based upon these results, we undertook quantitative real time RT-PCR analysis using a series of 5 paired littermate samples. There were clear differences between transgenic and wild type cells with respect to MMP gene and protein expression, consistent with the importance of metalloproteinases in connective tissue remodeling and their regulation by TGFβ. MMP3, -9, -10, -13, and TIMP1 were up-regulated in transgenic fibroblasts (Fig. 4A). Conversely, MMP7 and MMP11 were down-regulated (Fig. 4B). Although, the quantitative PCR results were consistent for all littermate pairs of fibroblasts examined, the Affymetrix genechip data were less clear: there was considerable discrepancy between the data from each paired comparison and together none reached the 2.5-fold threshold for differential expression used in this study. We suspect that these discrepant results may reflect the complexity of regulation of MMP and TIMP genes by TGFβ and the limitations of simple comparison of mRNA levels in unstimulated fibroblast cultures. To clarify whether differences in basal expression could be explained by differences in TGFβ pathway activation, additional time course analyses were performed using transgenic or wild type fibroblasts after stimulation with recombinant TGFβ1. Transcripts for MMP-3, MMP-9, MMP-13, and TIMP1 were strongly induced by 12 h in wild type fibroblasts (Fig. 4, C–F). In transgenic fibroblasts there was basal overexpression but no significant induction of these genes in response to TGFβ1. Unexpectedly, MMP-13 and MMP-3 transcripts, while elevated at 3 and 6 h compared with wild type, were suppressed to basal wild type levels in transgenic cells at 12- and 24-h time points. Together these observations suggest that despite the general refractoriness of transgenic fibroblasts to TGFβ, for some genes there is regulation and for these inhibitory pathways may be preferentially activated in transgenic fibroblasts in response to exogenous TGFβ1. Later experiments strongly suggest that dominant negative activity of TβRIIΔk transgene expressed at higher levels from 24 h was important in this context (see below).

Transgenic Fibroblasts Show Enhanced Remodeling and Contraction of Fibroblast-populated Collagen Lattices—Based upon the altered MMP expression and apparent contractile phenotype of transgenic fibroblasts implied by expression of a mature α-SMA containing cytoskeleton we examined contraction of FPCL by transgenic cells. First we used a tensiometer system that allows force generated by fibroblasts to be measured as they contract the lattice (Fig. 5A). Force generation in the fibroblast-populated collagen lattice has previously been shown to depend upon both remodeling of the lattice together with contraction of cells within the gel matrix. Lattice contraction continued at a much faster rate and with greater gel tension for transgenic fibroblasts compared with equal numbers of seeded littermate wild type fibroblasts. This is consistent with more myofibroblasts being present in cultures from transgenic skin. There was also greater contraction of free-floating collagen lattices by transgenic fibroblasts (Fig. 5B). Previously, it has been demonstrated for wild type dermal fibroblasts that free-floating gel contraction depends mainly upon lattice remodeling (31) that can be promoted by TGFβ (34) or blocked by inhibitors of MMPs such as GM6001 (35). Our results confirm these observations in wild type fibroblasts, but suggest that transgenic fibroblasts are refractory to recombinant TGFβ1 and also to GM6001 (Fig. 5C). High endogenous levels of TGFβ1 and MMP activity that cannot be inhibited by concentrations of GM6001 may underlie the refractoriness of...
Expression and Activation of TGF-β1/H9252 Is Enhanced in Transgenic Mice—To determine whether supernatants from transgenic fibroblasts contained higher levels of TGF-β1/H9252 bioactivity than those from wild type cells, a bioassay using mink lung epithelial cells harboring a TGF-β-regulated promoter-reporter construct was used. Transgenic fibroblast supernatants demonstrated more than 2-fold elevation of bioactive TGF-β1 compared with littermate wild type cultures (Fig. 6A). Specificity of

