Tenascin-C deficiency attenuates TGF-β-mediated fibrosis following murine lung injury

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Tenascin-C deficiency attenuates TGF-β-mediated fibrosis following murine lung injury. Am J Physiol Lung Cell Mol Physiol 299: L785–L793, 2010. First published September 10, 2010; doi:10.1152/ajplung.00385.2009.—Tenascin-C (TNC) is an extracellular matrix glycoprotein of unknown function that is highly expressed in adult lung parenchyma following acute lung injury (ALI). Here we report that mice lacking TNC are protected from interstitial fibrosis in the bleomycin model of ALI. Three weeks after exposure to bleomycin, TNC-null mice had accumulated 85% less lung collagen than wild-type mice. The lung interstitium of TNC-null mice also appeared to contain fewer myofibroblasts and fewer cells with intranuclear Smad-2/3 staining, suggesting impaired TGF-β activation or signaling. In vitro, TNC-null lung fibroblasts exposed to constitutively active TGF-β expressed less α-smooth muscle actin and deposited less collagen I into the matrix than wild-type cells. Impaired TGF-β responsiveness was correlated with dramatically reduced Smad-3 protein levels and diminished nuclear translocation of Smad-2 and Smad-3 in TGF-β-exposed TNC-null cells. Reduced Smad-3 in TNC-null cells reflects both decreased transcript abundance and enhanced ubiquitin-proteasome-mediated protein degradation. Together, these studies suggest that TNC is essential for maximal TGF-β action after ALI. The clearance of TNC that normally follows ALI may restrain TGF-β action during lung healing, whereas prolonged or exaggerated TNC expression may facilitate TGF-β action and fibrosis after ALI.

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Experimental animals. TNC-deficient mice of the 129Sv strain were established as previously described (11). Congenic mice heterozygous for the TNC-null allele were mated, and offspring were genotyped after weaning. TNC-deficient and wild-type littermates, or lung fibroblasts derived from these mice, were used in the studies described below. All animals were used in accordance with protocols approved by the University of California, San Francisco Committee on Animal Research.

Bleomycin-induced lung injury model. Age- and sex-matched TNC-deficient and wild-type mice received 60 μg bleomycin (MeadJohnson, Princeton, NJ) in 60 μl of sterile saline via intratracheal

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instillation; control mice received an equal volume of sterile saline. The trachea was cannulated either transorally or via surgical cutdown. Following treatment, mice were followed for 21 days, and survivors were killed for quantification of right lung collagen content. **Quantification of lung collagen.** After severing the abdominal aorta, the pulmonary vasculature was perfused with 20 ml of sterile saline via the right venticle. Right lungs were excised at the hilum, homogenized, and then incubated in 65% trichloroacetic acid (Fisher Scientific, Pittsburgh, PA) on ice. Following centrifugation and aspiration of the supernatant, the tissue pellet was resuspended in 12 N HCl and baked at 110°C overnight. The denatured tissue was then reconstituted in dH2O, and 200-µl aliquots were incubated with 500 µl of 1.4% chloramine T (Sigma-Aldrich) in 10% isopropanol and 0.5 M sodium acetate for 20 min at room temperature. Five-hundred microliters of 1 M 4-(dimethylamino)-benzaldehyde (Sigma-Aldrich) was then added, and the suspension was incubated for 15 min at 65°C. Absorbance at 540 nm was measured in triplicate for each sample. A standard curve was generated simultaneously using 0–500 µg/ml trans-4-hydroxy-l-proline (Sigma-Aldrich), starting with the chloramine T reaction.

