Transglutaminase 2 and Its Role in Pulmonary Fibrosis

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***Rationale*: Idiopathic pulmonary fibrosis (IPF) is a deadly progressive disease with few treatment options. Transglutaminase 2 (TG2) is a multifunctional protein, but its function in pulmonary fibrosis is unknown.**

***Objectives*: To determine the role of TG2 in pulmonary fibrosis.**

***Methods*: The fibrotic response to bleomycin was compared between wild-type and TG2 knockout mice. Transglutaminase and transglutaminase-catalyzed isopeptide bond expression was examined in formalin-fixed human lung biopsy sections by immunohistochemistry from patients with IPF. In addition, primary human lung fibroblasts were used to study TG2 function *in vitro*.**

***Measurements and Main Results*: TG2 knockout mice developed significantly reduced fibrosis compared with wild-type mice as determined by hydroxyproline content and histologic fibrosis score ($P < 0.05$). TG2 expression and activity are increased in lung biopsy sections in humans with IPF compared with normal control subjects. *In vitro* overexpression of TG2 led to increased fibronectin deposition, whereas transglutaminase knockdown led to defects in contraction and adhesion. The profibrotic cytokine transforming growth factor-β causes an increase in membrane-localized TG2, increasing its enzymatic activity.**

***Conclusions:* TG2 is involved in pulmonary fibrosis in a mouse model and in human disease and is important in normal fibroblast function. With continued research on TG2, it may offer a new therapeutic target.**

Keywords: fibroblast; myofibroblast; animal model; collagen; fibronectin

Fibrosis is characterized by the excess accumulation of fibroblasts and extracellular matrix proteins that destroy normal tissue architecture and function. This pathologic process can affect almost every organ of the body (1). Pulmonary fibrosis can result from a variety of inflammatory insults to the lung. Idiopathic pulmonary fibrosis (IPF), the most common form of pulmonary fibrosis, however, lacks a major classic inflammatory response, and thus antiinflammatory targeted therapies have failed. Instead, IPF is characterized by destruction of the air spaces and accumulation of fibroblasts and excess extracellular matrix in the interstitium (2). IPF is more common than once believed, with a prevalence between 16.3 and 42.7 cases per 100,000 persons (3). Because IPF currently lacks effective treatment options, the median survival time after diagnosis is less than 3 years (4, 5).

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Pulmonary fibrosis is a severe disease with few effective therapies. Transglutaminase 2 (TG2) protein is involved in the cross-linking of matrix proteins, and little is know about its role in the development of pulmonary fibrosis.

What This Study Adds to the Field

TG2 is up-regulated in idiopathic pulmonary fibrosis. Using a preclinical animal model, we identify profibrotic activities of the protein. Therefore, TG2 represents a new target for therapy of pulmonary fibrosis.

Fibroblasts have been implicated as a major participant in pulmonary fibrosis and are currently being studied as targets for therapy (6). Histologic sections of diseased lung from patients with IPF show clusters of proliferating fibroblasts termed "fibroblastic foci." These clusters of fibroblasts are composed primarily of myofibroblasts, contractile cells that express both fibroblast and smooth muscle cell markers such as α-smooth muscle actin (α-SMA) (7). Myofibroblasts are one of the main effector cells in fibrosis, as they are responsible for the excess production of extracellular matrix components, including collagen and fibronectin (8). *In vitro* differentiation of fibroblasts to myofibroblasts is driven by the cytokine transforming growth factor (TGF)-β, which is greatly increased in patients with IPF (4, 9).

Transglutaminases (TG) are a nine-member family of proteins, eight of which are enzymatically active, that catalyze post-translational bonds between proteins. The most studied reaction catalyzed by the transglutaminases is protein cross-linking through formation of Nε(γ-glutamyl) lysine bond called transamidation (10). The TGs all have a conserved cysteine residue in the active site and the transamidation activity requires high calcium concentrations. Transglutaminase 2 (TG2), also known as tissue transglutaminase, is the most widely expressed member of the transglutaminase family. TG2 is set apart from the other members of the family by its widespread tissue distribution, its presence in many different subcellular compartments, and its multiple functions. TG2 is found in many cell types throughout the body including

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fibroblasts, macrophage, smooth muscle cells, hepatocytes, red blood cells, cardiac myocytes, neurons, chondrocytes, and kidney cells (11). TG2 protein can be found in the cytosol, on the cell surface, in the nucleus, and in the extracellular space (12). In the cytosol the transamidation activity of TG2 is suppressed by the high guanosine triphosphate and low calcium concentration, and TG2 instead functions as a G-protein, Gαh (13). In the extracellular space, with a high calcium concentration, the transamidation activity of TG2 can become activated after injury or disruption, leading to cross-linking between many different extracellular proteins (14–18). On the cell surface, TG2 plays an additional role by binding to the β-subunit of integrins and acting as a coreceptor for extracellular fibronectin. This activity is also independent of transamidation enzymatic activity (19).

There are several ways in which the multiple functions of TG2 may promote tissue fibrosis. TG2 can cross-link extracellular collagen and fibronectin, making them more resistant to breakdown (20). In the cytosol, the G-protein function of TG2 has effects on cell survival and cell cycle progression (21, 22). When bound to integrins on the cell surface and fibronectin in the extracellular matrix, TG2 enhances cell adhesion and mobility independent of its transamidation activity (19, 23, 24). TG2 has also been shown to enhance the production and maturation of fibrillar fibronectin, again in an enzymatically independent manner (25). In the liver and kidney, TG2 promotes fibrosis in rat and mouse models, and TG2 expression is up-regulated in human kidney fibrosis (26–31). There have been no studies to date that look directly at the role of TG2 in pulmonary fibrosis. One early study demonstrated an increase in transglutaminase activity in a rat model of pulmonary fibrosis (32). There have been recent investigations of TG2 in the settings of cystic fibrosis and lung cancer, but no studies involving TG2 knockout mice or inhibitors. Although many aspects of fibrogenesis are shared between organs, there are still multiple differences, and thus it is important for pathogenesis in each organ system to be investigated (1). The examination of TG2 in the lung is an important first step if we hope to translate these results to the clinic.

