Fibroblast-specific Expression of a Kinase-deficient Type II Transforming Growth Factor β (TGFβ) Receptor Leads to Paradoxical Activation of TGFβ Signaling Pathways with Fibrosis in Transgenic Mice

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To better understand the role of disrupted transforming growth factor β (TGFβ) signaling in fibrosis, we have selectively expressed a kinase-deficient human type II TGFβ receptor (TβRII∆k) in fibroblasts of transgenic mice, using a lineage-specific expression cassette subcloned from the pro-α2(Ⅰ) collagen gene. Surprisingly, despite previous studies that characterized TβRII∆k as a dominant negative inhibitor of TGFβ signaling, adult mice expressing this construct demonstrated TGFβ overactivity and developed dermal and pulmonary fibrosis. Compared with wild type cells, transgenic fibroblasts proliferated more rapidly, produced more extracellular matrix, and showed increased expression of key markers of TGFβ activation, including plasminogen activator inhibitor-1, connective tissue growth factor, Smad3, Smad4, and Smad7. Smad2/3 phosphorylation was increased in transgenic fibroblasts. Overall, the gene expression profile of explanted transgenic fibroblasts using cDNA microarrays was very similar to that of littermate wild type cells treated with recombinant TGFβ1. Despite basal up-regulation of TGFβ signaling pathways, transgenic fibroblasts were relatively refractory to further stimulation with TGFβ. Thus, responsiveness of endogenous genes to TGFβ was reduced, and TGFβ-regulated promoters-reporter constructs transiently transfected into transgenic fibroblasts showed little activation by recombinant TGFβ1. Responsiveness was partially restored by overexpression of wild type type II TGFβ receptors. Activation of MAPK pathways by recombinant TGFβ1 appeared to be less perturbed than Smad-dependent signaling. Our results show that expression of TβRII∆k selectively in fibroblasts leads to paradoxical ligand-dependent activation of downstream signaling pathways and causes skin and lung fibrosis. As well as confirming the potential for nonsignaling receptors to regulate TGFβ activity, these findings support a direct role for perturbed TGFβ signaling in fibrosis and provide a novel genetically determined animal model of fibrotic disease.

Transforming growth factor β (TGFβ) isoforms β1–β3 are important regulators of embryonic and postnatal cell differentiation and proliferation. They are also potent profibrotic factors in vitro. Transgenic and gene-targeted mutant mice have proven valuable for investigating the important roles of the TGFβ family in growth and development. For example, mice lacking TGFβ1 show substantial embryonic lethality, and those that are born develop a fatal disseminated inflammatory illness within the first postnatal month. Conversely, mice lacking TGFβ2 all die neonatally with defective epithelial-mesenchymal interaction, whereas mice lacking TGFβ3 show a perinatal lethal phenotype with cleft palate. These different knock-out phenotypes suggest somewhat distinct biological functions in vivo, although all three TGFβ isoforms signal though the same specific receptor complex, so differential effects are likely to reflect patterns of ligand expression or the influence of accessory receptors at the cell surface. Ligand engagement by the type II high affinity receptor (TβRII) appears to be a major limiting step in cellular activation, and mice lacking this receptor die early in embryonic development from abnormal yolk sac vascularization.

There is tight regulation of extracellular TGFβ ligand bioavailability, dependent upon its release from preformed large latent complexes in which TGFβ is noncovalently associated with its propeptide fragment, latency-associated peptide and covalently linked to one of several latent TGFβ-binding proteins. Release of active ligand from the large latent complex occurs through chemical, thermal, or proteolytic activity. In vivo thrombospondin-1 and a number of matrix metalloproteinases determine stability of the large latent complex, and interestingly thrombospondin-1 null mice develop a phenotype reminiscent of TGFβ1 null animals, suggesting that extracellular cleavage of latent TGFβ complexes may be a key mechanism regulating ligand activity. A number of accessory proteins

Received for publication, January 21, 2003, and in revised form, April 16, 2003
Published, JBC Papers in Press, April 21, 2003, DOI 10.1074/jbc.M300636200

Printed in U.S.A.
facilitate binding of TGFβ ligand to its high affinity receptors. Of these accessory receptors, betaglycan is widely expressed, whereas endoglin has a restricted pattern of expression, being present predominantly on endothelial cells. Downstream signaling pathways activated by TGFβ are becoming increasingly well delineated. At the cell surface, ligand engagement by TpRII allows phosphorylation of serine residues within the type I receptor (TpRII). This activates receptor kinase activity, leading to a series of downstream events. Much interest has focused on defining the role of the Smad family of proteins in type I receptor (TpRII) complexes that determine many of the effects of TGFβ, particularly to fibroblasts, but not other cell types, during embryonic development and postnatally (9). This enhancer therefore provides a mechanism for TGFβ signaling in vivo. This is in part due to confounding effects of TGFβ on nonfibroblastic cells. Recently a potent fibroblast-specific transcriptional enhancer has been delineated within the far upstream region of the mouse pro-α2(I) collagen gene (8). In previous work, we have shown that expression of reporter transgenes linked to TGFβ (6), and these may control levels of transcriptional coactivators or other transcription factors that interact with Smad proteins to determine the diverse effects of TGFβ on target cells (7).

Although there is considerable evidence that expression or function of TGFβ isoforms is altered in fibrotic disease, supported by its potent profibrotic activity in tissue culture, there have been relatively few studies directly examining the consequences of sustained disruption of TGFβ signaling in vivo. This is in part due to confounding effects of TGFβ on nonfibroblastic cells. Recently a potent fibroblast-specific transcriptional enhancer has been delineated within the far upstream region of the mouse pro-α2(I) collagen gene (8). In previous work, we have shown that expression of reporter transgenes linked to this enhancer recapitulate expression of type I collagen in fibroblasts, but not other cell types, during embryonic development and postnatally (9). This enhancer therefore provides a unique tool by which genetic perturbation can be targeted specifically to fibroblasts.

