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

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We have generated transgenic mice expressing a kinase-deficient type II transforming growth factor- β (TGF β) receptor selectively on fibroblasts (T β RII Δ k-fib). These mice develop dermal and pulmonary fibrosis. In the present study we explore activation of TGF β 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 T β R1 kinase (ALK5) activity, and that transgenic fibroblasts show enhanced expression and activation of TGF β together with increased levels of wild type T β RII. Moreover, we confirm that transgene expression is itself regulated by TGF β and that expression at low levels facilitates signaling, whereas high level expression is inhibitory. For a subset of TGF β responsive genes basal up-regulation is normalized or suppressed by exogenous recombinant TGF β 1 at time points coincident with increased transgene expression. These findings explain the profound refractoriness of T β RII Δ k-fib fibroblasts to exogenous TGF β 1, 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 TGF β receptor function may lead to ligand-dependent activation of downstream signaling, and provides further evidence of a pivotal role for sustained TGF β overactivity in fibrosis.

A growing body of evidence implicates overactivity of transforming growth factor β (TGF β)¹ 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 TGF β signaling pathways (6, 7), altered expression of high and low affinity TGF β receptors (8–11), and autocrine overproduction of several TGF β -regulated genes (12, 13). In addition, several recent studies suggest altered expression of TGF β 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 TGF β -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 TGF β 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 TGF β receptors. Surprisingly, we achieved our goal of sustained activation of TGF β signaling by expressing a kinase-deficient type II TGF β receptor (T β RII Δ k) in fibroblasts. Initial characterization of a novel transgenic mouse strain in which T β RII Δ k is expressed selectively on fibroblasts by linking it a lineage-specific promoter demonstrated development of skin and lung fibrosis (21). Our findings were unexpected because kinase-deficient TGF β 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 T β RII Δ k-fib mice is delineated. We determine that expression of the mutant receptor leads to multilevel activation of the TGF β ligand-receptor axis and that

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¹ The abbreviations used are: TGF β , transforming growth factor β ; α -SMA, α -smooth muscle actin; MMP, matrix metalloproteinase; FPCL, fibroblast-populated collagen gel lattice; SSc, systemic sclerosis; PAI-1, plasminogen activator inhibitor; PBS, phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid; RT, reverse transcriptase; TIMP, tissue inhibitor of metalloproteinase.

activation is dependent upon endogenous $T\beta RI$ 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 $TGF\beta$ and that low level expression of the kinase-deficient $T\beta RII$ activates $TGF\beta$ signaling, whereas higher expression levels are inhibitory. We hypothesize that the profound refractoriness of explanted transgenic dermal fibroblasts to recombinant $TGF\beta 1$ 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 $TGF\beta$ ligand or receptor activity are unpredictable, and animal models such as the $T\beta RII\Delta k$ -fib strain are likely to be valuable tools in the development and evaluation of targeted antifibrotic therapies.

EXPERIMENTAL PROCEDURES

Transgenic Mice—The generation and initial characterization of the transgenic mice strains used in this work has been previously described. In brief, a fibroblast-specific expression cassette was subcloned from the upstream region of the pro- $\alpha 2(I)$ 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 $T\beta RII\Delta k$ -fib was generated by subcloning the cDNA encoding the extracellular and transmembrane portion of the human type II $TGF\beta$ receptor into the *SalI* 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 sequence. 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 $T\beta RII\Delta k$ -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 mg/ml streptomycin, and cultured in a humidified atmosphere of 5% CO_2 in air. Fibroblasts were subcultured at confluence and used between passages 2 and 5. Fibroblast monolayer cultures were incubated for 16–24 h in serum-free culture medium prior to experiments examining agonist effects. Transgene expression levels were routinely measured by the Galactolight™ biochemical assay of β -galactosidase activity (Tropix Inc., Bedford, MA), with normalization of assay data for cell number by assessing DNA concentration in cell or tissue lysates (23).

Transfection of Neonatal Fibroblasts—To assess $TGF\beta 1$ signaling pathway activity or responsiveness a series of $TGF\beta$ -regulated promoter-reporter constructs were introduced into transgenic or wild type cells by transient transfection, and the effect of recombinant $TGF\beta 1$ on reporter gene expression determined. Constructs used for these experiments have been previously described, including *PAI-1* (24), *Col1a2* (23), and a trimeric sequence delineated as the $TGF\beta$ response element of the *PAI-1* promoter, designated *3TP* (25). All were linked to a firefly luciferase reporter and plasmids were transfected into neonatal fibroblasts using Lipofectamine Plus™ (Invitrogen) according to the manufacturer's instructions. Assessment of luciferase activity was by the Dual Light™ reporter gene system (Tropix Inc.) with a 1:100 pSV40-*LacZ* control plasmid. For experiments 70% confluent fibroblast monolayers were used in 24-well tissue culture plates. To determine the effect of $T\beta RII\Delta k$ co-expression on activity, the cDNA encoding $T\beta RII\Delta k$ was subcloned into pCDNA3 (Invitrogen) and cotransfected at concentrations between 10 and 200 ng/tissue culture well with each of these $TGF\beta$ -regulated promoter-reporter constructs. Later, to investigate the responsiveness of the fibroblast-specific promoter-enhancer cassette driving $T\beta RII\Delta k$ a series of experiments were performed in which a pCMV-*Smad7* was transfected into transgenic fibroblasts from the 6-kb *LacZ* mouse strain or wild type fibroblasts cotransfected with

a plasmid in which the minimal endogenous promoter was replaced by a non- $TGF\beta$ responsive herpes simplex virus-thymidine kinase promoter as described previously (23).

Western Blot Analysis of Fibroblast Protein Expression—To examine biochemical or functional differences between transgenic or wild type fibroblast cultures and compare responsiveness to recombinant $TGF\beta 1$ 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, $T\beta RI$, $T\beta RII$, and phosphorylated *Smad2/3* (Santa Cruz Biotechnology, Santa Cruz, CA) using specific primary antibodies. Mouse-specific primary antibodies were used to ensure that endogenous $T\beta RII$ rather than truncated $T\beta RII\Delta k$ 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 specific transcripts were determined by Northern blotting, following separation of RNA on 1% agarose gels containing 2.2 M formaldehyde and capillary transfer to Hybond N+ membrane (Amersham Biosciences). Filters were hybridized and probed with cDNA fragments specific for pro- $\alpha 2(I)$ collagen and CTGF mRNAs as previously described (12). 18 S rRNA band intensity was used to confirm equal gel loading. Probes were labeled with [α - ^{32}P]dCTP to a specific activity of 10^9 dpm/mg, using the Megaprime™ random priming method (Amersham Biosciences). Levels of transcripts were determined from signal intensity measured by autoradiography.