To examine the role of TG2 in pulmonary fibrosis, we have used a multipronged approach using a mouse model of fibrosis, human lung biopsy sections from patients with pulmonary fibrosis, and cultured primary human lung fibroblasts. Our data show that TG2 plays a major role in both pulmonary fibrosis and pulmonary fibroblast biology, and represents an interesting new therapeutic target for this devastating disease. Some of the results of these studies have been previously reported in the form of abstracts (33, 34).

METHODS

Cells

Primary human lung fibroblasts were derived and grown as previously described (35). Human lung biopsy sections were taken from patients with IPF (usual interstitial pneumonia) and nonfibrotic control subjects. Written informed consent was obtained from all patients and all studies were approved by the University of Rochester Institutional Review Board.

Mice

Tgm2$^{-/-}$ mice were obtained from Robert Graham (36) and bred at the University of Rochester. Mice were backcrossed to C57Bl/6J mice at least 10 generations. Age-matched C57BL/6J mice were used as controls (Jackson Laboratory, Bar Harbor, ME). Mice were administered bleomycin by oropharyngeal aspiration and tissue harvested as described in the online supplement. All animal studies were approved by the University of Rochester Committee on Animal Research.

Histology

Formalin-fixed paraffin-embedded sections were rehydrated and stained as described (37). Immunohistochemistry was performed as described (37) using primary antibodies to TG2 (Thermo, Freemont, CA), pan-cytokeratin (Abcam, Cambridge, MA), Nε(γ-glutamyl) lysine isopeptide bond (Abcam), α-SMA (Sigma-Aldrich, St. Louis, MO), or Von Willebrand Factor (Dako, Carpinteria, CA). Visualization of antibodies is described in the online supplement.

Western Blot

Cytosolic and membrane fractions were prepared as described (38). Whole cell lysates were prepared using NP-40 lysis buffer with protease inhibitors (Sigma-Aldrich). Extracellular matrix fractions were prepared as described (39). Gel electrophoresis and Western blotting were performed as described in the online supplement.

Flow Cytometry

Cells were removed from plates using 2 mM ethylenediaminetetraacetic acid and stained with anti-TG2 and Alexa Fluor 488 conjugated secondary antibody (Invitrogen, Carlsbad, CA) as described (40). Cells were analyzed on a FACSCanto machine (BD Biosciences, San Jose, CA) in concert with FlowJo Software (Tree Star, Ashland, OR).

Reverse Transcriptase–Quantitative Polymerase Chain Reaction

RNA was collected using the RNAEasy kit from cells or mouse lung as directed by the manufacturer (Qiagen, Valencia, CA). RNA was analyzed as described in the online supplement.

Immunocytochemistry

Cells were stained with antibody to TG2 and/or fibronectin and visualized as described in the online supplement. For TG2 activity, cells were cultured with pentylamine-biotin (Thermo) or FITC-cadaverine (Invitrogen) and visualized as described in the online supplement (15). Slides were imaged on a Zeiss Axio Imager Z.1 Microscope using Axio Imaging software (Zeiss, Oberkochen, Germany).

TG2 Knockdown and Overexpression

Lung fibroblasts were transduced with an adenovirus vector expressing either wild-type TG2 or mutant TG2 (C277S) and used as described in the online supplement (41). Sh-RNA lentiviral vectors targeting TG2 and a scrambled Sh-RNA were used as described (42), and cells were sorted for green fluorescent protein (GFP) expression using a FACSAria machine (BD Biosciences). For collagen gel contraction assays, transduced cells were seeded in gels of rat-tail collagen, (Roche, Basel, Switzerland), floated in media, and weighed after 48 hours (43). Cells for scratch assay were grown to confluency and wounded with a pipet tip. Migration was tracked on a Zeiss Axio Observer A.1 microscope (Zeiss).

Statistical Analysis

Differences between data sets were determined using analysis of variance with Tukey post test, or Mann-Whitney *t* test as indicated using Prism (Graphpad, La Jolla, CA). *P* values are as listed in the legends.

RESULTS

Lung Expression of TG2 is Increased in the Bleomycin Model of Pulmonary Fibrosis

To examine TG2 expression in the bleomycin mouse model of pulmonary fibrosis, we performed immunohistochemistry of lung tissue from wild-type mice treated with 2.5 U/kg bleomycin and killed on Day 21. Compared with lungs from mice given phosphate-buffered saline, bleomycin-treated mice showed an increase in TG2 protein levels in the lung (Figure 1).

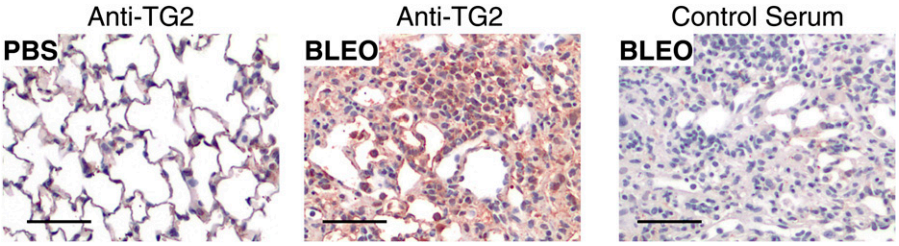

Figure 1. Transglutaminase 2 (TG2) is increased in the lungs of mice treated with bleomycin (BLEO). C57BL/6 mice were given 2.5 U/kg bleomycin by oropharyngeal aspiration. Twenty-one days after treatment the left lung was inflated and fixed in formalin. Lung sections were stained with an antibody to TG2 and developed with Nova Red. Scale bars = 50 μm. Sections shown are representative of four mice per group. PBS = phosphate-buffered saline.