In the present study, we have used this enhancer to selectively express a kinase-deficient mutant type II TGFβ receptor (TpβRII(k)). This construct encodes the extracellular and transmembrane portion of the human TpβRII. It may therefore engage free TGFβ ligand but cannot directly lead to phosphorylation of TpRII to initiate downstream signaling (10). Such a truncated receptor has previously been characterized as a competitive antagonist for TGFβ1 and, when expressed at high levels in vitro, operates as a dominant negative inhibitor of TGFβ activity (11). We predicted that fibroblast-specific expression of TpβRII(k) would selectively disrupt TGFβ signaling in these cells without affecting other type I collagen-producing cells or nonmesenchymal lineages. As expected, fibroblasts cultured from mice expressing the mutant receptor were refractory to exogenous TGFβ1, but surprisingly they also demonstrated a constitutive biochemical phenotype reminiscent of TGFβ1 activation, and adult transgenic mice developed dermal and pulmonary fibrosis. As well as providing insight into the potential regulatory effects of nonsignaling TGFβ receptors and the long term effect of sustained TGFβ overactivity in vivo, these transgenic mice provide a novel genetically determined model for fibrotic disease.

**EXPERIMENTAL PROCEDURES**

**Generation of Transgenic Mice**—The cDNA encoding a truncated TpβRII protein comprising amino acids 24–184 of human TpβRII, including extracellular and transmembrane domains and a few residues of the cytoplasmic portion of the receptor, was used in this study (10). The coding sequence was excised from its source plasmid (pSV-TpβRII(k) using HindIII and XbaI and subcloned into pBluescript KS+/-.. This was excised using HindIII and EcoRV, treated with Klenow fragment, and cloned into the expression vector pCD3 at a blunted SaI site. The expression vector pCD3 includes a 6-kb upstream fragment of the mouse pro-α2(I) collagen (Colα2) gene, between –19.5 and –13.5 kb upstream of the transcription start site, linked to a minimal Colα2 promoter (–350 to +35 bp). This enhancer-promoter cassette has previously been shown to strongly direct expression of reporter transgenes to fibroblasts in embryonic development and postnatally (8, 9). The vector also includes the intron and polyadenylation signal from the murine protamine-1 gene (12, 13). An internal ribosome entry site from the Encephalomyocarditis virus was subcloned at the 5′NruI fragment of the internal ribosome entry site-LacZ-containing plasmid pW18 (14). This was introduced into pRM-6kb-LacZ plasmid (9) using directional cloning between the same restriction sites. The final transgene construct is shown in Fig. 1A.

Transgenic mice were generated according to standard methods. In brief, the transgene construct was linearized by digestion with ScaI, and the backbone bacterial sequence was removed using NruI. The fragment was gel-purified and electrophoresed prior to ultracentrifugation (60 min at 4 °C). After dilution to 2 ng/ml final concentration in microinjection buffer (15), the DNA solution was microinjected into fertilized B6D2 F2 oocytes. These were transferred into CD1 foster mothers and examined at embryonic day 15.5 or allowed to reach full term for examination of postnatal time points. Progeny were backcrossed with wild type mice to establish lines. Mice were genotyped by PCR of genomic DNA extracted from tail biopsies of neonatal pups or from placentas of founder embryos, using primers specific for the β-galactosidase reporter gene (16) (5′-CGGATAACCGGACTGAAA-3′ and 5′-TAATCCAGACTCTGCTATAC-3′) to yield a 500-bp product. Amplification was undertaken by 35 cycles of 60 s of annealing at 56 °C, 60 s of extension at 72 °C, and 60 s of extension at 72 °C.

Later, transgene expression was determined in whole mount embyros using 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) staining for β-galactosidase activity (see below). Co-expression of this marker gene provided a simple method for confirming that genotypically transgenic animals expressed the transgene and also facilitated later histological and biochemical analysis of derived transgenic mouse lines. Expression of the β-galactosidase marker was also measured by biochemical assay. Fresh tissue samples were homogenized for 90 s and centrifuged at 3000 × g for 5 min, and β-galactosidase activity in the tissue extract or in lysed tissue culture cells was measured by GalactoLight™ chemiluminescence assay, according to the manufacturer’s instructions (Troppix Inc., Bedford, MA). In brief, 20 μl of extract supernatant was added to 100 μl of reaction buffer/GalactoLight™ substrate mixture and luminescence was measured after a 30-min incubation. Wild type tissue extract was included in each assay to control for background β-galactosidase activity. Biopsy wet weight or DNA content of the tissue extract was used for standardization of raw data. DNA content was measured by fluorometry using the Hoechst 33258 fluorochrome in a dedicated filter fluorometer (16) (Hoefer TKO 100; Amersham Biosciences).

**Histological Analysis**—For histological analysis, 6 × 6-mm samples of adult mouse tissues or whole mount embryos (embryonic day 15.5) were processed for X-gal staining. Briefly, after fixation for 60 min (0.1 m sodium phosphate, pH 7.3, 5 mm EDTA (pH 8.0), 2 mm magnesium chloride, 0.2% glutaraldehyde, 0.3% formaldehyde), samples were rinsed for 90 min and stained with X-gal staining solution (1 mg/ml) at room temperature, as described previously. Tissue was processed for light microscopy by increasing concentrations of ethanol and stored in 80% ethanol at –20 °C. Tissues were dehydrated and paraffin wax-embedded, and 7-μm sections were cut and counterstained with hematoxylin and eosin. Masson’s trichrome and Van Gieson elastin staining was performed to further assess extracellular matrix deposition.

**Analysis of Skin Collagen Content**—Noncross-linked fibrillar collagen content of biopsy specimens was determined using the Sirius red staining assay (Bioceil Co., Ltd., UK). Punch skin biopsies (2 mm) were obtained from shaved areas of skin from the lower back of age- and sex-matched transgenic (n = 7) or nontransgenic (n = 7) littermates, and time points of 6, 14, and 24 weeks were examined. Biopsies were finely chopped and homogenized in 0.5 M acetic acid with 1:20 (w/w) peptin overnight at 4 °C. The samples were centrifuged, and 100 μl of supernatant was analyzed using the Sirius red dye reagent. Dye-collagen complexes were resolubilized and assayed using a microplate reader at 540 nm. Standard curves were used to determine exact collagen content per biopsy. This assay measures the noncross-linked collagen content and has been shown to reflect recently synthesized collagen in fresh tissue samples (17) and closely correlate with hydroxyproline content.
(18). Data were expressed as mean collagen content per biopsy corrected for DNA content as described above.