**Histology and immunohistochemistry.** Mice were killed at the time points indicated in the figures. Lungs were fixed with 4% paraformaldehyde in PBS under 15–20 cmH2O pressure. Following fixation, lungs were embedded in paraffin, and 5-µm sections were prepared. For histology, sections were stained with hematoxylin/eosin or Mason’s trichrome. For immunohistochemistry, sections were first rinsed in 3% H2O2 to block endogenous peroxidase activity. The anti-human TNC antiserum was a gift from Harold Erickson (Duke Univ.) and was used at 1:100 dilution. Rabbit anti-human phosphoSmad-2/3 (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted 1:100 for use according to the Vector ABC Elite protocol (Vector Laboratories, Burlingame, CA), using the DAB chromogen (Vector) as a substrate for HRP. Mouse anti-human α-smooth muscle actin (DakoCytomation, Glostrup, Denmark) was diluted 1:50 for use according to the Dako ARK protocol (DakoCytomation).

**TGF-β activation assay.** The effect of TNC on activation of latent TGF-β was assayed using a previously described coculture system (30). In this system, stably transfected SW480 cells carrying a β1 integrin transgene were cocultured for 24 h with mink lung epithelial reporter cells stably expressing a luciferase reporter gene driven by a TGF-β responsive element of the plasminogen activator inhibitor-1 promoter. As described, the β6-transfected cells activated latent TGF-β, causing a sevenfold increase in luciferase activity compared with untransfected SW480 cells. TNC protein (1–10 µg/ml) was tested both as a substrate on which cells were plated and as an addition after cells were plated. TNC did not prevent adherence of either cell line. The effect of the polyclonal TNC antibody described above (2–10 µg/ml) was tested by addition immediately after cocultures were plated.

**Fibroblast cell lines.** To establish fibroblast cell lines, the air space of TNC-deficient and wild-type lungs were filled with PBS, minced, and submerged in DME-H21 supplemented with 10% FCS and the following growth factors: 1X Insulin-Transferrin-Selenium (Invitrogen, Carlsbad, CA), 5 µg/ml rhPDGF (R&D), and 10 µg/ml rhEGF (R&D). Proliferating fibroblasts migrated onto plates over 4–5 days at which point tissue fragments were removed and adherent cells were removed by trypsin treatment and replated in DME with the additives above. After expansion, cells were frozen, and experiments were performed between passages 3 and 10.

**Assessment of myofioblast phenotype.** Ten thousand fibroblasts were seeded into precoated plastic tissue culture wells as described above. To induce quiescence, the cells were cultured for 1–5 days in FCS-free, growth factor-supplemented DME-H21. Over the following 24 h, the cells were cultured in FCS- and growth factor-free medium in either the presence or the absence of 1 ng/ml rhTGF-β1. Type I collagen was measured in the cell layer fraction of confluent monolayers of dermal fibroblasts essentially as previously described (3). Briefly, confluent cultures of fibroblasts were labeled for 30 h with [1-4-H]proline (New England Nuclear) at 10–3 µCi/ml of medium containing 0.15 mM sodium ascorbate, 0.1 mM δ-aminopropionitrile fumarate (Sigma), and 10% FBS (Gibco BRL). Collagens were extracted from the cell layer with pepsin in 3% acetic acid at 4°C for 12 h and separated on 7.5% polyacrylamide gels. The gels were fixed, dehydrated, and impregnated for 3 h with 20% 2,5-diphenyloxazole (Sigma) in DMSO. After fluorography, the radioactivity in the α1(I) and α2(I) bands was determined by excision of the bands, hydrolysis in Scintigist tissue solubilizer (Fisher) at 37°C overnight, and scintillation counting. Results were normalized for cell number by counting cells treated identically in replicate wells.

**Quantitative PCR assay.** Total RNA was prepared from fibroblast cell cultures using the TRizol reagent. First-strand synthesis and subsequent qPCR was carried out using the RT2 Profiler system (SA Bioscience). Reverse transcription and PCR reagents and optimized primers were purchased (SA Biosciences) for TGF-β pathway targets and five housekeeping genes (Hprt1, Hsp90, Gapdh, Actb, and Gusb). Reverse transcription and PCR was carried out on a LightCycler (Roche) using Sybr Green for detection according to the manufacturer’s recommendation. Relative expression was determined using the ΔΔCT method (22). Expression ratios were calculated as 2ΔΔCT.