| Affymetrix ID   | Accession No. | Wild type | TβRIIΔk | Relative expression | -Fold change | Gene |
|-----------------|---------------|-----------|---------|---------------------|-------------|------|
| 98833_at        | X66402        | 247.5     | 385     | I                   | 1.6         | MMP3 |
| 168521_at       | X72795        | 45.4      | 72.0    | I                   | 1.6         | MMP9 |
| 94724_at        | Y13185        | 10.7      | 132.7   | I                   | ND          | MMP10 |
| 100015_at       | Z12604        | 133.4     | 95.4    | NC                  | 0.7         | MMP11 |
| 100016_at       | M82831        | 122.2     | 33.5    | D                   | 0.3         | MMP12 |
| 100484_at       | X66473        | 79.7      | 102.6   | I                   | 1.3         | MMP13 |
| 160118_at       | AF022432      | 1899.9    | 1527.3  | NC                  | 0.8         | MMP14 |
| 92461_at        | AB021224      | 105.9     | 159.2   | NC                  | 1.5         | MMP17 |
| 101464_at       | V00755        | 2286.0    | 3851.8  | I                   | 1.6         | TIMP1 |
| 160116_at       | A1850047      | 269.5     | 156.4   | NC                  | 0.6         | TIMP2 |
| 16051_at        | AV156389      | 969.2     | 1853.4  | I                   | 1.9         | TIMP3 |

* Designation of expression in two independent experiments using Affymetrix U74v2 genechips. Transcripts were designated as unchanged (UC), down-regulated (D), or up-regulated (U) in transgenic versus wild type samples by the Affymetrix™ MAS5.0 software program.

b This transcript was not present in wild type samples and so -fold change is not determined (ND).

**FIG. 4.** Perturbed MMP expression and regulation in transgenic fibroblasts. A, basal up-regulation or down-regulation of key MMP genes in transgenic (tg) fibroblasts was determined by quantitative real-time RT-PCR. Transcript quantitation was measured relative to 18S rRNA amplification from the same sample and data are expressed as a ratio (-fold change) above mean basal expression for paired wild type (wt) fibroblast samples. Data summarize at least three independent experiments using fibroblasts from 5 littermate pairs. B, basal overexpression but paradoxical down-regulation by TGFβ1 in transgenic fibroblasts was confirmed in three independent experiments using fibroblasts from transgenic or wild type littermate pairs (n = 3) treated with recombinant murine TGFβ1 (4 ng/ml) after overnight serum starvation. For TIMP1 and MMP-9 there was transient induction in transgenic fibroblasts with later return to basal expression. In contrast, MMP-3 and MMP-13 were suppressed to basal wild type levels at later time points.
the assay for TGFβ was confirmed using anti-TGFβ antibody (1D11) or an isotype-matched control monoclonal (13C4) (data not shown). Supernatants from transgenic fibroblasts also contained higher total TGFβ levels. These results were validated by examining the amount of active TGFβ present in transgenic fibroblast skin biopsies. Immunolocalization using a panspecific anti-TGFβ antibody confirms increased dermal expression of TGFβ1 in transgenic fibroblasts compared with littermate wild type biopsies (Fig. 6B). An isotype-matched control antibody (13C4) was negative, confirming specificity of this staining. The data for total TGFβ were later confirmed using a non-murine anti-TGFβ1 primary antibody in formalin-fixed biopsy sections (data not shown).

In view of our finding that a number of metalloproteinases known to activate latent TGFβ complex were elevated in transgenic fibroblast cultures, we asked whether increased TGFβ bioactivity in culture supernatants might be MMP-dependent. In a series of three independent experiments there was consistent reduction in active but not total TGFβ activity in supernatants from transgenic fibroblast cultures treated with GM6001 (Fig. 6C). As this reduction was relatively modest in comparison with the elevated TGFβ activity in these supernatants it is likely that other mechanisms may also activate latent TGFβ in transgenic cultures. The link between expression of a kinase-deficient type II TGFβ receptor and increased TGFβ bioactivity appears, therefore, to include increased expression of TGFβ1 as well as greater activation of latent TGFβ complex, which at least partly appears MMP dependent.

The TβRIIΔk-fib Transgene Is TGF-β Regulated—We hypothesized that expression of the TβRIIΔk-fib transgene was also regulated by TGFβ and suspected that low level expression of the kinase-deficient type II receptor may facilitate basal TGFβ signaling via endogenous receptors, whereas higher expression levels were likely to be inhibitory. This was confirmed in a series of cotransfection experiments using wild type fibroblasts harboring TGFβ responsive reporter constructs and different amounts of pCMV-TβRIIΔk. At low levels there was increased expression of a TGFβ-regulated reporter, while higher pCMV-TβRIIΔk concentrations of reporter gene expression returned to basal, or was suppressed. Data shown for a 3TP-luciferase reporter (Fig. 7) are summarized in three experiments using replicate wells of wild type neonatal dermal fibroblasts and are representative of the results of a series of
parallel studies \( (n = 3) \) using PAI-1-Luc or ColIa2-Luc. Cotransfection of a non-TGFβ-regulated reporter construct was used to correct for transfection efficiency in these studies.