**Fibroblast Culture**—Fibroblast cultures were derived from skin biopsies from lower back of neonatal transgenic or control littermate mice. Cells were cultured in the presence of antibiotics and passed at confluence. Transgene expression levels were routinely measured by biochemical assay of β-galactosidase activity. Proliferation of dermal fibroblasts cultured from transgenic mice or from nontransgenic littermates was compared by direct cell counting. Following 24 h of culture in low serum (0.5% fetal calf serum) medium, 10^6 cells were seeded into each well of replicate six-well tissue culture plates. At 24-h intervals, the cell layer was recovered and resuspended. Cell number was assessed by direct counting in duplicate samples for a series of cultures (n = 4) derived from two different transgenic or wild type littermates. Data from these experiments were combined for comparison of growth rates.

**Flow Cytometric Analysis of TGFβ Receptor Expression**—Expression of cell surface TGFβ receptors was analyzed and quantified by flow cytometry. Confluent cultures of neonatal fibroblasts were detached using Ca^2+Mg^2+-free EDTA (10 mM) for 10 min at 4 °C, and single cell suspensions were incubated with the primary antibodies at 10 μg/ml for 60 min at 4 °C, followed by a species-specific fluorochrome-conjugated secondary antibody for 30 min at 4 °C. Cells were then washed and fixed in freshly prepared 1% paraformaldehyde in PBS. An isotopematched irrelevant primary monoclonal antibody was used as a control for nonspecific binding. Fluorescence intensities of staining were measured by FACScalibur (Becton Dickinson, Twickenham, UK), analyzing data from 10^5 cells after gating on the basis of their size and granularity. Biotinylated antibodies specific for murine type I and type II TGFβ receptors (R & D Systems Ltd., Oxford, UK) were used to measure endogenous receptor expression. The transgene product was detected using a directly fluorescein-conjugated mouse monoclonal antibody specific for the extracellular portion of the human TβRII (R&D Systems), showing less than 10% cross-reactivity with the mouse receptor. Total TGFβ1 binding was assessed by the Fluorokine™ kit (R&D Systems), using a biotinylated TGFβ1 conjugate in place of a primary antibody.

**Genetic Expression**—For the TGFβ-regulated gene plasminogen activator-inhibitor-1 (PAI-1), fluorescent real time PCR (Taquin) was used to confirm basal and TGFβ-regulated differences in gene expression. These experiments demonstrated constitutive overexpression and refractoriness to further activation by recombinant TGFβ1, and this was confirmed by Western blot experiments and using cDNA microarrays. Initial time course experiments suggested that more comprehensive analysis of gene expression a 12-h time point would be informative. For this, total mRNA was prepared from confluent cultures of early passage neonatal dermal fibroblasts using the Triazol™ RNA extraction kit, following the manufacturer's protocol. Constitutive patterns of gene expression for transgenic fibroblasts or cells cultured from transgenic founders were compared with wild type neonatal dermal fibroblasts using TGFβ1 ligand (4–10 ng/ml final concentration). Expression analysis was performed using Clontech Atlas Mouse 1.2 arrays, incorporating oligonucleotides specific for 1176 mouse gene transcripts. Hybridization was performed according to the manufacturer's instructions. Briefly, after DNase I treatment of the total DNA, 5 μg of each paired sample (TGFβ1-treated or -untreated, or transgenic and nontransgenic) was incubated with the sequence-specific primer mix and reverse transcriptase. The resulting cDNA probes were labeled by incorporation of [α-32P]dATP and simultaneously hybridized to the microarrays. After hybridization, membranes were washed, and radioactivity determined by a PhosphorImager (Amersham Biosciences). Digital image analysis was performed using the Clontech Atlas Image™ software. After alignment of individual gene spots on the microarray image file, differentially expressed genes were identified by normalization for global gene expression. Relative and absolute differences in gene expression profiles were compared. Significant differential expression was defined by at least a 2-fold consistent difference between samples (p < 0.05). To determine constitutive differences in gene expression, basal expression patterns were compared for wild type or transgenic cells. The pattern of TGFβ1-modulated gene expression in wild type fibroblasts was compared with constitutive differences in gene expression for transgenic and wild type cells. Correlation analysis was performed to compare differences in gene expression and statistical significance was determined using statistical software and a Monte Carlo simulation.

**Western Blot Analysis**—To examine biochemical or functional differences between transgenic or wild type fibroblast cultures and compare responsiveness to recombinant TGFβ1 ligand, a series of Western blot experiments were performed. Gene products that are known to be TGFβ-regulated were initially examined in time course experiments. Additional protein targets were selected based on cDNA microarray data. Cell layer lysates and tissue culture supernatants were examined from independent strains derived from transgenic or nontransgenic littermates (n = 4). To determine TGFβ1 responsiveness, parallel cultures were treated for 12 h with recombinant TGFβ1 as outlined above. Supernatants were concentrated by ammonium sulfate precipitation to selectively enrich samples for secreted matrix proteins. After SDS-PAGE electrophoresis, 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 Associates, Birmingham, AL), fibronectin (Santa Cruz Biotechnology, Santa Cruz, CA), and keratinocyte growth factor (FGF7) (R&D Systems) were used. Cell layer lysates were probed using antibodies directed against Smad3, phospho-Smad2/3, Smad4, Smad7, vimentin (all from Santa Cruz Biotechnology), and connective tissue growth factor (CTGF, antibody supplied by FibroGen Inc.). Total and phosphorylated p38MAPK and ERK1/2 were detected by specific antibodies following activation of fibroblast monolayers using recombinant TGFβ1 at time points between 15 min and 24 h.

We hypothesized that basal differences in gene and protein expression might be dependent upon extracellular TGFβ and tested this using a soluble TβRII fusion protein that incorporates two ligand binding domains from the rabbit type II TGFβ receptor linked by the Fc portion of rabbit IgG. By binding active but not latent TGFβ, this has shown to be a powerful potent and highly selective competitive antagonist (21). Initial studies confirmed that a concentration of 5 μg/ml completely blocked promotion of type I collagen synthesis by recombinant TGFβ1 (4–10 ng/ml) in normal fibroblasts, and this concentration was used in subsequent experiments on transgenic cells.