**Statistical analysis.** Survival curves after bleomycin instillation were generated and compared using Kaplan-Meier statistics. Single comparisons between wild-type and TNC-null animals or cells were analyzed by t-testing with the significance threshold reduced to 0.005 to correct for multiple comparisons.

For analysis of TGF-β-stimulated Smad expression and phosphorylation, cells were stimulated with 0.5 ng/ml rhTGF-β1 for up to 30 min. After removing the culture medium, the cells were scraped from dishes in 1 ml of ice-cold hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF) and dounced 10 times (Kontes #19 teflon/glass dounce). These samples were used either for whole cell studies or were processed for nuclear studies as follows: nuclei were pelleted at 80 g (1,000 rpm, 4°C, microfuge), washed with 1 ml of hypotonic buffer, pelleted again at 80 g, and resuspended in Laemmli sample buffer containing β-mercaptoethanol. Equal volumes of whole cell extract or equal numbers of nuclei (as determined by hemocytometer count) were loaded onto 4–12% Bis-Tris polyacrylamide gels (Invitrogon). After membrane transfer, samples were exposed to 1:250 dilutions of rabbit anti-human Smad-2/3 antibody (Santa Cruz Biotechnology), mouse anti-human Smad-2 (Cell Signaling Technology, Boston, MA), or rabbit anti-human Smad-3 antibody (Cell Signaling). Membranes then were treated with the appropriate peroxidase-linked secondary antibodies (Pierce Biotechnology, Rockford, IL) before development with SuperSignal West Femto Substrate (Pierce). After development, the membranes were analyzed using the public domain NIH Image program (developed at the National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

**Lung fibroblast collagen assay.** We established and maintained fibroblast cultures as previously described (3). Equal numbers of wild-type and TNC-null cells were plated in replicate wells. To induce quiescence, the cells were cultured overnight in FCS-free, growth factor-supplemented DME-H21. Over the following 24 h, the cells were cultured in FCS- and growth factor-free medium in either the presence or the absence of 1 ng/ml rhTGF-β1. Type I collagen was measured in the cell layer fraction of confluent monolayers of dermal fibroblasts essentially as previously described (3). Briefly, confluent cultures of fibroblasts were labeled for 30 h with [1-4-H]proline (New England Nuclear) at 10–3 µCi/ml of medium containing 0.15 mM sodium ascorbate, 0.1 mM δ-aminopropionitrile fumarate (Sigma), and 10% FBS (Gibco BRL). Collagens were extracted from the cell layer with pepsin in 3% acetic acid at 4°C for 12 h and separated on 7.5% polyacrylamide gels. The gels were fixed, dehydrated, and impregnated for 3 h with 20% 2,5-diphenyloxazole (Sigma) in DMSO. After fluorography, the radioactivity in the α1(I) and α2(I) bands was determined by excision of the bands, hydrolysis in Scintigist tissue solubilizer (Fisher) at 37°C overnight, and scintillation counting. Results were normalized for cell number by counting cells treated identically in replicate wells.

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**Statistical analysis.** Survival curves after bleomycin instillation were generated and compared using Kaplan-Meier statistics. Single comparisons between wild-type and TNC-null animals or cells were analyzed by t-testing. Dose-response and time-course data were analyzed by analysis of variance with post hoc t-testing as appropriate. Statistical differences for quantitative PCR were determined using unpaired t-testing with the significance threshold reduced to P < 0.005 to correct for multiple comparisons.
RESULTS