Tissue TG Knockout Mice Develop Reduced Fibrosis after Bleomycin Challenge

To determine if TG2 expression is required for the development of pulmonary fibrosis, we examined the role of TG2 in the preclinical bleomycin mouse model of fibrosis. The fibrotic response of TG2 knockout mice (*Tgm2*$^{-/-}$) was compared with wild-type mice 21 days after administration of 2.5 U/kg bleomycin. Tgm2$^{-/-}$ mice treated with bleomycin had less fibrosis, as seen in representative hematoxylin and eosin and trichrome-stained lung sections (Figures 2A and 2B). This difference in fibrosis was quantified by an Aschroft scoring system on hematoxylin and eosin– and trichrome-stained lung sections. The mean fibrosis score of the wild-type mice was 4.6 out of 8 as compared with 3.0 in the knockout mice (Figure 3A). There was a statistically significant reduction in hydroxyproline content from 41.2 μg/right lung in the wild-type to 28.5 μg/right lung in the knockout mice (Figure 3B). Hydroxyproline is a surrogate marker for collagen content, and thus the knockout mice have reduced collagen accumulation in the lungs, indicating reduced fibrosis. Gene expression for various fibrotic markers was measured by real time reverse transcriptase–polymerase chain reaction (RT-PCR). Collagen and fibronectin expression were significantly increased 9- and 19-fold, respectively, when wild-type mice were treated with bleomycin (Figures 3C and 3D). Fibronectin expression in TG2 knockout bleomycin-treated mice was significantly reduced when compared with wild-type mice. Collagen expression was also decreased in TG2 knockout mice with a *P* value of 0.066. Matrix metallo proteinase (MMP)-9 expression was significantly decreased in both wild-type and knockout mice with bleomycin treatment but there was no difference between the two (*see* Figure E1A in the online supplement). There was a significant increase in active TGF-β protein in the bronchoalveolar lavage (BAL) fluid when mice were treated with bleomycin, but there was no difference between wild-type and knockout mice (Figure E1B). BAL cell counts and differentials were also examined. At Days 3, 7, or 21 after bleomycin administration there was no difference in either total cell counts (Figure E2A) or differential cell counts (Figures E2B–E2E).

TG2 Is Increased in the Lungs of Patients with IPF

Next, we examined TG2 expression in the lungs of patients with IPF. Formalin-fixed lung sections were obtained from lung biopsies performed on patients with a histologic diagnosis of usual interstitial pneumonia and a clinical diagnosis of IPF. Normal lung sections were taken from patients undergoing biopsy for a lung nodule ultimately found to be benign. Immunohistochemical detection of TG2 showed an overall increase in TG2 staining in the lung sections from patients with IPF (Figure 4A). The TG2 expression in the fibrotic lung was present both in the interstitial fibrotic areas and adjacent to fibroblastic foci, with TG2 staining intensely at the periphery of the foci (Figure 4B, Figure E3B). Compared with the intracellular α-SMA staining, TG2 staining was excluded from the center of the foci and primarily in the extracellular space around the fibroblastic foci. There was also a high level of expression of TG2 in alveolar macrophages (Figure E3A). There appeared to be low or minimal expression of TG2 in pulmonary epithelial cells as seen by lack of colocalization between TG2 and pan-cytokeratin (Figure E3C). In comparison to the expression in the IPF lung sections, there was much less TG2 expressed in the control lungs. TG2 expression in control healthy lung tissue appeared to be primarily located in the narrow interstitial spaces and areas with higher extracellular

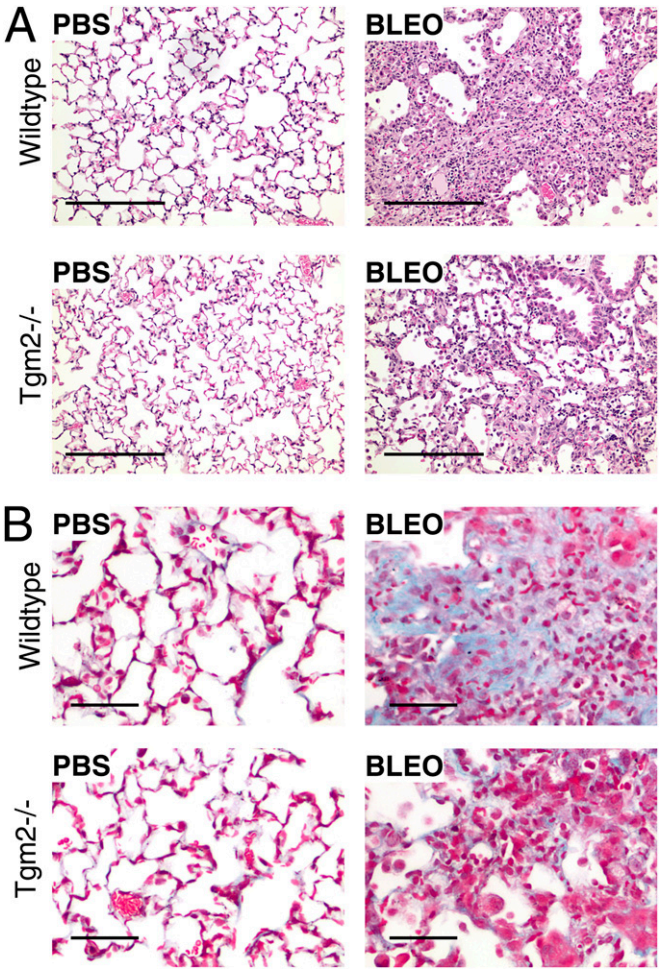

Figure 2. Transglutaminase 2 knockout mice (Tgm2$^{-/-}$) develop reduced fibrosis after bleomycin (BLEO) challenge. Male 8-week-old Tgm2$^{-/-}$ mice or C57BL/6 control mice were treated with 2.5 U/kg bleomycin. Twenty-one days after bleomycin administration, the fibrotic response was analyzed. (*A*) Representative hematoxylin and eosin–stained formalin-fixed paraffin-embedded sections from the left lung; scale bar equals 200 μm. (*B*) Representative sections were stained with Gomori Trichrome. Collagen appears as *blue*. Scale bar = 50 μm. PBS = phosphate-buffered saline.