**Transient Transfection Assays of Gene Activation**—To assess TGFβ responsiveness, a series of TGFβ-regulated promote-reporter constructs were introduced into transgenic or wild type cells by transient transfection, and the effect of recombinant TGFβ1 on reporter gene expression was determined. Constructs used for these experiments were previously described, including PAI-1 (22), collagen (Col) (19), fibronectin (5), and a trimeric sequence delineated as the TGFβ response element of the PAI-1 promoter, designated 3TP (23). 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 a dual luciferase reporter gene system (Promega Corp., Madison, WI) with a 1:100 test plasmid/control pTKRenilla luciferase ratio. For experiments, 70% confluent fibroblast monolayers were used in 24-well tissue culture plates. After 12 h, serum-supplemented medium was added to each well, and 12 h later TGFβ1 was added. Initial experiments used concentrations between 1 and 100 ng/ml and time points of 6–24 h for wild type neonatal dermal fibroblasts and determined that treatment of cells at between 4 and 10 ng/ml for 16 h gave consistent responses of the firefly luciferase gene expression after correction for transfection efficiency.

To examine whether overexpression of wild type high affinity TGFβ receptors might influence ligand responsiveness or basal properties of wild type or transgenic fibroblasts, cDNA encoding full-length type I and type II receptors regulated by a CMV promoter were cotransfected with reporter constructs into wild type or transgenic littermate fibroblasts (see Ref. 9 for a detailed description of these reporter expression constructs). Effects of recombinant TGFβ1 (4 ng/ml) on a 3TP-regulated reporter gene (see above) were assessed in a series of three independent experiments and similar experiments performed using a Col1a2-luciferase construct.

**RESULTS**

**Adult TβRⅡi/h Transgenic Mice Develop Skin and Lung Fibrosis**—From a series of pronuclear injections, 13 PCR-positive founders were obtained. Seven of these founders (54%) showed bacterial β-galactosidase activity by X-gal staining of whole mount embryos (embryonic day 15.5), confirming that the integrated transgene was expressed. Of the expressing founders, there was a range intensity of X-gal staining, as observed in reporter mice previously (9), but all expression was in fibroblastic tissues, including stromal cells of specialized organs and at sites of membranous ossification in the skull. (Fig. 1B). Histological examination confirmed expression in...
meninges, fascia, dermis, and fibroblastic cells within developing limbs (Fig. 1, C–G). Two transgenic lines (designated F107 and M113) were established from female and male founders. Both lines demonstrated consistent expression in the skin and other fibroblastic structures, and one of these (M113) was used for detailed histological and biochemical studies.

Transgenic mice appeared normal postnatally, but from 6 weeks to 25% of animals failed to thrive and lost weight. Systematic histological examination of these mice identified patchy areas of altered lung histology with reduced airspace and increased cellular connective tissue (Fig. 2, A and B) and extracellular matrix (Fig. 2, C and D) compared with littermate wild type animals. In around 10% of mice, this change was extensive, and affected adults were euthanized by 16 weeks of age. Contrasting with the sporadic lung abnormalities, all transgenic mice manifested increased thickness of the dermis by 12 weeks of age. This was especially apparent over the lower back with adherence of skin to underlying fascial layers. Histologically, the dermis in a series of age 14-week-old male mice was thickened (Fig. 2, E and F) with loss of the subcutaneous adipose layer. To quantify these changes, biopsies were analyzed using a biochemical assay for noncross-linked fibrillar collagen content, and transgenic mice demonstrated 50% greater collagen content (p < 0.01; Student’s unpaired t test) after correction for biopsy wet weight (Fig. 3 A). Similar results were obtained after adjustment for DNA content in skin extracts as a surrogate for cell number (data not shown).

Fibroblast Growth and TGFβ Receptor Expression—To better understand the mechanism underlying development of skin and lung fibrosis, dermal fibroblasts from neonatal or adult transgenic mice were cultured. These showed sustained transgene expression, determined by biochemical assay of β-galactosidase in fibroblast lysates compared with littermate wild type fibroblasts. Expression persisted over successive passages in culture (Fig. 3 B) and was severalfold higher in neonatal cells than those cultured from adult mice. Neonatal cultures were therefore used for more detailed analysis. Significantly greater cell proliferation was apparent for transgenic fibroblasts from 48 h after seeding (mean ± S.D. doubling time was 55 ± 3 h for transgenic, compared with 72 ± 7 h for wild type; p = 0.008). Representative growth curves are shown in Fig. 3C.

Expression of the mutant receptor protein by transgenic fibroblasts was confirmed by flow cytometry. Total TGFβ1 binding was more than 60% greater for transgenic cells (Fig. 4A), and these (but not fibroblasts from littermate wild type

![Fig. 1. Fibroblast-directed expression of a kinase-deficient human type II TGFβ receptor. A, the cDNA encoding TβRIIΔk is regulated by a minimal Col1a2 promoter linked to a fragment of the far upstream (~19.5 to ~13.5 kb) Col1a2 enhancer. Bacterial β-galactosidase (LacZ) is co-expressed via an internal ribosome entry site (IRES). An intron and polyadenylation sequence of mouse protamine 1 is also included. B, whole mount preparation of a transgenic embryo (embryonic day 15.5) stained by X-gal. Representative histological sections of the embryo are shown in C–G. m, meningeal staining; d, dermal staining.](http://www.jbc.org/)

![Fig. 2. Pulmonary and dermal fibrosis in adult transgenic mice. Adult transgenic mice demonstrated patchy fibrosis within lung parenchyma in 25% of animals from 6 weeks old. Representative littermates are shown for mice aged 16 weeks stained by hematoxylin and eosin (A and B) or at higher power by Masson’s trichrome method (C and D). The latter confirms increased extracellular matrix expression in affected tissue. The skin of adult mice was thickened, and representative sections (hematoxylin and eosin) for littermate male mice aged 14 weeks (E and F) show increased dermal thickness and loss of subcutaneous fat.](http://www.jbc.org/)
animals) were recognized by an antibody specific for extracellular epitopes of human T/H9252 RII (Fig. 4B), confirming mutant receptor (T/H9252 RII/h) expression. Parallel studies examining expression of endogenous murine type I or type II receptors between wild type and transgenic fibroblasts (Fig. 4, C–E) showed a very modest increase in T/H9252 RII expression (111% nontransgenic) and a slight reduction in murine T/H9252 RII expression (89% nontransgenic). In replicate studies, only differences in total TGFβ1 binding and T/H9252 RII/h expression were statistically significant (p < 0.05).