Pharmacological Inhibitor Studies—Pharmacological inhibitors and anti- $TGF\beta$ antibodies were used to explore the role of key components of the ligand-receptor axis in determining the abnormal properties of transgenic fibroblasts. A novel, highly specific, inhibitor of the $T\beta RI$ kinase (SD-208, Scios Inc., Fremont CA) was used to ask whether blocking endogenous $TGF\beta$ signaling would abrogate the increase in $TGF\beta$ -dependent gene and protein expression in transgenic cells. SD-208 has an IC_{50} of 49 nmol/liter based on direct enzymatic assay of $TGF\beta RI$ kinase (ALK5) activity with a specificity of >100-fold against $TGF\beta RII$ and at least 17-fold over members of a panel of related protein kinases including p38a, p38b, p38d, JNK1, EGFR, MAPKAPK2, MKK6, ERK2, PKC, PKA, PKD, CDC2, and CaMKII. Preliminary experiments defined the effective concentration producing specific ALK5 inhibition. For these experiments fibroblasts were cultured to confluence, incubated in a low serum medium overnight, and then stimulated with recombinant $TGF\beta 1$ (4 ng/ml) for between 30 min and 24 h, in the presence or absence of the novel inhibitor. For studies examining regulation of the fibroblast-specific expression cassette by $TGF\beta 1$, mithramycin was used to inhibit Sp1 (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 $TGF\beta$ (1D11) on transgene expression in $T\beta RII\Delta k$ -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 $TGF\beta 1$ -induced differences in gene expression. Total RNA was extracted and treated with RNase-free DNaseI (Roche, Lewes, UK) for 10 min at 37 °C and heat inactivation at 70 °C for 15 min. 1 μ g of total RNA was reverse transcribed using 2 μ g of random hexamers (Amersham Biosciences) and 200 units of Superscript II reverse transcriptase (Invitrogen). Quantitative PCR were done on the ABI Prism 7700 (Applied Biosystems, Warrington, UK) according to previously described methods, with each reaction containing 5 ng of reverse transcribed RNA in 25 μ l. 18 S rRNA (primers and probes from Applied Biosystems) were used as an endogenous control to account for differences in the extraction and reverse transcription of total RNA.

Affymetrix Genechip Experiments—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 U74Av2 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 fluidics station and chip reader following the AffymetrixTM 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 M 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 to 80,000 cells and 1.2 mg of collagen/ml. The collagen-cell suspension (1 ml) was added to each well, and allowed to solidify for 1 h. After polymerization, 1 ml of MCDB medium was added to each well, causing detachment of the lattice from the tissue culture plastic, yielding free-floating FPCLs.

Measurement of tension across a three-dimensional, free-floating fibroblast-populated collagen lattice was performed as described previously (31, 32). Briefly, with 1×10^6 cells/ml collagen gel, we measured the force generated across the collagen lattice, by using a tensioning culture force monitor that is capable of measuring the minute forces exerted by cells within a collagen lattice over 24 h as fibroblasts attach, spread, and migrate. The rectangular fibroblast-seeded collagen gel was cast and floated in medium, tethered to two flotation bars on either end of the short edges, in turn attached to an anchor point at one end and a force transducer at the other. Cell-generated tensional forces in the collagen gel were detected by the force transducer and logged into a personal computer. Graphical readings are produced every 10 min, averaged from 600 readings (1 per second), providing a continuous output of force (Dynes) generated (31).

Immunostaining of Cells and Tissues—Cells were seeded at 4×10^3 cells/well in Dulbecco's modified Eagle's medium, 10% normal calf serum into chamber slides (Labtek, Nunc; Fisher Scientific UK, Loughborough, UK), grown to subconfluence, made quiescent in serum-free Dulbecco's modified Eagle's medium overnight, and exposed to control media (Dulbecco's modified Eagle's medium) or recombinant TGF β 1 (4 ng/ml) for between 3 and 24 h. Parallel experiments were performed with SD-208 to block T β RI kinase (ALK5) activity. The cell monolayer was washed three times with PBS, and fixed in 4% formaldehyde for 5 min. Cells were washed three times in PBS before permeabilization and after each later step. Permeabilization was performed using 0.1% Triton in 50 mM PIPES (pH 7.0), 90 mM HEPES (pH 7.0), 0.5 mM MgCl₂, 0.5 mM EGTA, and 75 mM KCl for 30 s, at room temperature. Chamber slides were incubated with primary antibody specific for phosphorylated Smad2/3 (Santa Cruz Biotechnology, Santa Cruz, CA) to examine receptor-regulated Smad activity and localization. Other studies used anti-SMA specific antibody (Santa Cruz Biotechnology). After incubation with primary antibody or irrelevant control, serum slides were treated with a fluorescein-labeled anti-mouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), each for 60 min at room temperature. Nuclei were stained for 1 min in 1 μ g/ml 4,6-diamidino-2-phenylindole (Molecular Probes, Eugene, OR). After extensive washing with PBS, a single drop of CitiFluor AF1 (Chem Lab, Canterbury, UK) was added and cells were then visualized and photographed using an Axioskop Z fluorescence microscope (Carl Zeiss, Jena, Germany). For immunolocalization of TGF β in neonatal mouse, skin samples from T β RII Δk transgenic or wild type littermates were skin snap frozen. Serial frozen sections (5 μ m) were cut on a cryostat at -30 °C and air-dried for 1 h. Sections were fixed in ice-cold acetone and covered with 3% hydrogen peroxide for 10 min in the dark to block endogenous peroxidase activity. Slides were then blocked with 20% normal horse serum, and incubated with monoclonal antibody against TGF β (1D11) for 1 h at room temperature. Primary antibody was localized using a biotin/streptavidin-based amplifying system. After washing with PBS, sections were incubated with biotinylated rabbit anti-mouse IgG diluted in PBS (7.5 g/ml, BA-9500; Vector Laboratories) for 30 min,

rinsed, and finally incubated with Vectastain Elite STR-ABC reagent (Vector Laboratories) for 30 min. Immunolocalization was visualized using 3-amino-9-ethylcarbazole chromogen and H₂O₂ as substrate (SK-4200; Vector Laboratories). Sections were then washed in tap water, counterstained with Carrazzi's hematoxylin, and mounted with Gel-mount (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.

TGF β Bioassay Using Mink Lung Epithelial Cells—To examine TGF β bioactivity in fibroblast culture supernatants we used a validated bioassay incorporating mink lung epithelial cells stably transfected with a PAI-1 luciferase reporter construct. The assay was performed as described previously (24). For each supernatant, aliquot basal activity (active TGF β) and activity after heating the supernatant at 80 °C for 10 min (total TGF β) was compared. Samples were analyzed in triplicate. Briefly, 1.6×10^4 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 TGF β 1 and neutralizing anti-TGF β 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 number at the time of harvesting the supernatant was confirmed by direct counting.

Statistical Analysis—For quantitative variables, mean (\pm 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 T β RII Δk 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 TGF β 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 TGF β 1 (Fig. 1A). We also observed constitutive expression of CTGF (CCN2, FISP12) protein by transgenic fibroblasts compared with littermate wild type cells. To confirm that activation of the TGF β signaling pathway determined constitutive overexpression of pro(I) collagen or CTGF, SD-208, a novel T β RI-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- α 2(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 T β RII Δk -fib transgenic mice (Fig. 1D).