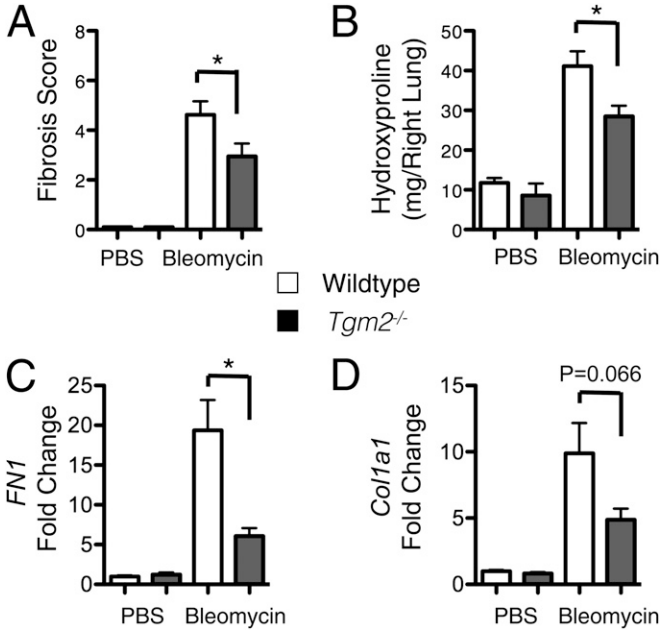

Figure 3. Transglutaminase 2 knockout mice have reduced histologic fibrosis score, hydroxyproline, and fibrosis-associated genes after bleomycin challenge. Mice were treated as in Figure 2. (*A*) Fibrosis was scored on hematoxylin and eosin and trichrome sections from 0 to 8 using an Aschroft scoring system. (*B*) Hydroxyproline content was measured in the right side of the lung. One representative experiment of three performed at different doses of bleomycin is shown. mRNA was isolated from the lungs, analyzed for (*C*) fibronectin (*FN1*) and (*D*) collagen 1 (*Col1a1*), and normalized to 18s RNA. n = 4 control groups, n = 7 bleomycin groups, error bars represent mean ± SEM; $*P < 0.05$ by analysis of variance (*A, C, D*) or Mann Whitney Student *t* test (*B*). PBS = phosphate-buffered saline.

matrix content, such as around larger vessels and airways (Figures 4A and 4B).

Immunohistochemical staining for the Nε(γ-glutamyl) lysine isopeptide bond that is catalyzed by TG2 was also performed on normal and fibrotic lung sections. There is an increase in the expression of the isopeptide bond in fibrotic lung sections concomitant with an increase in protein expression (Figure 4C). The expression pattern of the isopeptide bond is similar to that of TG2 protein expression, with the areas of highest density contained in the fibrotic interstitium. This result suggests that in addition to higher expression of TG2 in pulmonary fibrosis, there is also increased enzymatic activity.

Membrane-Associated TG2 Is Increased *In Vitro* by TGF-β

The subcellular localization of TG2 plays an important role in its function (44). As TGF-β is a major profibrotic cytokine in both humans and mice (4, 45), we examined the effect of TGF-β on both the expression and the localization of TG2 in primary human lung fibroblasts. As seen by Western blot, TGF-β leads to an increase in TG2 in the membrane fraction with little change in cytosolic TG2 (Figure 5A). The increased membrane-associated TG2 was confirmed by flow cytometry for surface TG2 staining. Surface staining of TG2 increased from a mean fluorescence intensity of 925 in the untreated cells to a mean fluorescence intensity of 2,410 when cells were treated with TGF-β (Figure 5B). Interestingly, treatment of primary human lung fibroblasts with TGF-β did not change the amount of TG2 mRNA (Figure 5C) as detected by real time quantitative RT-PCR. Collagen I mRNA expression, a marker for myofibroblast differentiation, was significantly

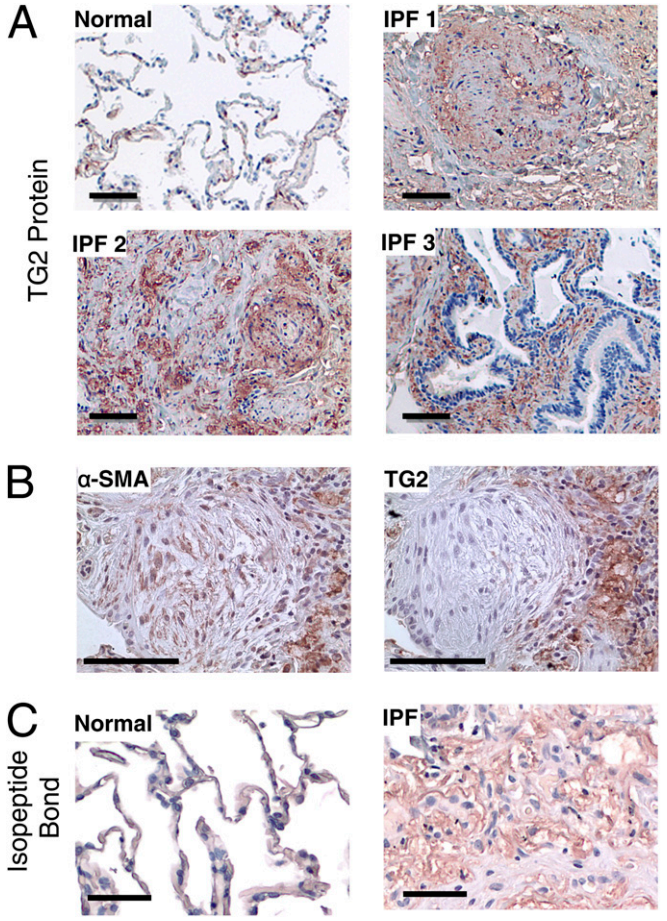

Figure 4. Transglutaminase 2 (TG2) protein and isopeptide bond are expressed in idiopathic pulmonary fibrosis (IPF). Samples of lung biopsies taken from six patients with IPF and four nonfibrotic control subjects. (*A*) Immunohistochemical staining for TG2; scale bar = 100 μm. (*B*) High-power serial sections of a fibroblastic focus stained for α-smooth muscle actin (α-SMA) and TG2; scale bar = 100 μm. (*C*) Immunohistochemical staining for transglutaminase catalyzed Nε(γ-glutamyl) lysine bond; scale bar = 50 μm. Slides were developed with Nova Red. Representative images from three patients with IPF and one control patient are shown.

induced. The expression of another myofibroblast marker, α-SMA, which increases with TGF-β treatment, was also tested by Western blot and real time RT-PCR to confirm TGF-β activity and myofibroblast differentiation (data not shown). These data all suggest that TGF-β increases membrane TG2 expression without altering its gene expression or cytosolic levels.