Earlier experiments performed in the type I tight skin mouse suggested that fibroblast-specific reporter gene expression might be up-regulated by TGFβ (23). This was confirmed in the present study using dermal fibroblasts cultured from TβRIIΔk-fib mice. Thus, levels of co-expressed β-galactosidase were significantly increased by recombinant TGFβ1, with maximum induction at 24 h. There was a subsequent fall in reporter gene expression back to basal expression levels at 48- and 72-h time induction at 24 h. A construct in which the 6-kb upstream enhancer fragment region was linked to a minimal thymidine kinase promoter was used in transient transfection to confirm that the TGFβ responsive elements are likely to be within the upstream fibroblast-specific enhancer element (Fig. 8D), although it is likely that TGFβ responsive elements within the proximal ColIa2 promoter may also be involved.

Collectively, our results demonstrate activation of the TGFβ axis at multiple levels in transgenic mice expressing TβRIIΔk selectively on fibroblasts. Fibroblast gene and protein expression in the transgenic mice shares many hallmark features of chronic fibrosis. Because TβRIIΔk functions as a dominant negative inhibitor of TGFβ signaling at high expression levels, and the fibroblast-specific expression cassette is also partially regulated by TGFβ, basal differences in gene expression are normalized or reversed by recombinant TGFβ1 for a subset of genes in vitro. In vivo, this mechanism may underlie balanced activation of TGFβ signaling in the TβRIIΔk-fib strain and is likely to protect mice from the deleterious consequences of uncontrolled TGFβ overactivity in growth and development.

**DISCUSSION**

A large body of indirect evidence supports a role for increased TGFβ bioactivity in the pathogenesis of fibrosis. To obtain more direct evidence we have used fibroblast-directed transgenesis to assess the potential contribution of altered TGFβ signaling in fibroblasts. This experimental strategy allows altered TGFβ signaling in fibroblasts to be examined while minimizing effects in other cell types. The present study extends our analysis of a novel genetically determined mouse model of fibrosis, the TβRIIΔk-fib strain. In our initial description we reported that a kinase-deficient human type II TGFβ receptor leads to fibrosis in transgenic mice (21). This surprising finding raised additional questions concerning the role of secondary signaling mediators in determining the biochemical phenotype, and the mechanism by which a kinase-deficient type II TGFβ receptor might activate downstream signaling pathways. In the present study we have analyzed altered gene expression in TβRIIΔk-fib transgenic fibroblasts and confirmed the pivotal role of endogenous TβRII receptor kinase (ALK5). We also show that the TβRIIΔk-fib transgene is itself regulated by TGFβ. Interestingly, the transgene facilitates TGFβ signaling via ALK5 at low expression levels but is inhibitory at higher levels, thereby establishing balanced up-regulation of TGFβ signaling in transgenic fibroblasts. Together our data reaffirm the importance of TGFβ activation in fibrosis and demonstrate that perturbation of receptor expression may result in multilevel activation of the TGFβ ligand-receptor axis.