We hypothesized that expression of T β RII Δk on fibroblasts may alter basal levels of endogenous wild type TGF β receptors. A series of Western blot experiments using mouse-specific primary antibodies confirmed substantially increased levels of wild type T β RII in transgenic fibroblast lysates. This provides a plausible explanation for increased TGF β signaling in these cells. Levels of wild type T β RI 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 T β RII Δk -fib cells to recombinant TGF β 1, while there was a marked nuclear

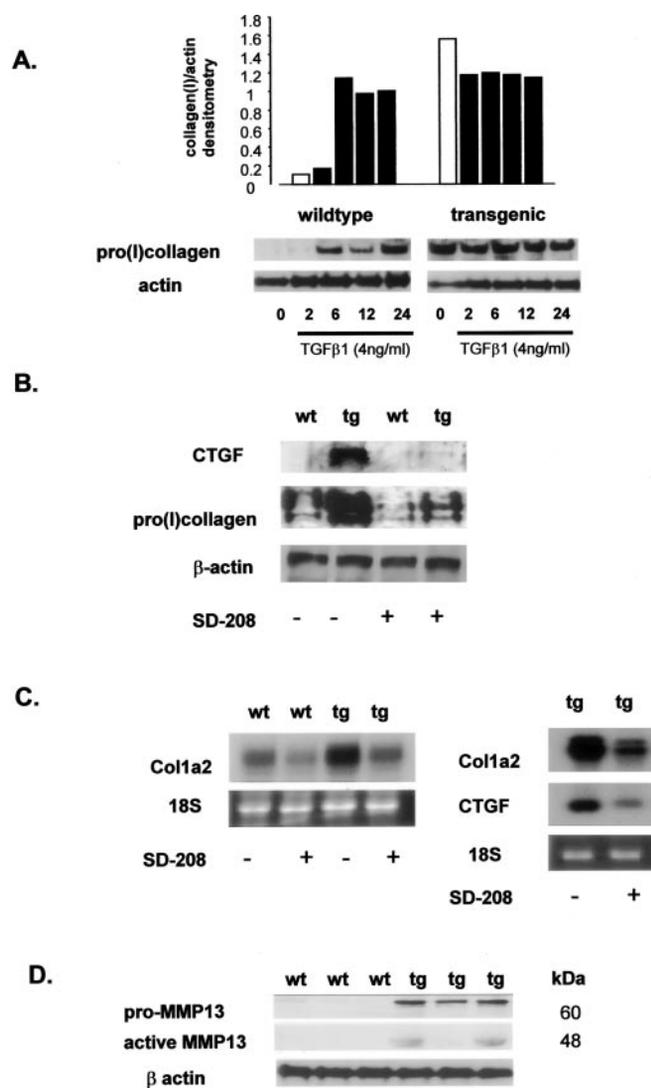


FIG. 1. Biochemical phenotype of $T\beta RII\Delta k$ -fib dermal fibroblasts. *A*, pro(I) collagen overexpression and refractoriness to TGF β 1 demonstrated by Western blot analysis of dermal fibroblast supernatants after stimulation by recombinant murine TGF β 1 (4 ng/ml). *B*, collagen and CTGF protein overexpression is abrogated by T β R1 kinase (ALK5) inhibition (SD-208). *C*, Northern blot analysis shows *Col1a2* and *CTGF* mRNA overexpression is abrogated by T β R1 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.

localization of pSmad2/3 in wild type fibroblasts 10 min after TGF β 1 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 T $\beta RII\Delta k$ -fib, and also its dependence upon ALK5 activation because it is abrogated by SD-208 (Fig. 2*F*).

Global Expression Profiling of $T\beta RII\Delta k$ Transgenic Fibroblasts—Based upon the similarity in phenotype between the T $\beta RII\Delta k$ -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 TGF β 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 ex-

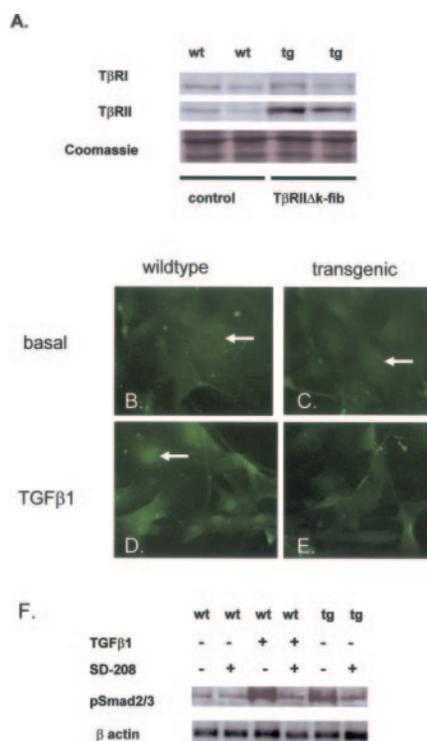


FIG. 2. $T\beta RII\Delta k$ -fib dermal fibroblasts have increased levels of endogenous T βRII and altered phosphorylation of Smad2/3. *A*, Western blot analysis of littermate fibroblast pairs confirms increased levels of endogenous T βRII in transgenic (*tg*) cells using a mouse-specific primary antibody. Levels of T $\beta R1$ 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 T $\beta RII\Delta k$ -fib cells (*C*). There is strong staining of wild type nuclei 10 min after incubation with recombinant TGF β 1 (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 T $\beta RII\Delta k$ -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.

pression 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-A, and *Cyr61*, and perhaps most significantly TGF β 1). Two of the most overexpressed transcripts, *PAI-1* (14.7-fold) and *CCN2/CTGF* (11.5-fold), are prototypic markers of TGF β activation, consistent with our hypothesis that these transgenic fibroblasts have sustained activation of TGF β signaling pathways. Other TGF β 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*.

TABLE I
Up-regulated genes in $T\beta RII\Delta k$ transgenic fibroblasts compared with wild type littermates (Affymetrix U74v2)