TGF-β Increases Extracellular TG2 Protein Expression and Activity

Because cell surface TG2 increases with TGF-β treatment, we also examined extracellular TG2 expression and activity. Extracellular TG2 was visualized using immunofluorescence on unfixed, unpermeabilized cells. Treatment with TGF-β led to an increase in the extracellular TG2 staining seen by immunocytochemistry (Figure 6A). Consistent with the Western blot data, when cells were permeabilized and stained to detect intracellular TG2, there was no change in total TG2 levels (data not shown). Because of the increase in the cell surface expression of TG2, it was important to determine if transglutaminase activity also increases. To examine TG2 activity, primary human lung fibroblasts

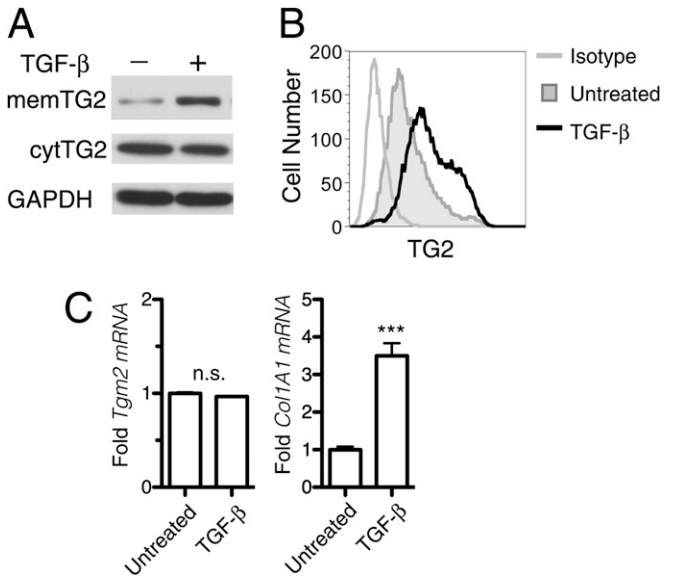

Figure 5. Transforming growth factor (TGF)-β drives an increase in cell surface transglutaminase 2 (TG2) expression. Human lung fibroblasts were treated with TGF-β (5 ng/mL). (*A*) Protein extracts were separated into particulate (memTG2) and cytosolic (cytTG2) fractions and analyzed by Western blot for TG2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) after 72 hours. (*B*) Surface expression of TG2 was analyzed by flow cytometry 72 hours after TGF-β treatment. (*C*) Expression of TG2 and collagen I mRNA were measured by quantitative reverse transcriptase–polymerase chain reaction after 24 hours of TGF-β treatment. n = 4 per group ***$P < 0.001$.

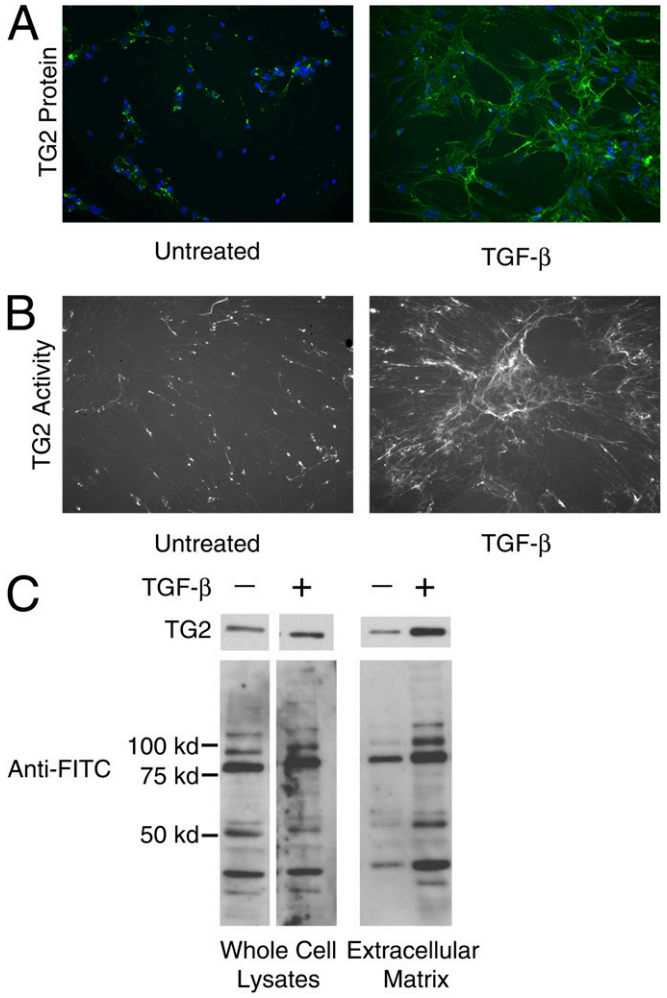

Figure 6. Transforming growth factor (TGF)-β treatment leads to an increase in extracellular transglutaminase 2 (TG2) and a corresponding increase in activity. (*A*) Human lung fibroblasts (HLFs) grown in chamber slides were stained with an antibody to TG2 (*green*) and 4′,6-diamidino-2-phenylindole (DAPI) (*blue*) after 72 hours of TGF-β (5 ng/mL) treatment. (*B*) HLFs were grown in the presence of pentylamine-biotin or fluorescein isothiocyanate (FITC) cadaverine and TGF-β (5 ng/mL) for 72 hours. Cells were stained with strep-Alexa Fluor 594, fixed, and stained with DAPI. (*C*) HLFs grown in the presence of FITC-cadaverine were collected as whole cell lysates or extracellular matrix proteins. Proteins were then analyzed by Western blot with antibodies against TG2 and against FITC.