Initial biochemical characterization of the TβRIIΔk-fib mouse strain suggested increased activation of intracellular signaling pathways downstream of TGFβ. The present study confirms perturbation of Smad-dependent signaling with altered expression and localization of phosphorylated Smad2/3 in transgenic compared with wild type littermate fibroblast cultures. Global gene expression profiling defines a cohort of dysregulated genes in this mouse strain and confirms that dermal fibroblasts have a TGFβ-activated phenotype, and share many hallmark features seen in chronic fibrosis. Similar approaches have been applied to whole tissue or explanted cell cultures from human fibrotic diseases including SSC and pulmonary fibrosis (38) and a number of animal models of fibrosis (16). While recognizing that -fold-change in mRNA may not neces-
sarily correlate with biological importance, we chose to focus initially upon the most dysregulated genes. A relatively stringent cut off of at least 2.5-fold up- or down-regulation was selected. Fold differences at this level were invariably determined as significant ($p < 0.001$) by the MAS5.0 analysis software. Our findings provide robust confirmation that $\alpha$-RII transgenic fibroblasts in culture show a TGF-$\beta$-activated phenotype with the most highly overexpressed genes being PAI-1 and CTGF. Gene expression profiles provide a large volume of data but there are well recognized limitations to interpretation. First, there are technical considerations because some oligonucleotide sets used on the Affymetrix gene-chip may have poor discriminatory potential (39). This may explain the disparity between the gene array results and those obtained from other methods including quantitative real-time RT-PCR and immunolocalization of protein using Western blot or immunostaining of cultured cells. In addition, only two pairs of fibroblast strains were examined by genechip analysis, whereas for PCR and protein analysis far more samples were evaluated. There was some variability in results between experiments although trends of differential expression were consistent. There is disparity between protein and mRNA data for a number of gene products. For example, basal expression of pro-$\alpha$-II collagen is $\sim 10$-fold greater in transgenic than wild type fibroblasts, whereas mRNA expression levels were only 2-fold greater by Northern blot analysis. Although this extends our earlier observation of high levels of type I collagen protein secretion by this transgenic strain, it suggests that this is not solely dependent upon increased mRNA or indeed transcriptional activation. Confirmation that CTGF mRNA was highly overexpressed supports previous results showing protein up-regulation (21). The significance of this is unclear, although a body of evidence suggests that CTGF, especially in combination with TGF-$\beta$, is fibrogenic (2). It may be, however, that CTGF is

**FIG. 8.** Fibroblast-specific transgene expression is regulated by TGF-$\beta$. A, recombinant TGF-$\beta$1 (4 ng/ml for 16 h) stimulated transgene expression (coexpressed LacZ) in $\alpha$-RII transgenic fibroblasts explanted from the skin of mice aged 3 days, 10 days, or 6 weeks. B, in vivo administration of a pan-specific TGF-$\beta$ neutralizing antibody reduces transgene expression in neonatal fibroblasts. Data summarize a series of independent experiments comparing the expression of LacZ at 6 and 10 days after treatment with anti-TGF-$\beta$1 (1D11) or an isotype-matched control antibody (13C4). Expression increased between 6 and 10 days in control mice but was substantially reduced in mice treated with 1D11. C, transgenic fibroblasts cultured from 6-kb LacZ reporter transgenic mice show TGF-$\beta$ responsiveness of the reporter transgene that is Sp1- and Smad-dependent because mithramycin or cotransfected pCMV-Smad7, but not curcumin, attenuated LacZ induction. D, the 6-kb far-upstream enhancer linked to a thymidine kinase promoter driving LacZ is also TGF-$\beta$ responsive, confirming that elements within the upstream promoter are responsive to TGF-$\beta$. Expression is adjusted relative to a control plasmid (see text). Expression in panels A–C is corrected for lysate DNA concentration as a surrogate for cell number. Data summarize three independent experiments.
a marker rather than mediator of the fibrotic phenotype. There is considerable evidence that CTGF is elevated in a large number of fibrotic diseases including SSC (1). It is interesting that in this transgenic model CTGF overproduction appears to require continued activation of the TβRI kinase, contrasting with reports suggesting that in SSC up-regulation of CTGF may be TGFβ independent (28, 38, 40).

The TβRIIΔκ-fib mouse strain provides a valuable tool for elucidating the regulation of metalloproteinase gene expression by TGFβ. Our quantitative RT-PCR results suggest that a subset of MMPs and TIMPs are abnormally expressed by TβRIIΔκ-fib transgenic fibroblasts, whereas the same cohort of MMP genes is up-regulated by TGFβ in control strains. Although MMPs are critical for connective tissue breakdown and remodeling they are also important regulators of cytokine bioactivity and receptor expression. Thus, it is possible that MMP overexpression, including MMP3 and MMP9, both of which activate latent TGFβ complex, could contribute to fibrogenesis. Consistent with this the broad spectrum MMP inhibitor GM6001 reduced levels of active TGFβ in transgenic fibroblast culture supernatants. Previous reports have suggested that MMP-dependent TGFβ activation is important in regulating osteoblast differentiation (29) and our results suggest that similar mechanisms may pertain to fibroblasts.