Affymetrix ID	Accession No.	Wild type ^a	$T\beta RII\Delta k$ ^a	-Fold change	Descriptions
94147_at	M33960	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
98865_at	U52554	64.7	512.9	7.9	Hyaluronan synthase 2
94378_at	U94828	113.6	878.2	7.7	<i>Mus musculus</i> retinally abundant regulator of G-protein signaling mRGS-r
96055_at	X59520	88.4	517.5	5.9	Cholecystokinin
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 plasminogen activator receptor <i>M. musculus</i> muPAR1
100554_at	AF053367	108.95	501.85	4.6	PDZ and LIM domain 1 (elfin) carboxyl-terminal LIM domain protein
92730_at	L07264	79.15	364.45	4.6	Heparin binding epidermal growth factor-like growth factor
93883_at	K03235	224.8	1000.9	4.5	Proliferin
94383_at	D50586	79.2	351.8	4.4	Tissue factor pathway inhibitor 2
103039_at	X79003	74.1	326.5	4.4	Integrin α 5 (fibronectin receptor α)
101393_at	AJ001633	206.8	792.6	3.8	Annexin III
92571_at	D85904	100.35	369.8	3.7	Heat shock protein 4, mouse mRNA for apg-2
92310_at	M96163	439.3	1473.0	3.4	Serum inducible kinase (SNK)
92777_at	M32490	88.15	284.6	3.2	Cyr61, insulin-like growth factor binding protein 10
101551_at	X78989	76.8	246.1	3.2	Testin
101979_at	AF055638	167.5	531.25	3.2	Growth arrest and DNA-damage-inducible 45 γ
99942_s_at	U28923	155.5	491.95	3.2	Smooth muscle calponin gene
97535_at	D87661	180.4	549.65	3.0	mRNA for 14-3-3 eta
100026_at	U42443	237.15	721.8	3.0	MECA39
160829_at	U44088	106.15	315.4	3.0	FasL synthesis, pleckstrin homology-like domain, family A, member 1
100381_at	M12347	199.3	577.85	2.9	Actin, α 1, skeletal muscle
101918_at	M17298	62.95	182.2	2.9	Transforming growth factor- β 1
102298_at	M17298	55.85	161.0	2.9	Nerve growth factor, β
96701_at	U35823	230.45	660.9	2.9	Ubiquitin-like 1 (sentrin) activating enzyme E1B
96657_at	L10244	353.5	1011.9	2.9	Spermidine/spermine N^1 -acetyltransferase
160102_at	Z237164	132.95	375.2	2.8	<i>M. musculus</i> chaperonin subunit 8 (theta) Cctq
94932_at	M29464	180.75	496.4	2.7	Platelet-derived growth factor α
101973_at	y15163	132.15	357.9	2.7	Cbp/p300-interacting transactivator
102887_at	U94331	91.8	245.9	2.7	Osteoprotegerin (OPG)
94288_at	J03482	241.3	645.3	2.7	Histone H1 gene
95580_at	U39473	83.85	223.6	2.7	Histidyl-tRNA synthetase
104601_at	X14432	161.4	423.3	2.6	Thrombomodulin
99158_at	U58888	159.4	416.3	2.6	Osteoclast stimulating factor 1
94826_at	Y11460	96.2	250.55	2.6	β 4 Integrin interactor
101096_s_at	AF203482	80.55	209.15	2.6	Nidogen 2

^a 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.

TABLE II
Down-regulated genes in $T\beta RII\Delta k$ transgenic fibroblasts (Affymetrix U74v2)

Affymetrix tag	Accession No.	$T\beta RII\Delta k$	Wild type ^a	-Fold change	Gene
95545_at	U22399	1218.1	53.8	22.6	Cyclin-dependent kinase inhibitor 1C
94813_at	X65128	1299.9	226.2	5.7	Growth arrest specific 1
101095_at	L23769	516.65	95.3	5.4	Microfibril-associated glycoprotein
93028_at	X58196	3997.2	759.35	5.3	H19 fetal liver mRNA
93511_at	L38971	511.05	115.6	4.4	Integral membrane protein 2
92365_at	X99572	682.3	165.4	4.1	Secreted frizzled related protein sFRP-2 (Sfrp2)
93503_at	U88567	206.5	52.65	3.9	Lysosomal-associated protein transmembrane 5
99010_at	AB24538	415.45	116.75	3.6	Immunoglobulin superfamily containing leucine-rich repeat)
96195_at	U57686	194.85	59.05	3.3	Embryonal Fyn-associated substrate p130 Cas-related protein Sin
102691_at	A1848518	308.85	97.7	3.2	UI-M-AP1-agf-e-02-0-UI.s2
103030_at	L31397	651.95	207.1	3.1	Dynamin
103031_at	L31397	954	322.95	3.0	Dynamin
160511_at	L12029	657.65	227.3	2.9	Chemokine (C-X-C motif) ligand 12, stromal cell-derived factor 1
103492_at	AF077738	293.55	105.5	2.8	Metalloproteinase CPX-1
93324_at	M58566	579.8	212.9	2.7	Zinc finger protein 36
98088_at	X13333	443.15	163.2	2.7	CD14 antigen
93353_at	AF013262	1215.55	448.6	2.7	Lumican
93343_at	J03952	1709.9	642.85	2.7	Glutathione transferase
9531_at	A1854771	193.75	73.1	2.7	UI-M-BH0-aka-c-10 ⁻⁰ -UI.s1
99622_at	U20344	395.15	152.5	2.6	Kruppel-like factor 4 (gut), gut-enriched Kruppel-like factor
102395_at	Z38110	931.85	364.7	2.6	PMP22 mRNA for peripheral myelin protein

^a Mean expression determined from two independently performed experiments using the Affymetrix U74v2 genechips. Genes were determined as present in all samples and designated significantly down-regulated ($p < 0.001$) in both transgenic samples compared with wild type littermates.

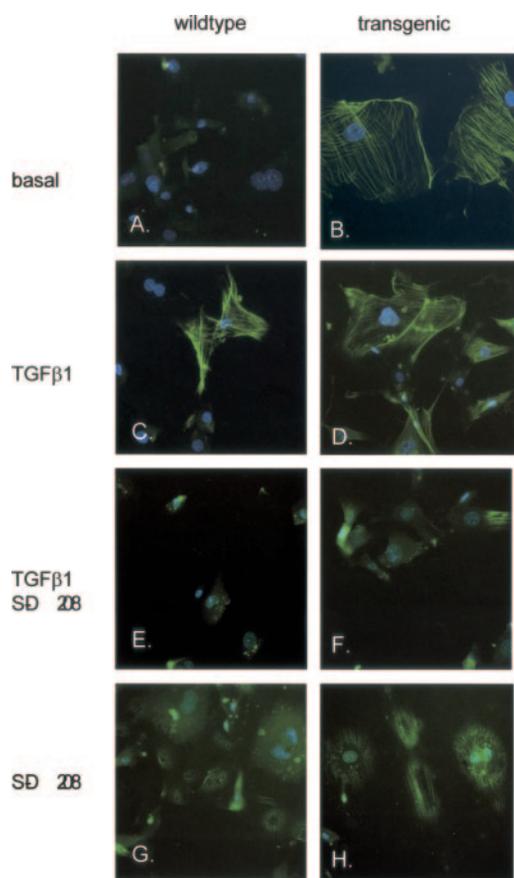


FIG. 3. Constitutive myofibroblast formation *in vitro* is abrogated by a specific ALK5 inhibitor. Constitutive formation of α -SMA containing myofibrils in transgenic fibroblasts confirms myofibroblast differentiation in serum-starved transgenic fibroblasts (A and B). Similar expression is induced in wild type cells from 3 h by recombinant TGF β 1 (4 ng/ml) (C and D). The ALK5 inhibitor SD-208 blocks α -SMA induction (E and F) and abrogates constitutive myofibril formation in transgenic fibroblasts (G and H).