were grown in media containing pentylamine biotin, a TG2 substrate. With an increase in TG2 activity, there will be an increase in the amount of pentylamine incorporated into the extracellular matrix that can then be visualized using a streptavidin-bound fluorescent probe. Treatment with TGF-β led to an increase in TG2 activity as seen by increased pentylamine incorporation into the extracellular matrix (Figure 6B). This increase in activity was also visualized by Western blot. Cells were grown with another TG2 substrate, cadaverine, which was bound to fluorescein (FITC). A primary antibody against FITC was used to detect all proteins that had been enzymatically cross-linked to FITC-cadaverine by TG2. With TGF-β treatment, TG2 expression was increased in the extracellular matrix fraction (Figure 6C). The amount of FITC-cadaverine that was enzymatically linked to proteins was also increased. In the whole cell lysates, there was very little change in TG2 expression or cadaverine-bound fluorescein incorporation with TGF-β treatment. This shows that in addition to increasing membrane localization of TG2, TGF-β increased the deposition of TG2 into the extracellular matrix and increased TG2 activity.

Knockdown of TG2 in Primary Human Lung Fibroblasts Leads to Functional Defects

To examine the role of TG2 in pulmonary fibroblast function, TG2 was knocked down using an SH-RNA lentivirus vector containing GFP, and cells were sorted for GFP expression (42). This strategy resulted in very efficient 85% knockdown of TG2 protein levels (Figure 7A and data not shown). Knockdown did not affect the ability of fibroblasts to differentiate to myofibroblasts as determined by α-SMA expression (Figure 7A). To further examine the role of TG2 in matrix organization and contraction, we compared the ability of these TG2-deficient primary human lung fibroblasts (Sh-TG2) to contract collagen gels with cells infected with a scrambled Sh-RNA lentivirus vector (Sh-Scram). When fibroblasts contract collagen gels in three dimensions, they squeeze out water, decreasing the weights of the gels. TG2 knockdown strongly inhibited collagen gel contraction in untreated cells with a 50% reduction in contraction by weight at 48 hours. TGF-β treatment stimulated increased collagen gel contraction in Sh-Scram fibroblasts but not in TG2 knockdown fibroblasts (Figure 7B). The difference between control and Sh-TG2 cells points toward an essential role for TG2 in the normal fibroblast function of matrix production, organization, and contraction.

Cell migration is important in fibrogenesis; therefore, we studied the migration of Sh-TG2 cells as compared with control primary human lung fibroblasts. To test this in our primary cells, we compared the mobility of Sh-TG2 cells with control fibroblasts in a scratch assay. Cells were grown to confluency, scratched with a pipet tip, and monitored for 72 hours. Forty-eight and 72 hours

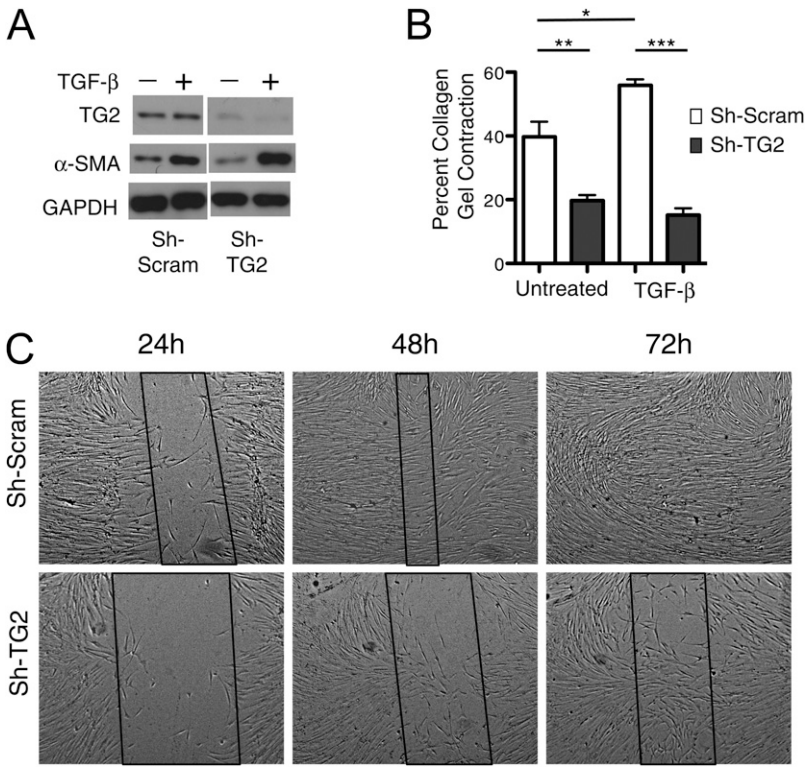

Figure 7. Human lung fibroblasts (HLFs) deficient in transglutaminase 2 (TG2) have functional defects. HLFs were infected with a lentivirus that encodes GFP and either a TG2 (Sh-TG2) or a scrambled (Sh-Scram) Sh-RNA. Cells were then sorted on GFP expression and used at passage 6 to 10. (*A*) Western blot for TG2, α-smooth muscle actin (α-SMA), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression with or without transforming growth factor (TGF)-β. (*B*) Equal numbers of cells were seeded in a collagen gel, floated in media, and then weighed after 48 hours. Weights were compared with no cell control gels and percent contraction was calculated. (*C*) Confluent cell layers were wounded with a pipet tip and migration was tracked over 72 hours. n = 3 per group; error bars represent mean ± SEM; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ by analysis of variance.

after the scratch, the Sh-TG2 cells lag in migration into the scratched area when compared with control cells (Figure 7C).