In earlier work we showed that a number of basally up-regulated genes in TβRIIΔκ-fib fibroblasts were suppressed 24 h after stimulation with recombinant TGFβ1 (21). Here, analysis of the time course for induction of MMP genes in transgenic fibroblasts shows that for some transcripts a transient increase in mRNA is followed by suppression. This downregulation of TGFβ-responsive genes suggests a TGFβ-inducible suppressor mechanism. A number of endogenous inhibitors may be relevant but the transgene product is also a very plausible candidate. Importantly, although transgene expression returns to its basal level in fibroblasts after initial induction by recombinant TGFβ1, similar to transcripts such as TIMP1 or PAI-1 (21), expression is not abolished completely, unlike other transcripts such as MMP-13 (Fig. 4B). This suggests that although TGFβ1-dependent transcriptional activators can modulate activity of the fibroblast-specific transgenic promoter other, presumably lineage-specific, transactivating factors determine constitutive activity. If transgene expression were abolished this might have abrogated the in vivo phenotype of the TβRIIΔκ-fib strain.

Experiments using SD-208 strongly suggest that activation of the type I TGFβ receptor ALK5 is a key determinant of the profibrotic phenotype in the TβRIIΔκ-fib transgenic mouse strain. Moreover, examination of endogenous receptor levels in transgenic fibroblasts suggest that levels of wild type TβRII are substantially increased compared with littermate wild type strains. This provides a plausible mechanism whereby basal signaling is activated. Our data suggest that low levels of TβRIIΔκ expression facilitate basal TGFβ signaling, whereas higher levels of expression are inhibitory. We confirm in vitro that the fibroblast-specific expression cassette is up-regulated by TGFβ in fibroblast cultured from transgenic mice and extend earlier work by showing that up-regulation involves both Smad-dependent signaling and also Sp1, but not AP-1 activity. Our in vivo studies show that a pan-isomorph specific anti-TGFβ monoclonal reduced expression of a fibroblast-specific reporter gene. Together, our results elucidate the mechanism underlying balanced up-regulation of TGFβ signaling pathways in the TβRIIΔκ transgenic mouse strain and provide insight as to how altered TGFβ signaling can sustain a fibrotic phenotype. Thus, expression of the transgenic receptor on fibroblasts at levels similar to that of the endogenous type II receptor activates TGFβ signaling, probably by facilitating activation of the TβRI kinase. The molecular mechanism by which co-expression of a kinase-deficient type II TGFβ receptor on fibroblasts activates signaling is uncertain. Gene expression profiling studies did not identify significant differences between transgenic or wild type levels of mRNA encoding TβRI or TβRII and so increased biosynthesis seems unlikely. Another possibility is that at low transgene expression levels heterodimers form with wild type TβRII and that the resulting complexes signal more efficiently, have greater ligand affinity, or are internalized and degraded more slowly than those homodimeric receptor complexes. The latter would be consistent with increased TβRII levels in transgenic fibroblast lysates. Interactions between pathway-specific ubiquitin ligases and heterodimeric receptor complexes are likely to be different from wild type receptor homodimers (41).

Recent reports suggest that the ratio of type I (TβRI) to type II (TβRII) receptors may determine basal activity of the TGFβ signaling pathway (42) and the mutant receptor may increase the ratio of TβRII/TβRII in transgenic fibroblasts. At higher

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**Fig. 9. Schematic summarizing multilevel activation of the TGFβ ligand-receptor axis in TβRIIΔκ-fib transgenic mice.**

- **A. Low level transgene expression**
  - Ligand-dependent facilitation of TGFβ signaling
  - TGFβRI
  - TGFβRII
  - MMP-3
  - TIMP-1
  - Paradoxical suppression of TGFβ1 upregulated genes
  - Transgene upregulation by TGFβ1

- **B. High level transgene expression**
  - Dominant negative activator antagonizes TGFβ ligand
  - Recombinant TGFβ1
  - TGFβRI
  - TGFβRII
  - MMP-3
  - TIMP-1
  - Increased active TGFβ signaling

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concentrations homodimers of mutant receptor are likely to form and these may inhibit responsiveness by direct competition for ligand with wild type receptors. A schematic summarizing the potentially contrasting effect of different levels of transgene expression is shown in Fig. 9.