**Transgenic Fibroblasts Have a TGFβ1-activated Phenotype**—To examine expression of TGFβ-regulated genes in transgenic fibroblasts, fluorescent RT-PCR (Taqman), Western blot analysis of gene products, and cDNA microarray expression profiling were used. A series of independent experiments

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**Fig. 3.** Transgene expression is associated with increased collagen production and fibroblast proliferation. A, noncross-linked fibrillar collagen content was determined colorimetrically using the Sircol™ assay in a series (n = 14) of littermate transgenic or nontransgenic mouse skin biopsies. Representative data for 14-week-old mice are shown. B, transgene expression was confirmed in explanted fibroblasts using a biochemical assay for co-expressed β-galactosidase. First (P1) and second (P2) passage cells were compared. Nontransgenic activity is not above lysis buffer control (blank). Data are mean ± S.D. for triplicate samples in a representative experiment. C, after overnight serum deprivation, the growth curve for transgenic fibroblasts shows significantly increased cell number from 48 h. Data (mean ± S.E.) summarize triplicate samples from two independent experiments. ***, p < 0.01; ***, p < 0.001.

**Fig. 4.** Expression of TβRIIΔk alters fibroblast surface binding of TGFβ1. Total TGFβ1 binding to neonatal fibroblasts was measured flow cytometrically using biotinylated recombinant TGFβ1 (A). Binding of anti-human T/H9252 RII by transgenic cells confirmed expression of the mutant receptor (B). Mouse-specific antibodies were used to examine endogenous receptor levels (T/H9252 RII-/mouse; C and D). Each panel shows specific antibody binding to transgenic (red) or nontransgenic (black) fibroblasts. An isotope-matched control antibody was included to control for nonspecific binding (gray). E, transgenic fibroblast average fluorescence intensity is expressed as a percentage of nontransgenic for ligand binding and each receptor. Total TGFβ1 binding and expression of mutant human receptor (T/H92RII-h) were increased on transgenic cells. Modest differences in mouse receptor levels (T/H92RII-m and T/H92RII-m) were not statistically significant. Data are representative of three independent experiments.
confirmed that transgenic fibroblasts were relatively refractory to up-regulation of PAI-1 mRNA by TGFβ1, with only a 2-fold increase compared with basal level in transgenic cells compared with more than 25-fold induction in wild type fibroblasts. However, basal levels of the PAI-1 transcript were 15-fold expressed genes in transgenic fibroblasts showing a similar pattern in wild type cells after TGFβ1 activation. This is shown graphically for a series of 30 genes consistently expressed by neonatal fibroblasts (Fig. 6A). Individual transcripts, normalized wild type relative to expression, confirmed the pattern seen for PAI-1 and CTGF in earlier time course experiments for both up-regulated and down-regulated genes (Fig. 6, B and C).

Western blotting largely confirmed the changes observed for TGFβ-regulated transcripts and representative data are summarized in Fig. 7. Fibronectin expression by wild type cells was strongly induced by TGFβ1, whereas transgenic cells had elevated basal levels. Induction was seen in transgenic cells treated with TGFβ1, but the relative response was less than for wild-type fibroblasts. For keratinocyte growth factor (FGF7), there was substantial down-regulation of the protein level in culture media from wild type cells after treatment with TGFβ1, and transgenic fibroblasts had a significantly lower level of FGF7 than control cells, with a proportionately smaller suppressive effect of TGFβ1. Type I procollagen was strongly induced by TGFβ1 in wild type cells and transgenic fibroblasts showed high levels of type 1 collagen gene expression that were comparable with those seen in wild type cells after treatment with TGFβ1, these was not further influenced by exogenous TGFβ1. Transgenic fibroblast lysates contained increased Smad7, a receptor-regulated inhibitory signaling protein, but further induction by TGFβ1 was not observed. This is in marked contrast to the results obtained for wild type cells. Similarly, for Smad3, there was an elevated basal protein level in whole cell extracts with no further induction by TGFβ1, compared with several-fold induction in nontransgenic control fibroblasts. Activation of Smad signaling was further confirmed by demonstration of increased phosphorylated Smad2/3. For the nonreceptor-regulated intermediate Smad4, transgenic samples showed around 2-fold higher levels compared with wild type littermates, and, as expected, these were independent of TGFβ1 exposure. Despite the changes in vimentin gene expression observed in transgenic fibroblasts or in wild type cells in response to TGFβ1 (Fig. 6A), protein expression did not differ. Vimentin levels therefore provided a loading control for Western blot analysis. Overall, Western blot data confirm that transgenic fibroblasts show a TGFβ-activated phenotype with diminished responses to exogenous TGFβ1, although the degree of response varied between gene products, with fibronectin retaining significant responsiveness, especially at the protein level (Fig. 7A). Initial results are consistent with a marked disruption of Smad-dependent signaling pathways. Later results examined the MAPK pathway that is also activated in fibroblasts after TGFβ1 stimulation. Time course analysis for total and phosphorylated p38MAPK and ERK1/2 suggests very low levels of phosphorylated forms of these MAPK family members in unstimulated wild type fibroblasts. TGFβ1 induced phosphorylation, which was maximal at 30 min for ERK1/2 and between 1 and 4 h for p38MAPK in control cells. Transgenic fibroblasts also responded to recombinant TGFβ1 with increased phosphorylation of p38MAPK and ERK1/2, but there were some differences from wild type cells. Thus, a greater level of phosphorylated protein was seen in unstimulated transgenic fibroblasts, and peak levels of phosphorylation occurred at later time points for ERK1/2 (Fig. 7B)

Thus, results of protein and mRNA studies suggested two
different but related biochemical phenotypes for transgenic fibroblasts. First, there was basal activation of TGFβ signaling pathways with consequent up-regulation or down-regulation of target genes. Second, transgenic fibroblasts were partially refractory to further modulation by recombinant TGFβ. A soluble dimeric TGFβ receptor was used to determine whether the basal activation of TGFβ-regulated genes was ligand-dependent. Results of a series of three independent experiments confirmed that Smad3 and type I procollagen overexpression was refractory to further modulation by recombinant TGFβ.