We confirmed our results from the TG2 knockdown primary human cells using primary mouse lung fibroblasts from TG2 knockout mice. In a collagen gel contraction assay, TG2 knockout fibroblasts did not contract the gels as efficiently as wild-type fibroblasts and, similar to the human TG2 knockdown, failed to respond to TGF-β with increased contraction (Figure E4A). In a scratch assay, TG2 knockout fibroblasts did not migrate as well as wild-type fibroblasts (Figure E4B). These results from primary mouse lung fibroblasts, which mirror those seen with TG2 knockdown in primary human lung fibroblasts, show that TG2 is important in proper function of both mouse and human fibroblasts.

Overexpression of TG2 Increases Deposition of Fibronectin and Matrix Organization

TG2 is an important regulator of extracellular matrix stability and has been shown to increase the deposition of fibronectin in transformed cells (25, 46). To study the role of TG2 expression in fibronectin deposition, TG2 was overexpressed in primary lung fibroblasts using an adenovirus vector. Overexpression of wild-type TG2 led to a significant increase in fibronectin deposition as determined by immunofluorescence and by Western blot (Figures 8A and 8B, Figure E5), and there was also increased fibronectin organization. When C277S, a TG2 mutant lacking transamidation activity, was overexpressed, fibronectin deposition was equal to the wild-type TG2 overexpression. This overexpression of TG2 did not affect fibroblast differentiation as α-SMA expression did not change (Figure 8B). Furthermore, overexpression of TG2 in primary human lung fibroblasts *in vitro* did not increase amounts of active TGF-β in the cell supernatants (data not shown). Thus TG2, through a mechanism independent of its transamidation enzymatic activity, promotes the deposition of fibronectin in the extracellular matrix. These results suggest that overexpression of TG2 should enhance collagen gel contraction in human lung fibroblasts. With overexpression of TG2 by adenovirus vector, we saw an increase in the amount of gel contraction compared with uninfected cells or cells infected with an empty control vector (Figure 8C). Interestingly, this ability to contract collagen gels was not reliant on the transamidation activity of TG2, as the C277S mutant produced the same increase in collagen gel contraction as the wild-type TG2.

DISCUSSION

In this study, we demonstrate for the first time that TG2, through multiple mechanisms, plays an important role in the development of pulmonary fibrosis. Surprisingly, for such a widely expressed protein, TG2 knockout mice do not display any overt phenotype at baseline (36). On challenge, however, defects in wound healing are evident (47). In our experiments, TG2 knockout mice treated with bleomycin were protected from fibrosis with reduced accumulation of scar tissue in histologic sections, reduced fibrosis scores, and reduced hydroxyproline accumulation at 21 days. The TG2 knockout mice also had reduced amounts of fibronectin and collagen mRNA at 21 days reflecting a decreased fibrotic phenotype. Although both wild-type and knockout mice treated with bleomycin showed reduced expression of MMP-9 compared with PBS-treated control mice, there was no difference in expression between wild-type and TG2 knockout mice. We hypothesize that TG2 knockout mice have a reduced fibrotic response due to reduced collagen production and increased collagen turnover due to failure of transglutaminase-catalyzed cross-linking.

We chose to study fibrosis at 21 days because the inflammatory response to bleomycin has largely resolved and the fibrotic response to bleomycin peaks around this time (48, 49). Although we cannot exclude the possibility that TG2 deficiency simply slows the development of fibrosis, we do not believe that this is likely because by Day 21 the fibrotic process is well established with significant decreases in profibrotic mRNA genes in the knockout mice. In addition, TG2 inhibition or knockout in liver and kidney fibrosis results in permanent and long-lived