TNC is highly expressed at sites of lung injury. TNC is minimally expressed in adult lung but is highly induced following several forms of lung injury in mice and humans (2, 20). We began by investigating the time course of TNC expression at baseline and after ALI. To do this, we administered 60 mU of bleomycin by intratracheal injection into pairs of wild-type mice and harvested their lungs 6, 14, or 21 days later for quantification of TNC. Figure 1A shows that TNC is absent from normal lungs, but is dramatically induced in wild-type mice exposed to bleomycin at 6 and 14 days. By 21 days, TNC has largely been cleared from the lung. As expected, TNC was not induced by saline installation (not shown) and was not expressed in TNC-null mice after bleomycin (Fig. 1A). Because peak expression was seen at 6 days, we examined TNC localization at that time point. Immunohistochemistry shows that TNC is deposited into the interstitial extracellular matrix of thickened alveolar septae specifically at sites of injury (Fig. 1, D–G) but not in adjacent areas of normal lung (Fig. 1, H and I). These data are consistent with previous detailed analyses of TNC expression after ALI (46, 57) and suggested to us that TNC may play a direct role in mediating the acute injury response to bleomycin.

TNC deficiency reduces interstitial collagen deposition. To investigate the role of TNC on collagen deposition, wild-type and TNC-null mice received 60 mU of bleomycin or saline by intratracheal injection and were followed for 21 days. All saline-treated control animals survived for 21 days. Following bleomycin administration, mortality was substantial, but not different, in the two groups (Fig. 2, A and B). Because less than

Fig. 1. Tenascin-C (TNC) induction and localization after lung injury. A: Western blot of whole lung extracts prepared before and 6, 14, or 21 days after bleomycin administration to wild-type and TNC-null mice (+/+ and −/−). The 190-kDa “large” isoform of TNC predominates at all time points after acute lung injury (ALI). At ×100 magnification, TNC staining in the matrix is robust in areas of lung injury (D and E), but not in adjacent areas of normal lung (H and I). At ×250 magnification (F and G), TNC is primarily localized to the interstitium of thickened alveolar septae in areas of injury. There is minimal TNC staining in saline-injected wild-type lungs (B and C) or in bleomycin-treated TNC-null lungs (J and K). Bars in fluorescence images represent 50 μm.
**TNC deficiency is associated with diminished TGF-β responses after ALI.** TGF-β plays a well-established role in producing the interstitial fibrosis that characterizes the chronic phase of bleomycin-induced lung injury (30). TGF-β action in the lung is primarily mediated through phosphorylation of the signaling molecules Smad-2 and Smad-3 (9, 32, 56). In fibroblasts, Smad phosphorylation and signaling promotes differentiation to the myofibroblast phenotype, which is characterized by expression of α-smooth muscle actin (α-SMA) (37). Therefore, we examined α-SMA expression and Smad phosphorylation as proxy indicators of TGF-β action in wild-type and TNC-null mice. Using an antibody that recognizes both phosphorylated forms of Smad-2 and Smad-3, we found that fibroproliferative foci in the lungs of bleomycin-exposed TNC-deficient mice appeared to contain fewer cells with intranuclear Smad-2/3 staining (Fig. 4, A and B). Likewise, the interstitium of wild-type lungs contained an abundance of α-SMA-positive cells that was much reduced in injured areas of TNC-deficient lungs (Fig. 4, C and D). These findings suggested to us that TGF-β action may be impaired in TNC-deficient lungs after ALI, so we proceeded to evaluate TGF-β action in vitro.

**TNC deficiency impairs TGF-β-induced activation of cultured fibroblasts.** While our in vivo studies suggest that TGF-β-mediated myofibroblast activation is impaired, they do not distinguish between impaired TGF-β activation and altered responsiveness to locally activated TGF-β. In the lung, TGF-β activation is primarily mediated through interaction of latent TGF-β with αvβ6 integrin on the surface of pulmonary epithelial cells (30). Using a previously reported in vitro test system for αvβ6-mediated latent TGF-β activation (30), neither exogenous TNC nor TNC antiserum had any effect on TGF-β activation of lung epithelial cells using the PAI-1 promoter as a reporter (not shown).