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 TGF β 1 by forming α -SMA-rich fibers, whereas there was no change in α -SMA expression by transgenic fibroblasts in response to TGF β 1 (Fig. 3, C and D). The novel T β R1 kinase (ALK5) inhibitor SD-208 inhibited α -SMA filament formation in both transgenic or wild type fibroblasts treated with TGF β (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- β 1 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 TGF β . *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 TGF β 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 TGF β pathway activation, additional time course analyses were performed using transgenic or wild type fibroblasts after stimulation with recombinant TGF β 1. 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 TGF β 1. 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 TGF β , for some genes there is regulation and for these inhibitory pathways may be preferentially activated in transgenic fibroblasts in response to exogenous TGF β 1. Later experiments strongly suggest that dominant negative activity of T β R1 Δk 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 TGF β (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 TGF β 1 and also to GM6001 (Fig. 5C). High endogenous levels of TGF β 1 and MMP activity that cannot be inhibited by concentrations of GM6001 may underlie the refractoriness of

TABLE III
Metalloproteinase gene expression in wild type and transgenic fibroblasts

Affymetrix ID	Accession No.	Wild type	$T\beta RII\Delta k$	Relative expression ^a	-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 ^b	MMP10
100015_at	Z12604	133.4	99.4	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	3651.8	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

^a Designation of expression in two independent experiments using Affymetrix U74v2 genechips. Transcripts were designated as unchanged (UC), down-regulated (D), or up-regulated (I) 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).

A.

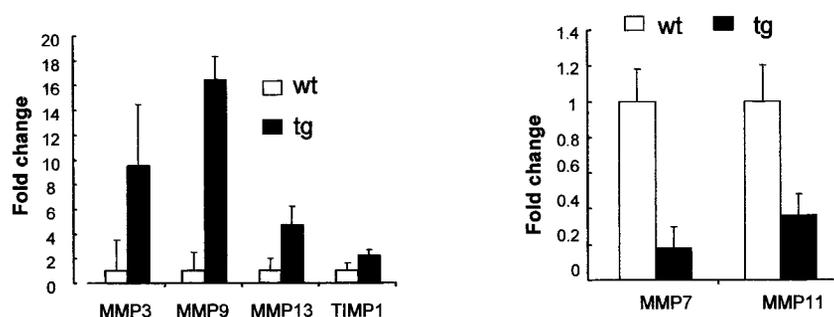
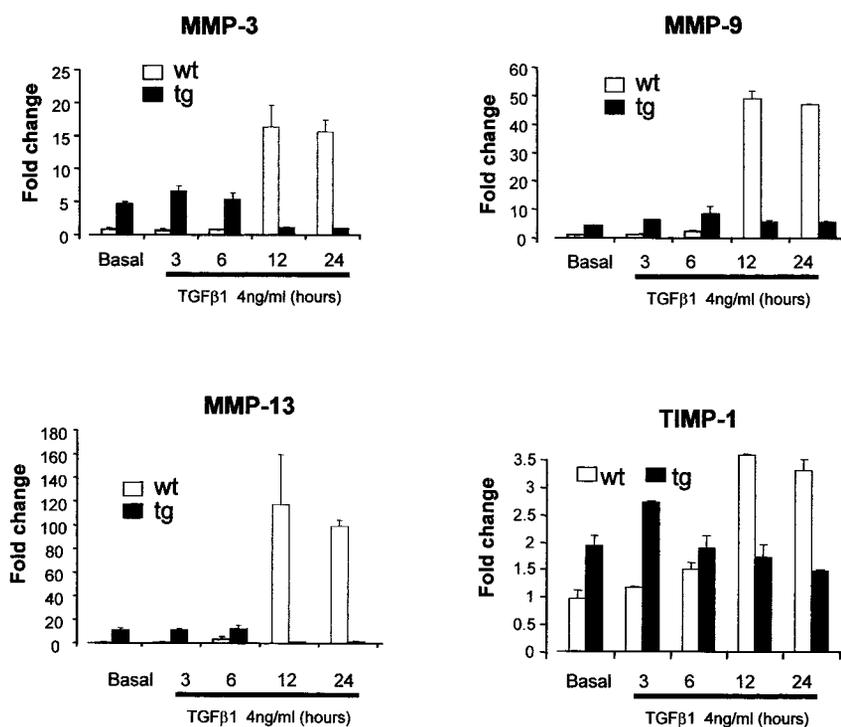


FIG. 4. Perturbed MMP expression and regulation in transgenic fibroblasts. A, basal up-regulation or down-regulation of key MMP genes in transgenic (*tg*) fibroblasts was determined by quantitative real-time RT-PCR. Transcript quantitation was measured relative to 18 S rRNA amplification from the same 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 $TGF\beta 1$ in transgenic fibroblasts was confirmed in three independent experiments using fibroblasts from transgenic or wild type littermate pairs ($n = 3$) treated with recombinant murine $TGF\beta 1$ (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.

B.



transgenic compared with wild type fibroblasts in this experimental system.

Expression and Activation of $TGF\beta$ Is Enhanced in Transgenic Mice—To determine whether supernatants from transgenic fibroblasts contained higher levels of $TGF\beta$ bioactivity

than those from wild type cells, a bioassay using mink lung epithelial cells harboring a $TGF\beta$ -regulated promoter-reporter construct was used. Transgenic fibroblast supernatants demonstrated more than 2-fold elevation of bioactive $TGF\beta$ compared with littermate wild type cultures (Fig. 6A). Specificity of

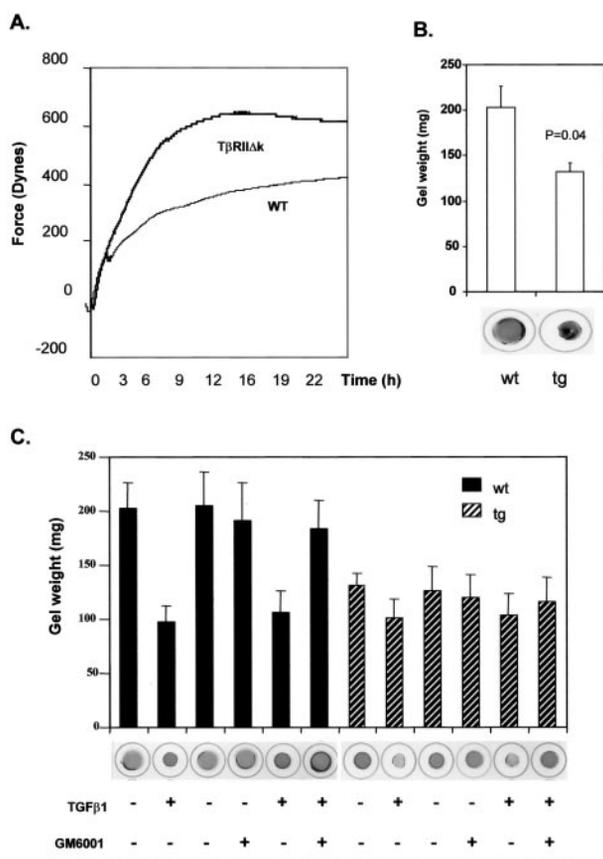


FIG. 5. Transgenic (*tg*) fibroblasts demonstrate enhanced remodeling and contraction of fibroblast-populated collagen lattices. *A*, transgenic fibroblasts show enhanced contraction of fixed FPCL over 24 h compared with littermate wild type (*wt*) fibroblasts seeded at equivalent density. *B*, there is also increased contraction of free-floating FPCL after 16 h. *C*, contraction of free-floating FPCL induced by TGF β 1 in wild type cultures is blocked by GM6001, confirming dependence upon MMP activity. Conversely, GM6001 does not inhibit the enhanced gel contraction shown by transgenic fibroblasts, suggesting that other mechanisms are also important. Data summarize a series of three independent experiments.

the assay for TGF β was confirmed using anti-TGF β antibody (1D11) or an isotype-matched control monoclonal (13C4) (data not shown). Supernatants from transgenic fibroblasts also contained higher total TGF β levels. These results were validated by examining the amount of active TGF β present in transgenic fibroblast skin biopsies. Immunolocalization using a panspecific anti-TGF β antibody confirms increased dermal expression of TGF β 1 in transgenic fibroblasts compared with littermate wild type biopsies (Fig. 6*B*). An isotype-matched control antibody (13C4) was negative, confirming specificity of this staining. The data for total TGF β were later confirmed using a non-murine anti-TGF β 1 primary antibody in formalin-fixed biopsy sections (data not shown).