**Table 1**

| TGFβ1-treated wild type | -Fold change | TβRIIΔk transgenic | -Fold change |
|-------------------------|--------------|--------------------|--------------|
| Up-regulation (+)       |              | Overexpression (+) |
| T-cell death-associated protein | 7.3          | TGFβ3 precursor    | 4.6          |
| Thrombospondin 1 precursor | 6.5          | Prolyl 450 kinase  | 3.8          |
| Osteopontin precursor   | 5.7          | Rab2 Ras-related protein | 3.1 |
| Fibronectin 1 precursor | 5.2          | G1 cyclin-Cdk kinase inhibitor | 3.0 |
| 84-kDa heat shock protein | 3.1          | Osteopontin precursor | 2.5 |
| Vimentin                | 2.9          | Biglycan           | 2.5          |
| Prolysin a              | 2.8          | N-Ras proto-oncogene | 2.1 |
| Fos-related antigen 2   | 2.8          | Vimentin           | 2.0          |
| Tyrosine-protein kinase ryk precursor | 2.8        | Interferon γ receptor | 2.0 |
| Thymosin β4             | 2.7          |                    |              |
| Transforming growth factor β1 | 2.7         |                    |              |
| Ets-domain protein Etk  | 2.4          |                    |              |
| Biglycan                | 2.2          |                    |              |
| Retinoic acid receptor γ-A | 2.1         |                    |              |
| YB1 DNA-binding protein | 2.0          |                    |              |
| Defender against cell death 1 (DAD1) | 2.0 |                    |              |
| Interferon γ receptor   | 2.0          |                    |              |
| Down-regulation (−)     |              |                    |              |
| AT motif-binding factor | 4.4          | AT motif-binding factor | 4.4 |
| Interleukin-1 receptor   | 3.6          | Engrailed homeobox protein | 3.5 |
| Keratinocyte growth factor (FGF-7) | 3.2        | Keratinocyte growth factor (FGF-7) | 3.5 |
| Fragile X mental retardation | 2.9       | Homeobox protein 11 | 3.0 |
| Breast cancer type 2 susceptibility | 2.4 | Involucrin | 2.8 |
| Rac a serin/threonine kinase | 2.3       | Fragile X mental retardation syndrome | 2.6 |
| Mycinoxin A precursor | 2.2          | Mycinoxin A precursor | 2.2 |
| Neural cadherin precursor | 2.0         | Ret proto-oncogene precursor | 2.2 |
| Interleukin-3 receptor   | 2.0          | Macrophage CSF 1 receptor | 2.1 |
| Frizzled homolog 6       | 2.0          | Signal-transducing adaptor molecule | 2.1 |
| Proenkephalin A precursor | 2.0         | Rac a serin/threonine kinase | 2.0 |
|                     |              | Thrombomodulin | 2.0 |
|                       |              | Breast cancer type 2 susceptibility | 2.0 |
|                       |              | Frizzled homolog 6 | 1.9 |

| Note | |
|------|---|
| - Fold up- or down-regulated compared with basal wild type littermate fibroblast gene expression. Normalized for global gene expression and averaged for two replicate arrays. |
| *Italicized* genes demonstrate similar expression for TGFβ1-treated wild type and unstimulated TβRIIΔk transgenic fibroblasts. |

Transgenic Fibroblasts Are Refractory to Exogenous TGFβ—Expression studies of TGFβ-regulated genes described above showed that transgenic fibroblasts were partially refractory to recombinant TGFβ. This could be due to a global change in TGFβ responsiveness or might reflect changes in regulation of individual genes or post-transcriptional events. To explore altered TGFβ responsiveness in detail, the activity of promoter fragments cloned from plasmogen activator inhibitor 1, fibronectin, and pro-collagen (Col1a2) genes was assessed in the presence or absence of recombinant TGFβ1 (Fig. 9A). All of these constructs showed substantial TGFβ1-dependent activation (mean ± S.E.) in wild type cells (552 ± 43% basal) but little up-regulation in transgenic cells (overall 84 ± 20% basal p < 0.05, Student’s unpaired t test). The results of a series of independent experiments are summarized in Fig. 9B. A modest but significant (p < 0.05) induction of PAI-1 receptor activity was consistently observed, confirming RT-PCR data and suggesting that some differential effects on TGFβ-regulated genes may become apparent in future studies. Basal activity for the other three reporter constructs were suppressed in transgenic fibroblasts. Contrary to Western blot and cDNA microarray data, no activation of the fibronectin promoter was observed, suggesting that mechanisms other than transcriptional activation may underlie fibronectin up-regulation in transgenic fibroblasts.

Overall, these results show that expression of a truncated kinase-deficient type II TGFβ receptor selectively on fibroblasts produces fibrosis in vivo and is associated with a profibrotic phenotype in explanted dermal fibroblasts with features of constitutive overexpression of a number of TGFβ-activated genes together with a blunted response to exogenous TGFβ1.

**DISCUSSION**

The goal of this study was to determine whether expression of a kinase-deficient human TβRII on fibroblasts led to sus-
tained alteration in TGFβ-regulated gene expression. Based upon reported experiments using similar constructs, we had predicted that expression of TGFβ up-regulated genes might be suppressed. In fact, the opposite occurred, with transgenic fibroblasts demonstrating features of TGFβ activation. Moreover, the development of dermal and pulmonary fibrosis in these mice provides strong direct evidence of the potential importance of TGFβ-dependent pathways in the development of fibrosis and illustrates how nonsignaling ligand-binding proteins may regulate TGFβ activity. This is consistent with recent reports suggesting that accessory proteins such as betaglycan or endoglin (24, 25) and a nonsignaling type I receptor (26) can modulate type I and type II TGFβ receptor function.