To test TGF-β responsiveness, we generated primary fibroblast cell lines from the lungs of wild-type and TNC-null littermates. Unstimulated wild-type lung fibroblasts produce large amounts of TNC in culture, whereas TNC-null cells do not, allowing for direct comparison of the effects of endogenously produced TNC. We then used these cells to test the ability of recombinant, constitutively active TGF-β to stimulate myofibroblast differentiation and collagen deposition. As shown in Fig. 5A, wild-type cells had a nearly linear dose-response to TGF-β with >70% of cells expressing α-SMA after 24 h of exposure to the highest concentration of TGF-β. In contrast, TNC-null cells had a flat dose-response with less than 30% of cells expressing α-SMA (P < 0.001). Similarly, as shown in Fig. 5B, TNC-null fibroblasts secreted less collagen into the cell layer matrix than did wild-type cells, both at baseline and in response to TGF-β. These findings mirrored observations in vivo, so we proceeded to examine TGF-β signaling in these cells.

Immunohistochemical staining of lung sections suggested altered Smad-2/3 signaling, so we began there. Using the same anti-phospho-Smad-2/3 antibody used for in vivo studies, we found that TNC-null cells had slightly reduced Smad-2/3 phosphorylation following exposure to TGF-β (Fig. 5C). Importantly, it also appeared that total Smad-2/3 expression was reduced in whole cell extracts of TNC-null cells, so we blotted these extracts with antibodies specific for Smad-2 and Smad-3. As shown in Fig. 5D, Smad-2 expression was similar in wild-type and TNC-null cells, but Smad-3 expression was dramatically reduced in TNC-null cells, completely explaining the reduced intensity of Smad-

30% of animals survived to 21 days following direct tracheal injection, we treated a second cohort of animals with transoral bleomycin. In our hands, this method delivered the drug exclusively to the right lung and resulted in nearly 100% survival in wild-type and TNC-null animals while still causing significant unilateral lung injury.

Right lungs were harvested from mice surviving for 21 days, and whole lung collagen content was determined using hydroxyproline as a proxy. Following direct intratracheal bleomycin, lung collagen content doubled in wild-type mice, whereas TNC-null mice increased collagen content by only 15% (Fig. 3A). TNC-null mice were similarly protected following tracheal administration of bleomycin, demonstrating that the protective effect was not the result of selection of TNC-null survivors with less fibrosis. The fibrotic effect of bleomycin delivered by the transoral route was somewhat diminished, possibly because this route ultimately delivers less bleomycin into the lower airways.

Lung sections were prepared from mice at 10 and 21 days after bleomycin injection. Masson’s trichrome staining, in which collagen stains blue, was suggestive of reduced interstitial fibrosis in TNC-null lungs by 10 days (Fig. 3, B and C). By 21 days, TNC-deficient lungs not only appeared to contain less collagen, but also seemed to have less severe embarrasment of the alveolar architecture (Fig. 3, D and E).
Replicate blots demonstrated a 70 ± 12% reduction in whole cell Smad-3 protein in TNC-null cells (n = 4, P < 0.01) but no change in Smad-2 protein.

We also used these Smad-2- and Smad-3-specific antibodies to evaluate TGF-β-stimulated nuclear translocation of these proteins. As shown in Fig. 5, E and F, blotting of purified nuclei demonstrated a 30% reduction in nuclear Smad-2 and a 65% reduction in nuclear Smad-3 following 30 min of TGF-β exposure. These findings suggest that impaired myofibroblast transformation and collagen deposition in TNC-null mice may result from impaired TGF-β signaling as a result of reduced expression of Smad-3 and reduced phosphorylation and nuclear translocation of both Smad-2 and Smad-3.

Finally, we examined the mechanism of decreased Smad-3 protein in TNC-null mice. We used quantitative RT-PCR to examine mRNA levels for Smads-2, -3, and -4 as well as TGF-β1, TGF-β2, and TGF-β receptors. Figure 6A shows fold-change differences between wild-type and TNC-null cells. As might be expected, Smad-3 mRNA is downregulated threefold in TNC-null cells (P < 0.001) at least partially explaining the paucity of Smad-3 protein in these cells, whereas Smad-2 and -4 are unchanged. Interestingly, non-Smad components of the TGF-β signaling system including TGF-β1 and TGF-β2 and TGF-β receptors 1 and 2 are slightly upregulated in TNC-null cells, perhaps as a compensatory mechanism.