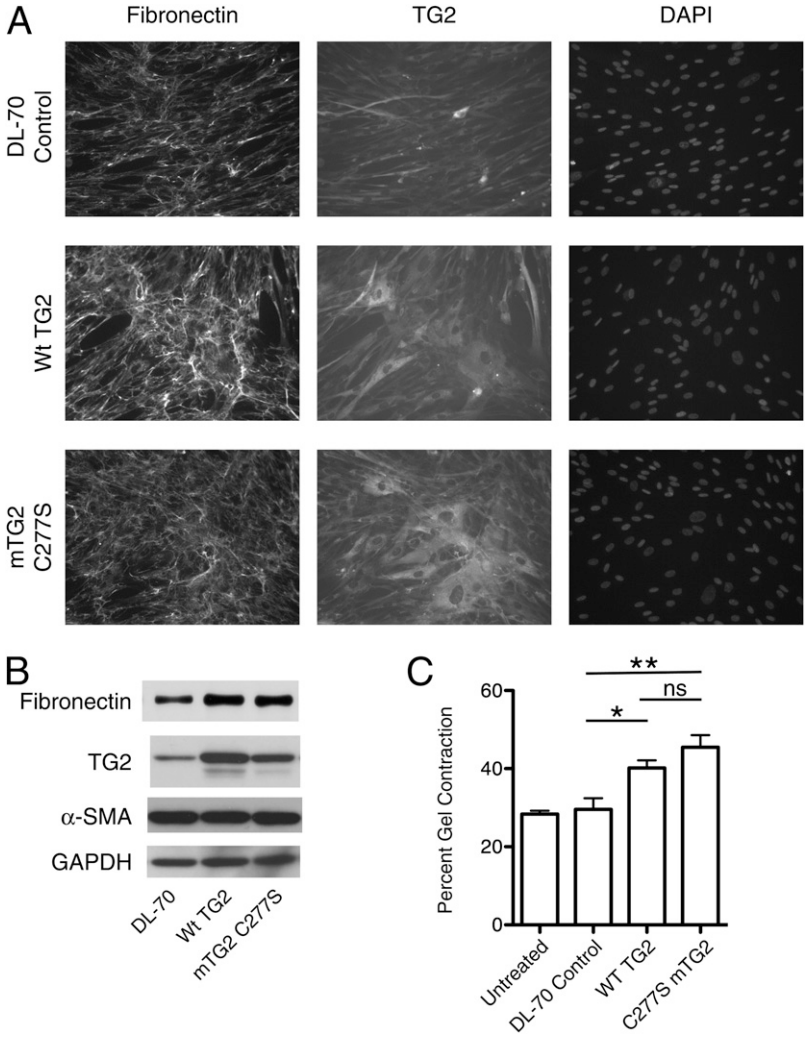

Figure 8. Overexpression of transglutaminase 2 (TG2) leads to an increase in fibronectin protein and increased collagen gel contraction. Primary human lung fibroblasts (HLFs) from nonfibrotic patients were infected with an empty control adenovirus (DL-70), an adenovirus containing the wild-type TG2 sequence (wt TG2), or an adenovirus with a transglutaminase activity null mutant (mTG2 C277S). (*A*) Cells were grown on chamber slides, fixed with methanol, and double stained for fibronectin and TG2. Mounting media containing 4′,6-diamidino-2-phenylindole (DAPI) was used to visualize the nuclei. (*B*) After infection with adenovirus, cells were serum starved for 24 hours and protein lysates were collected. Lysates were analyzed by Western blot for fibronectin, TG2, α-smooth muscle actin (α-SMA), and GAPDH. (*C*) Equal numbers of cells were seeded in a collagen gel, floated in media, and then weighed after 48 hours. Weights were compared with no cell control gels and percent contraction was calculated. n = 3 per group; error bars represent mean ± SEM; *$P < 0.05$, **$P < 0.01$, by analysis of variance.

decreases in fibrogenesis (27, 28, 30). Regardless, even a slowing of fibrogenesis in patients with IPF would be a worthy goal, as there are currently limited treatment options.

In patients with IPF, we identified increased overall expression of the TG2 protein in the lung. The dense fibrotic interstitial areas and areas around fibroblastic foci showed the highest expression of TG2. Using serial sections, we showed that TG2 was expressed in the extracellular space around fibroblastic foci and not by epithelial cells. Myofibroblasts in fibroblastic foci are major producers of excess extracellular matrix during fibrosis and the expression of TG2 likely functions to stabilize newly created collagen and fibronectin. Alveolar macrophages stain positively for TG2 expression and are known to play multiple roles in the development of IPF, including production of the fibrotic cytokine TGF-β (6).

The isopeptide bond between the side chains of glutamine and lysine residues, catalyzed by TG2, is highly resistant to degradation by collagenases and matrix metalloproteases (47). By rendering the extracellular matrix more resistant to breakdown, TG2 alters the balance between matrix production and degradation and ultimately leads to matrix accumulation. This balance appears to be affected by TG2 in IPF, as there is also an increase in the number of Nε(γ-glutamyl) lysine isopeptide bonds seen in lung tissue sections in these patients. The presence of the isopeptide bond in the fibrotic tissue shows that in addition to increased expression, TG2 is also enzymatically active.

TGF-β is a pleiotropic cytokine with myriad profibrotic activities (4). In primary human lung fibroblasts, we found that TGF-β increased TG2 on the cell surface and extracellular space but did not affect TG2 gene transcription. This increase in TG2 outside of the cell resulted in an increase in the enzymatic activity of the protein. Therefore TGF-β, in addition to directly increasing extracellular matrix production, can also indirectly affect extracellular matrix composition by increasing TG2 cross-linking activity. The mechanism by which TG2 transits outside of the cell and the manner in which TGF-β may control TG2 trafficking is currently unknown (47).

Conversely, TG2 has been shown to be involved in the activation of TGF-β through the anchoring of the latent TGF-β complex to the extracellular membrane, thus creating a potential positive feedback loop. The exact mechanism by which this occurs *in vivo*, or what role TG2 plays, is not completely understood (50). Although one study reported differences in the amount of active TGF-β in the kidney of TG2 knockout mice (31), another group found no difference in TGF-β activity with inhibition of TG2 in the kidney (28). We did not see differences in active TGF-β in the lavage fluid from the lungs of TG2 knockout mice.

TG2 is important in the normal *in vitro* activities of fibroblasts. We show here that TG2 knockdown leads to various defects in fibroblast function, including migration and contraction. Proper cell migration requires tight control of adhesion molecules such that if cells cannot properly adhere to a matrix, they will not migrate efficiently. In order for cells to migrate, they must also be able to release from the trailing edge (51). TG2 on the cell

surface enhances adhesion through its role as a fibronectin- and integrin-binding protein (52). If TG2 is lacking, the cells will not properly adhere to the extracellular matrix and thus will not migrate as efficiently. In the case of experimental nonphysiologic overexpression of TG2, excess TG2 reduces migration, likely through failure of the cells to release the trailing edge (24). Here we used a scratch assay to show that when primary human lung fibroblasts lack TG2, they are unable to migrate as effectively as control cells. In addition, when TG2 was knocked down, fibroblasts were both unable to contract collagen gels as well as control mice and unable to increase contraction in response to TGF-β.

Interestingly, not all the functions of TG2 are reliant on enzymatic activity. As we show here, overexpression of TG2 in primary human lung fibroblasts leads to an increase in the expression of fibronectin and increased gel contraction independent of enzyme activity (25, 46). The binding of TG2 to both integrin and fibronectin occurs irrespective of its enzymatic function (24, 52). This binding appears to be important for proper cell adhesion and motility as well as increased fibronectin multimerization (25, 53).

In the present studies we have identified that TG2 plays an important role in pulmonary fibrogenesis, and TG2 potentially represents an interesting target for therapy of IPF and other fibrotic disorders. We have shown that TG2 knockout mice develop reduced fibrosis, that TG2 is expressed in the fibrotic human lung, and that, *in vitro*, TGF-β controls the expression and function of TG2 in primary human lung fibroblasts. Although these studies show a connection between TG2 and fibrosis, further studies are necessary to completely understand the role of TG2 in fibrogenesis. In future studies we hope to further dissect the mechanism of how TG2 enhances fibrosis. Efforts to develop TG2-specific inhibitors are currently in progress (54), and we hope these drugs will translate into effective therapeutic options for our patients with IPF.

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