Although this is the first time that fibroblast-specific expression has been examined, our results should be considered in the context of previous studies of other transgenic mice harboring kinase-deficient type II TGFβ receptor constructs. When expressed at a high level, in vitro TβRIIΔk appears to operate as a dominant negative inhibitor of TGFβ; however, in vivo in transgenic mice it is likely to induce much more complex effects. Most reported phenotypes are consistent with perturbed TGFβ signaling, and this has been seen most clearly when tissue-specific or cell type-specific promoters have been used. Thus, expression in epidermal cells of the skin produced a hypertrophic thickened epidermis with keratinocytes showing increased growth rate and refractoriness to TGFβ1-induced growth inhibition (27). These mice were later shown to have enhanced sensitivity to chemical carcinogenesis (28). In pancreatic acinar cells, normal development was disrupted, and explanted cells were refractory to TGFβ1 but showed intact responses to activin (29). Interestingly, this study also found some paradoxical effects including up-regulated TGFβ1 expression in transgenic acinar cells leading to focal pancreatic fibro-
Fig. 8. Overexpression of type I procollagen and Smad3 by transgenic fibroblasts is TGFβ1-dependent. A soluble TGFβ1 receptor fusion protein (sol-TβRII) was used to ask whether basal differences in protein expression by transgenic fibroblasts reflected increased extracellular TGFβ activity. Elevated basal type I collagen and Smad3 levels (lane 4) were reduced toward those of wild type cultures after the addition of soluble TGFβ receptor fusion protein (lanes 2 and 6). Suppression from basal expression was also seen for wild type cells (lanes 2 and 3). Induction of both proteins in wild type cells by recombinant TGFβ1 (4 ng/ml) was blocked by soluble TGFβ receptor fusion protein (lane 8). Data (mean ± S.E.) are representative of three independent experiments.

sis, neoangiogenesis, and mononuclear cell infiltration. More recently, selective pancreatic expression using the trefoil peptide promoter increased susceptibility to cerulean-induced pancreatitis (30). Previous studies expressing TβRIIΔk in mesenchymal cells, whose growth or differentiation is likely to be promoted rather than inhibited by TGFβ stimulation, probably better reflect the present study. Expression in chondrocytes has been studied in mice with TβRIIΔk expressed under a metallothionein promoter (31). Skeletal degenerative changes in adult mice occurred from 4 months, mirroring the late phenotype seen in our study, with an excess of hypertrophic chondrocytes suggesting defective TGFβ response. Expression in osteoblasts via the osteocalcin promoter led to a complex phenotype with increased trabecular bone density (32), consistent with the effect of blocked TGFβ responses on osteoblast differentiation. In mammary stromal cells, TβRIIΔk was associated with impaired mammary gland development and differentiation (33), providing a mechanism for defective lactation observed in some of our founder transgenic mice (data not shown). By contrast, in mammary epithelial cells, the same construct increased tumor frequency and progression, as well as having distinctly different effects on mammary epithelium with alveolar hyperplasia (34). Expression in prostatic epithelial cells in mice results in glandular hyperplasia, with reduced ventral prostate apoptosis (35). Using a C-reactive protein promoter to direct expression of TβRIIΔk to hepatocellular cells

Fig. 9. Absence of TGFβ1-dependent promoter activation in TβRIIΔk transgenic fibroblasts. A, neonatal fibroblasts transiently transfected with TGFβ1-responsive promoter constructs (PAI-1, 3TP, Col1a2, and fibronectin (FN)) were stimulated with TGFβ1 (4 ng/ml). Cotransfection of a Renilla luciferase-expressing plasmid was used to correct for transfection efficiency (mean ± S.E. firefly/Renilla luciferase activity). Transgenic fibroblasts were much less responsive than littermate wild type cells. The upper panel shows raw data from a representative experiment. B, overall results from a series of four independent experiments are summarized. Basal activation was consistently elevated for PAI-1 but below wild type for the other three constructs. C, responsiveness of 3TP-Luc in transgenic fibroblasts was partially restored by transient transfection of wild type TGFβ1 receptor constructs. Overexpression of wild type TβRII (pCMV-TβRII) did not alter basal or TGFβ1-induced (pCMV-TβRII + TGFβ) activity in wild type cells but partially restored responsiveness to transgenic fibroblasts. Responsiveness was not restored by overexpression of type I TGFβ1 receptor (pCMV-TβRI + TGFβ), although transfection of expression vectors for both TβRII and TβRI (pCMV-TβRII + TGFβ) was similar to TβRII alone. All data are corrected for transfection efficiency using TK-Renilla luciferase and expressed as percentage of basal expression. Similar results were obtained in two further experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001 (Student’s t test).
accelerated chemically induced carcinogenesis (36), whereas expression in lens fibers led to bilateral cataracts with show definite migration and actin fiber assembly by cultured lens cells (37). Tissue-specific expression in stomach or intestinal epithelium increased mucosal proliferation and promoted carcinogenesis in these sites (38). In addition, several recent studies have used TpRIIΔk to try and unravel the complex role for TGFβ signaling in autoimmunity. Expression in T lymphocytes resulted in a lymphoproliferative disorder, similar to the TGFβ1 null mouse phenotype (39), and also increased susceptibility to autoimmune hepatitis (40). Overall, these data from previously reported transgenic mice harboring TpRIIΔk all suggest blunted ligand responsiveness, but it is unclear whether constitutive activation of TGFβ1 signaling that we also observe in fibroblasts is a general feature of TpRIIΔk expression.

Apparent disparate results from experiments using similar receptor constructs may reflect different levels of mutant receptor expression relative to endogenous receptors or the potentially complex ways in which the mutant protein influences ligand-dependent complex assembly and stability in different cell types. In our mice, transgene product increases TGFβ1 binding to neonatal fibroblasts by around 50%. Although there may be competition between mutant and wild type TpRII for active ligand, it seems unlikely that this is the predominant mechanism, particularly since overexpression of wild type receptors only restored partial responsiveness to transfected reporter constructs. It is more likely that the effects observed in transgenic fibroblasts result from the mutant receptor operating as an accessory ligand binding protein (24) or via a more subtle effect on receptor complex formation or stability or even by altering the orientation of wild type receptors within signaling complexes. Such changes have been shown to regulate basal or ligand-dependent TGFβ signaling activity (41). Another important mechanism regulating TGFβ signaling is the rate of ubiquitination and lysosomal or proteosomal degradation of receptors and receptor-ligand complexes. Recent data suggest that these processes, in which Smad proteins facilitate interaction with specific ubiquitin ligases such as Smurf2 regulate TGFβ activity or responsiveness (42). Incorporation of a mutant receptor may alter complex susceptibility to ubiquitination, and studies to determine this are ongoing.