Recent studies have demonstrated that much of the physiological regulation of TGF-β and BMP pathways occurs at the protein level mediated by degradation of receptors and nuclear or cytoplasmic Smads (including Smad-3) through the ubiquitin-proteasome system (8, 51). Therefore, we examined the effects of MG-132, a potent inhibitor of the ubiquitin-proteasome system, on Smad-3 protein levels. As shown in Fig. 6B, Smad-3 protein, normalized for β-actin, was 30% of wild-type level at baseline, but doubled following overnight incubation with MG-132 (P < 0.05). In contrast, Smad-3 protein fell slightly in wild-type cells exposed to MG-132. Hence, it appears that TNC regulates Smad-3 at both the RNA and protein levels.

**DISCUSSION**

Our findings implicate TNC as an important mediator of TGF-β-mediated fibrosis in the pathogenesis of the ALI syn-
In response to intratracheal bleomycin, TNC-null mice exhibited reduced interstitial fibrosis compared with wild-type mice, with markedly less collagen deposition in the lung interstitium following exposure to bleomycin. Protection against interstitial fibrosis seems to result from impaired TGF-β responsiveness in the absence of TNC because TNC-null fibroblasts showed similar impairment of myofibroblast transformation and collagen deposition as well as basal reduction in Smad-3 expression and impaired Smad-2 and Smad-3 phosphorylation and nuclear translocation when exposed to constitutively active TGF-β in vitro. Because Smad-3-deficient mice are also protected from bleomycin-induced pulmonary fibrosis (56), it is likely that the reduction in Smad-3 protein and nuclear translocation seen in TNC-null mice are sufficient to explain this phenotype. Thus, through its effects on TGF-β signaling, TNC acts as an important modulator of the host response in the ALI syndrome.

Definition of the physiological role of TNC in mammals has remained elusive (5). In the lung, it was postulated that TNC plays a role in organogenesis by facilitating branching morphogenesis (40, 54). However, the lungs of adult TNC-deficient mice are morphologically normal, with no evidence of altered airway ramification (11). Significant expression of TNC in the adult lung is limited to disease states characterized by inflammation and/or fibrosis (1, 2, 24, 33, 57, 58), but before our studies, the importance of TNC in the pathogenesis of the ALI syndrome was unknown. We believe that the altered fibrosis following bleomycin exposure in TNC-null mice is a direct result of TNC deficiency. As such, this is the first TNC-null phenotype described with clear relevance to human lung pathophysiology.

TGF-β dominates the chronic phase of ALI by stimulating fibroblast proliferation, transdifferentiation to the activated myofibroblast phenotype (7, 35), and matrix protein synthesis (20, 30). The absence of TNC restrains these events both in vivo and in vitro, apparently through alterations in TGF-β signaling. We have shown that nuclear translocation of both Smad-2 and Smad-3 are impaired and that Smad-3 protein levels are significantly reduced in TNC-null mice. Smad-3 protein appears to be regulated both at the level of mRNA expression and protein stability. The latter finding is perhaps not surprising given that many components of the TGF-β/BMP signaling pathways are regulated through the ubiquitin-protease system, including TGF-β receptors and Smads, including Smad-3 (8, 51). Recently ubiquitin-protease inhibition has been suggested as a potential treatment strategy in pulmonary fibrosis (48), but our observation suggests this might not be effective, consistent with observations in ALI in mice (10).