There is a growing body of evidence suggesting that myofibroblasts are key players in the development of fibrosis (43). For example, in SSc there are increased numbers of myofibroblasts in skin and lung tissue (5). Differentiation of myofibroblast subpopulation of cells may be a critical regulatory step in wound repair as well as pathological fibrosis. The present study suggests that myofibroblasts are important in determining the phenotype of the TβRIIα-k-fib strain. Moreover this appears to be dependent upon TπRI kinase activity because it is blocked by the inhibitor SD-208. The functional consequences of this are demonstrated by enhanced contraction of tethered FPCLs by transgenic fibroblasts. It is noteworthy that free floating collagen gel contraction by transgenic fibroblasts was not significantly reduced by MMP inhibition in contrast to wild type fibroblasts. This is surprising and suggests that other cell properties, independent of MMP overexpression, are also important.

Animal models provide a platform for evaluation of novel antifibrotic therapies and there is considerable interest in targeting TGFβ as a therapy for fibrosis. One strategy is to use soluble antagonists of TGFβ. Many studies have shown that anti-cytokine antibodies, soluble receptor fusion proteins, and factors that bind active TGFβ ligand such as LAP or decorin may have antifibrotic potential in animal models (1). There have been recent trials of anti-TGFβ monoclonal antibody therapy for human fibrosis including a study of anti-TGFβ1 in early stage systemic sclerosis (45). However, it has been suggested that this a key abnormality in SSc may not be overproduction of TGFβ ligand but a more subtle disruption of signaling or receptor expression. Our findings support using ligand-directed blocking strategies to treat fibrosis; however, this is likely only to be truly effective if, as in the TπRIIα-k-fib strain the primary determinant of the phenotype is itself regulated by TGFβ. Another potential approach is the use of small molecule inhibitors of downstream components of the TGFβ signaling cascade. A number of ALK5 inhibitors have been tested in vitro, although the advantage of specific versus broader spectrum inhibition of this family of receptor kinases remains to be determined (46). The present study confirms the potential for ALK5 inhibition using one of these antagonists, SD-208, to block key pathogenic mechanisms including collagen overproduction, CTGF expression, and myofibroblast differentiation. However, there have been few studies of such inhibitors in vivo and formal comparison with other TGFβ blocking strategies would be worthwhile. It is possible that only some of the important biological effects of TGFβ are mediated via ALK5 (47). For example, other BMP superfamily members may also be important and it has recently been suggested that ALK1 and ALK5 stimulation by TGFβ ligand may have antagonistic effects (44).

In summary, we have explored the biochemical phenotype of fibroblasts cultured from a novel transgenic mouse strain and confirm that key features of human SSc are reproduced. We show that fibroblast-specific TβRIIα-k expression can stimulate profibrotic pathways in a TGFβ-dependent manner, and elucidate the basis for balanced activation of TGFβ-regulated genes by demonstrating that the transgene promoter is regulated by TGFβ1, and that high level transgene expression antagonizes the stimulatory effect of lower level expression.

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42. Pannu, J., Gore-Hyer, E., Yamanaka, M., Smith, E. A., Robinchik, S., Dong, J. Y., Jablonowska, S., Blaszczyk, M., and Trojanowska, M. (2004) Arthritis Rheum. 50, 1566–1577
43. Gabbiani, G. (2003) J. Pathol. 200, 500–503
44. Goumans, M. J., Valdimarsdottir, G., Itoh, S., Lebrin, F., Larsson, J., Mum- mery, C., Karlsson, S., and ten Dijke, P. (2003) Mol. Cell. 12, 817–828
45. Denton, C. P., Merkel, P. A., Purst, D. E., Khanna, D., Emery, P., Hsu, V. M., Silliman, N., Streisand, J., Powell, J., Korn, J. H., Black, C. M., and Seibold, J. R. (2004) Arthritis Rheum. 50, Suppl. 34, Abstr.
46. Inman, G. J., Nicolas, F. J., Callahan, J. F., Harling, J. D., Gaster, L. M., Reith, A. D., Lapin, N. J., and Hill, C. S. (2002) Mol. Pharmacol. 62, 65–74
47. Lapin, N. J., Grygielko, E., Mathur, A., Butter, S., Bomberger, J., Tweed, C., Martin, W., Porowald, J., Lehr, R., Harling, J., Gaster, L., Callahan, J. F., and Olson, B. A. (2002) Mol. Pharmacol. 62, 58–64