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

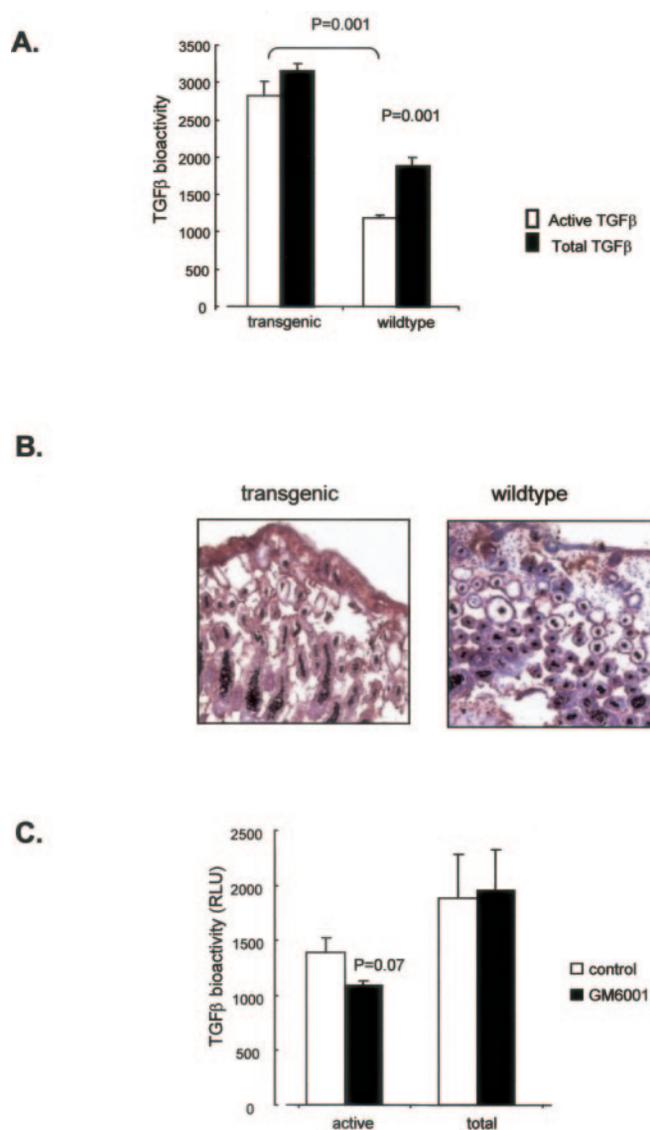


FIG. 6. TGF β expression and bioactivity is increased in transgenic mice. *A*, transgenic fibroblasts produce and activate TGF β more than wild type cells, assessed using a TGF β bioassay using mink lung epithelial cells stably transfected with a PAI-1-luciferase reporter construct. *B*, increased expression of TGF β isoforms in transgenic mouse skin stained using a monoclonal antibody specific for active TGF β isoforms (1D11). *C*, an MMP inhibitor (GM6001) reduces the level of active TGF β in transgenic fibroblast culture supernatant but does not affect total TGF β bioactivity. Data summarize three independent experiments.

pression of TGF β 1 as well as greater activation of latent TGF β complex, which at least partly appears MMP dependent.

The T β RII Δ k-fib Transgene Is TGF- β Regulated—We hypothesized that expression of the T β RII Δ k-fib transgene was also regulated by TGF β and suspected that low level expression of the kinase-deficient type II receptor may facilitate basal TGF β 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 TGF β responsive reporter constructs and different amounts of pCMV-T β RII Δ k. At low levels there was increased expression of a TGF β -regulated reporter, while higher pCMV-T β RII Δ k 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

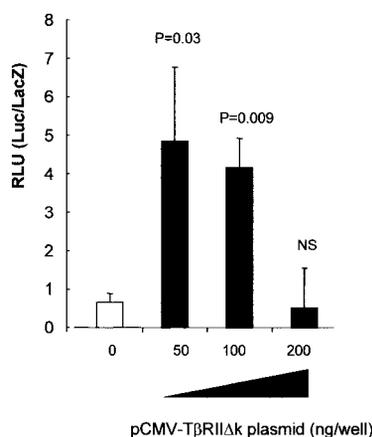


FIG. 7. **Low level expression of $T\beta RII\Delta k$ activates a TGF β -regulated promoter.** Data show activation of the *3TP-Luc* reporter construct and are representative of a series of three experiments using TGF β -regulated constructs. Correction for transfection efficiency is made using a cotransfected reporter construct driving *LacZ*, and results are expressed as relative light units (RLU) *Luc/LacZ*. Equal total DNA was transfected per well into subconfluent cultures of wild type dermal fibroblasts.

parallel studies ($n = 3$) using *PAI-1-Luc* or *Col1a2-Luc*. Co-transfection of a non-TGF β -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 TGF β (23). This was confirmed in the present study using dermal fibroblasts cultured from $T\beta RII\Delta k$ -fib mice. Thus, levels of co-expressed β -galactosidase were significantly increased by recombinant TGF β 1, 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 points, consistent with our observation for other TGF β -regulated transcripts including TIMP1 and MMP-9 (Fig. 4B). Induction at 24 h was maximal for fibroblasts cultured from 10-day-old mice but remained significant up to 6 weeks (Fig. 8A). To determine whether the constitutive level of transgene expression was regulated by TGF β *in vivo*, a series of neutralization experiments were undertaken using the pan-isoform-specific anti-TGF β antibody 1D11. This antibody, but not an isotype-matched control antibody of irrelevant specificity, reduced *in vivo* transgene expression by ~50% (Fig. 8B). Thus, for transgenic mice injected with the irrelevant 13C4 monoclonal there was an increase in expression of the transgene between genotyping biopsy performed at the 6-day and analysis at a 10-day time point. In contrast, mice injected with 1D11 expressed transgene at only around half the level of the 13C4-injected pups at 10 days. These results provide strong support for regulation of $T\beta RII\Delta k$ transgene expression by endogenous TGF β *in vivo* and suggest that anti-TGF β treatment may be an effective way of abrogating the mouse phenotype. Our previous work (23) identified the fibroblast-specific enhancer as a target for activation by TGF β 1. To circumvent potentially confounding effects of $T\beta RII\Delta k$ coexpression we investigated TGF β regulation of the fibroblast-specific expression cassette in fibroblasts cultured from 6-kb *LacZ* reporter mice in which the 6-kb fibroblast-specific enhancer was linked to a minimal endogenous promoter driving a *LacZ* reporter gene (described in detail in Ref. 23). To delineate the downstream pathways that are important in activating the transgene we used inhibitors of potential downstream pathways that have been implicated in mediating transcriptional activation of *Col1a2*, including Sp1, AP-1, and Smad signaling (36, 37). Thus transfection of an expression vector encoding Smad7 was used to inhibit Smad-

dependent signaling, mithramycin as an antagonist for Sp1, and curcumin to inhibit AP-1 signaling. Through this approach, we confirmed that TGF β 1 up-regulated the fibroblast-specific expression cassette and that this depended upon Sp1, and to a lesser degree, Smad activity, but was independent of AP-1 (Fig. 8C). 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 TGF β responsive elements are likely to be within the upstream fibroblast-specific enhancer element (Fig. 8D), although it is likely that TGF β responsive elements within the proximal *Col1a2* promoter may also be involved.