It is possible that our findings reflect autocrine or paracrine stimulation of fibroblasts due to an increased level of active TGFβ1 ligand in transgenic animals. As discussed above, there is a precedent for overproduction of TGFβ1 in pancreatic acinar cells expressing a similar transgenic receptor (29). Such autocrine overproduction of TGFβ1 might contribute to activation of transgenic fibroblasts and would be consistent with the antagonistic effect of soluble TGFβ fusion protein on transgenic cells, although many of the other potential mechanisms discussed above would also be ligand-dependent. It is also plausible that enhanced activation of preformed latent TGFβ complexes might result in increased in ligand concentration (3). The most highly overexpressed gene by microarray assessment was thrombospondin-1, a potent activator of latent TGFβ (4), and further gene expression analysis suggested that other activators, including a number of matrix metalloproteinases, were also up-regulated (data not shown). In view of the biochemical abnormalities identified in explanted fibroblasts, it is perhaps surprising that transgenic mice did not demonstrate a more dramatic gross phenotype. This may reflect the complex regulation and redundancy within TGFβ1 ligand-receptor axis, supported by the relatively benign consequences of long term antagonism of TGFβ responses using soluble antagonists (43, 44).

Despite transfection experiments suggesting profound refractoriness of transgenic fibroblasts to recombinant TGFβ1, our data for endogenous gene responses are less clear. Basal activation of TGFβ signaling is confirmed by increased levels of phosphorylated Smad2/3. However, it appears that MAPK pathways are still responsive in transgenic fibroblasts, and it is possible that Smad-dependent signaling may be more disrupted in transgenic fibroblasts than other downstream pathways. These other pathways are likely to mediate the residual TGFβ responses observed in our study for some gene products, including fibronectin and TGF7. A possible explanation for altered responses of transgenic fibroblasts is that the basal stimulation reduces their potential for further activation. In addition, TGFβ ligands induce a number of antagonistic downstream factors that limit further cellular activation, including Smad7, which is highly overexpressed in transgenic fibroblasts. This contradicts a recent report of reduced Smad7 levels in some fibrotic states (45). Smad7 overexpression raises the possibility that responses of Smad-dependent genes might be more completely blocked than those regulated by Smad-independent pathways, such as fibronectin, although our transfection data suggest a more global refractoriness, at least at the level of transcriptional activation.

These transgenic mice may provide a novel animal model for the human multisystem fibrotic disease systemic sclerosis (scleroderma, SSc), in which skin and sporadic pulmonary fibrosis develop. There is considerable evidence for overactivity of TGFβ or downstream secondary cytokines in SSc, and TGFβ-neutralizing strategies are currently being evaluated for therapy (46). Much of our understanding of the pathogenesis of SSc is based upon studies of the biology of explanted lesional fibroblasts, and it is intriguing that many aspects of the SSc fibroblast phenotype are reproduced in TpRIIΔk transgenic mice, including hallmark overexpression of CTGF (47). For SSc fibroblasts, there have been conflicting data regarding expression of TGFβ receptors. Overexpression was suggested by Kawakami et al. (48), and an autocrine loop dependent upon TGFβ1 has been proposed with suppression of type I collagen production by a neutralizing antibody to TGFβ1 (49), similar to the effect of soluble TpRII fusion protein that we have observed. More recent reports suggest transcriptional activation of TGFβ receptor genes in SSc (50) and altered expression of the accessory receptor endoglin (51), and studies of TGFβ1 ligand and receptor protein or mRNA have demonstrated increased expression in early stage lesions but not in the established fibrotic skin (52). Up-regulation of ligand in involved lung tissue may be more sustained (53).

Interestingly, an analogous member of the TGFβ superfamily of receptors, the bone morphogenetic protein receptor type 2, has been identified as a causal mutation underlying some cases of familial pulmonary arterial hypertension (54) and also some sporadic cases (55). A large number of different mutations have been identified, mostly within the intracellular portion of the receptor, that reduce receptor kinase activity or cause premature termination of the protein. Pulmonary arterial hypertension is characterized by proliferative changes in the vasculature that include medial and adventitial fibrosis. The current study demonstrates that the absence of kinase activity can lead to paradoxical activation of downstream TGFβ signaling pathways in vivo, and smooth muscle cells from pulmonary arteries of patients heterozygous for mutations in the signaling domain of bone morphogenetic protein receptor type 2 show altered responses to TGFβ1 as well as bone morphogenetic proteins (56). There is, however, a distinct functional difference between mutations that truncate the cytoplasmic tail of the bone morphogenetic protein receptor type 2 protein compared...
with those that delete kinase activity (57). Although significant pulmonary hypertension develops in around 15% of cases of SSc, mutations in bone morphogenetic protein receptor type 2 have not been found in SSc-associated pulmonary arterial hypertension (58, 59), supporting a hypothesis that other defects in TGFβ receptor expression or function may be more important in SSc (60).

In conclusion, our findings show that genetically determined perturbation of TGFβ signaling in fibroblasts can induce skin and lung fibrosis. These mice provide a potentially valuable model for studies of human fibrosis, including SSc, probably best reflecting the established fibrotic phase of this disease. Other animal models for SSc (61) exist, but none have such a clearly targeted disruption of TGFβ signaling in fibroblasts. The development of significant lung disease in only a proportion of cases parallels human SSc and merits further analysis. Other internal organs are also likely to be affected, and these will be examined in detail in future studies, which should determine the specific molecular basis of altered responsiveness and altered pathway activation in TgRRIαK transgenic fibroblasts.

Acknowledgments—We are grateful to Zhaoping Zhang for generating transgenic mice and to Heidi Eberspecher for assisting with histological examination of embryos. Dr. Daniel Pennington performed flow cytometric analysis of fibroblast TGFβ receptor expression. Soluble TgRII fusion protein was provided by Dr. Philip Gotwals, and Professor Dylan Edwards assisted with Taqman RT-PCR analysis of PAI-1 gene expression. Dr. Michael Schneider kindly provided the cDNA encoding TgRRIαK. Plasmids for expressing TgRI and TgRII and reporter constructs for TgRRIαK signaling were gifts from Dr. Joann Massague and Dr. Daniel Rifkind, respectively.

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Fibroblast-specific Expression of a Kinase-deficient Type II Transforming Growth Factor β (TGFβ) Receptor Leads to Paradoxical Activation of TGFβ Signaling Pathways with Fibrosis in Transgenic Mice

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J. Biol. Chem. 2003, 278:25109-25119.
doi: 10.1074/jbc.M300636200 originally published online April 21, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M300636200

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