Collectively, our results demonstrate activation of the TGF β axis at multiple levels in transgenic mice expressing $T\beta RII\Delta k$ selectively on fibroblasts. Fibroblast gene and protein expression in the transgenic mice shares many hallmark features of chronic fibrosis. Because $T\beta RII\Delta k$ functions as a dominant negative inhibitor of TGF β signaling at high expression levels, and the fibroblast-specific expression cassette is also partially regulated by TGF β , basal differences in gene expression are normalized or reversed by recombinant TGF β 1 for a subset of genes *in vitro*. *In vivo*, this mechanism may underlie balanced activation of TGF β signaling in the $T\beta RII\Delta k$ -fib strain and is likely to protect mice from the deleterious consequences of uncontrolled TGF β overactivity in growth and development.

DISCUSSION

A large body of indirect evidence supports a role for increased TGF β bioactivity in the pathogenesis of fibrosis. To obtain more direct evidence we have used fibroblast-directed transgenesis to assess the potential contribution of altered TGF β signaling in fibroblasts. This experimental strategy allows altered TGF β 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 $T\beta RII\Delta k$ -fib strain. In our initial description we reported that a kinase-deficient human type II TGF β 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 TGF β receptor might activate downstream signaling pathways. In the present study we have analyzed altered gene expression in $T\beta RII\Delta k$ -fib transgenic fibroblasts and confirmed the pivotal role of endogenous $T\beta RI$ receptor kinase (ALK5). We also show that the $T\beta RII\Delta k$ -fib transgene is itself regulated by TGF β . Interestingly, the transgene facilitates TGF β signaling via ALK5 at low expression levels but is inhibitory at higher levels, thereby establishing balanced up-regulation of TGF β signaling in transgenic fibroblasts. Together our data reaffirm the importance of TGF β activation in fibrosis and demonstrate that perturbation of receptor expression may result in multilevel activation of the TGF β ligand-receptor axis.

Initial biochemical characterization of the $T\beta RII\Delta k$ -fib mouse strain suggested increased activation of intracellular signaling pathways downstream of TGF β . 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 TGF β -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-

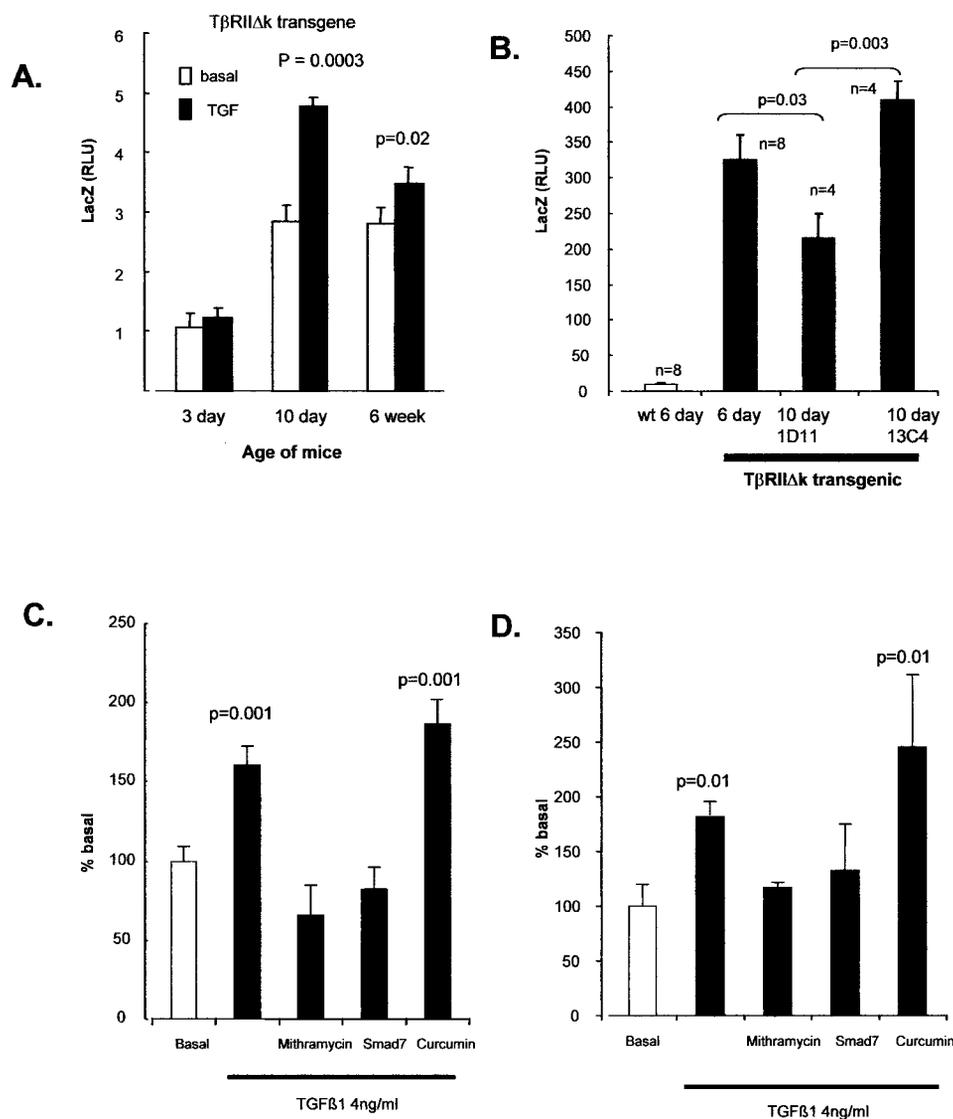


FIG. 8. Fibroblast-specific transgene expression is regulated by TGF β 1. A, recombinant TGF β 1 (4 ng/ml for 16 h) stimulated transgene expression (coexpressed *LacZ*) in $T\beta RII\Delta k$ -fib fibroblasts explanted from the skin of mice aged 3 days, 10 days, or 6 weeks. B, *in vivo* administration of a pan-specific TGF β 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 β 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 β 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 β responsive, confirming that elements within the upstream promoter are responsive to TGF β 1. 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.

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 $T\beta RII\Delta k$ -fib transgenic fibroblasts in culture show a TGF β -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 genechip 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- α 2(I) 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 β , is fibrogenic (2). It may be, however, that CTGF is

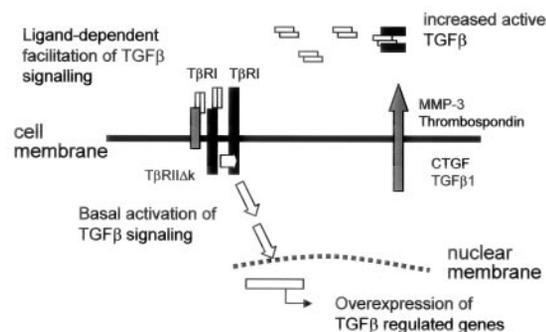
a marker rather than mediator of the fibrotic phenotype. There is considerable evidence that CTGF is elevated in a large number of fibrotic diseases including SSc (1). It is interesting that in this transgenic model CTGF overproduction appears to require continued activation of the $T\beta RI$ kinase, contrasting with reports suggesting that in SSc up-regulation of CTGF may be $TGF\beta$ independent (28, 38, 40).

The $T\beta RII\Delta k$ -fib mouse strain provides a valuable tool for elucidating the regulation of metalloproteinase gene expression by $TGF\beta$. Our quantitative RT-PCR results suggest that a subset of MMPs and *TIMPs* are abnormally expressed by $T\beta RII\Delta k$ -fib transgenic fibroblasts, whereas the same cohort of MMP genes is up-regulated by $TGF\beta$ 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 $TGF\beta$ complex, could contribute to fibrogenesis. Consistent with this the broad spectrum MMP inhibitor GM6001 reduced levels of active $TGF\beta$ in transgenic fibroblast culture supernatants. Previous reports have suggested that MMP-dependent $TGF\beta$ 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 $T\beta RII\Delta k$ -fib fibroblasts were suppressed 24 h after stimulation with recombinant $TGF\beta 1$ (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 down-regulation of $TGF\beta$ responsive genes suggests a $TGF\beta$ -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 $TGF\beta 1$, 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 $TGF\beta 1$ -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 $T\beta RII\Delta k$ -fib strain.

Experiments using SD-208 strongly suggest that activation of the type I $TGF\beta$ receptor ALK5 is a key determinant of the profibrotic phenotype in the $T\beta RII\Delta k$ -fib transgenic mouse strain. Moreover, examination of endogenous receptor levels in transgenic fibroblasts suggest that levels of wild type $T\beta RII$ 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 $T\beta RII\Delta k$ expression facilitate basal $TGF\beta$ signaling, whereas higher levels of expression are inhibitory. We confirm *in vitro* that the fibroblast-specific expression cassette is up-regulated by $TGF\beta$ 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-isoform specific anti- $TGF\beta$ monoclonal reduced expression of a fibroblast-specific reporter gene. Together, our results elucidate the mechanism underlying balanced up-regulation of $TGF\beta$ signaling pathways in the $T\beta RII\Delta k$ transgenic mouse strain and provide insight as to how altered $TGF\beta$ 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

A. Low level transgene expression



B. High level transgene expression

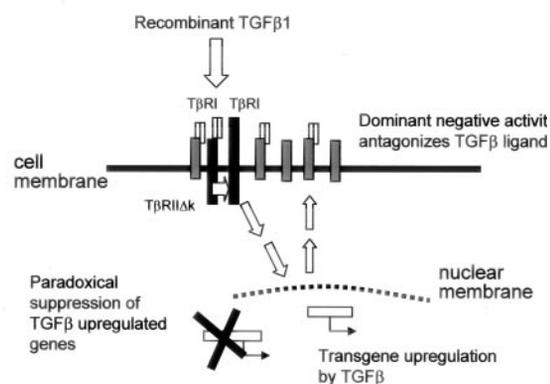


FIG. 9. Schematic summarizing multilevel activation of the $TGF\beta$ ligand-receptor axis in $T\beta RII\Delta k$ -fib transgenic mice. *A*, expression of the kinase-deficient type II $TGF\beta$ receptor at low levels facilitates activation of the endogenous type I $TGF\beta$ receptor, at least in part by increasing levels of wild type $T\beta RII$. Downstream consequences include up-regulation of $TGF\beta 1$ and other gene products that promote $TGF\beta$ activity or activate the latent $TGF\beta$ complex. This results in net activation of $TGF\beta$ signaling. *B*, in response to recombinant $TGF\beta 1$ there is significant elevation of transgene expression. Higher level transgene expression is inhibitory and blocks signaling. Thus for transcripts up-regulated at early time points by $TGF\beta 1$, such as *TIMP1*, there is transient response in transgenic cells, but for transcripts induced 12 h (such as *MMP-3* and *MMP-13*) when the transgene is also up-regulated, suppression is observed (see Fig. 3B). High level transgene expression does not suppress the fibroblast-specific promoter completely suggesting that other $TGF\beta$ -independent pathways also govern activity of this lineage-specific construct.

$TGF\beta$ signaling, probably by facilitating activation of the $T\beta RI$ kinase. The molecular mechanism by which co-expression of a kinase-deficient type II $TGF\beta$ 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 $T\beta RI$ or $T\beta RII$ and so increased biosynthesis seems unlikely. Another possibility is that at low transgene expression levels heterodimers form with wild type $T\beta RII$ 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 $T\beta RII$ 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 ($T\beta RI$) to type II ($T\beta RII$) receptors may determine basal activity of the $TGF\beta$ signaling pathway (42) and the mutant receptor may increase the ratio of $T\beta RI:T\beta RII$ in transgenic fibroblasts. At higher

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 a 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 $T\beta RII\Delta k$ -fib strain. Moreover this appears to be dependent upon $T\beta R I$ 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 $TGF\beta$ as a therapy for fibrosis. One strategy is to use soluble antagonists of $TGF\beta$. Many studies have shown that anti-cytokine antibodies, soluble receptor fusion proteins, and factors that bind active $TGF\beta$ ligand such as LAP or decorin may have antifibrotic potential in animal models (1). There have been recent trials of anti- $TGF\beta$ monoclonal antibody therapy for human fibrosis including a study of anti- $TGF\beta 1$ in early stage systemic sclerosis (45). However, it has been suggested that a key abnormality in SSc may not be overproduction of $TGF\beta$ 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 $T\beta RII\Delta k$ -fib strain the primary determinant of the phenotype is itself regulated by $TGF\beta$. Another potential approach is the use of small molecule inhibitors of downstream components of the $TGF\beta$ 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 $TGF\beta$ blocking strategies would be worthwhile. It is possible that only some of the important biological effects of $TGF\beta$ 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 $TGF\beta$ 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 $T\beta RII\Delta k$ expression can stimulate profibrotic pathways in a $TGF\beta$ -dependent manner, and elucidate the basis for balanced activation of $TGF\beta$ -regulated genes by demonstrating that the transgene promoter is regulated by $TGF\beta 1$, and that high level transgene expression antagonizes the stimulatory effect of lower level expression.

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