The altered TGF-β responsiveness we saw in TNC-null mice has important pathophysiological implications for the ALI syndrome. TNC is not normally present in the lung interstitium, is highly expressed during the acute phase of ALI, and is then cleared during the healing phase. This temporal pattern of expression is highly correlated with TGF-β action in vivo, whereas abnormal expression of TNC is highly correlated with lung fibrosis in both mouse models and human disease (20, 33, 57, 58). We hypothesize that clearance of TNC from the lung interstitium is an important physiological event that normally restrains TGF-β action after ALI and that prolonged pulmonary expression of TNC facilitates TGF-β action and fibrosis.

It is not yet clear how the absence of TNC leads to altered Smad signaling and TGF-β responsiveness, but several possibilities exist. First, recent studies have revealed the importance of cell adhesion generally (47), and αvβ3 integrin specifically, in the modulation of the myofibroblast phenotype (42, 43). In response to TGF-β, interstitial lung fibroblasts upregulate their
expression of this adhesion receptor (16, 34) blockade, which prevents the transdifferentiation of lung fibroblasts in vitro (42). TNC is a well-known ligand of \(^{38}/\)H9251/\(^{38}/\)H9252/\(^{38}/\)H9253 integrin (38, 52, 53). Upon its ligation with vitronectin, \(^{38}/\)H9251/\(^{38}/\)H9252/\(^{38}/\)H9253 integrin associates with and may activate the type II TGF-\(^{38}/\)H9252/\(^{38}/\)receptor to promote myofibroblast differentiation and proliferation (43). It is not known whether all \(^{38}/\)H9251/\(^{38}/\)H9252/\(^{38}/\)H9253 ligands possess this activity, but based on our observations, we speculate that TNC may facilitate myofibroblast differentiation and proliferation through its interaction with \(^{38}/\)H9251/\(^{38}/\)H9252/\(^{38}/\)H9253 integrin.

Second, activated myofibroblasts elaborate excessive amounts of matrix, as well as specific isoforms of other matrix proteins (20). One such isoform of fibronectin, fibronectin EIIIA (FN EIIIA), is an essential element of the chronic phase matrix. Functional blockade of the FN EIIIA domain prevents perpetuation of the myofibroblast lineage (44). Interestingly, considerable homology exists between the amino acid sequence of the EIIIA domain and that of the third fibronectin type three repeat (25) that is included in the long isoform of TNC expressed after ALI. Given this homology, which includes a functionally critical isoleucine residue (26), it is conceivable that the presence of TNC in the chronic phase matrix serves to support the myofibroblast phenotype in a fashion analogous to FN EIIIA.

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**Fig. 5. Reduced TGF-\(^{beta}/\) signaling in TNC-null fibroblasts.**

A: lung fibroblasts were exposed to varying concentrations of TGF-\(^{beta}/\), and the fraction of \(^{alpha}/\)SMA-positive cells was determined for wild-type (●) and TNC-deficient (○) cultures. Each data point represents the mean of 4 wells from each of 2 independent cell lines for each genotype. B: collagen deposited by wild-type (black bars) and TNC-null (white bars) cells (n = 4).

C: Western blots of Smad-2/3 (whole cell extract) and phospho-Smad-2/3 (nuclear fraction) after TGF-\(^{beta}/\) stimulation. D: Western blots of whole cell extracts showing reduced Smad-3 expression in TNC-null fibroblasts compared with wild type. E and F: quantification of Smad-2 and Smad-3 in nuclear extracts after TGF-\(^{beta}/\) stimulation (mean of 4 replicates). \(*P < 0.05; **P < 0.01; ***P < 0.001 in all panels."

**Fig. 6. Reduced Smad-3 mRNA and protein stability in TNC-null fibroblasts.**

A: quantitative RT-PCR analysis of TGF-\(^{beta}/\) signaling components in wild-type and TNC-null fibroblasts. Mean and 95% confidence intervals for log2 expression ratios of TNC-null over wild-type mRNA are shown for each target. A positive ratio reflects increased expression in TNC-null vs. wild type, and negative ratio reflects decreased expression. Significance threshold (*) was reduced to \(P < 0.005\) to compensate for multiple comparisons. N = 4 for each target. B: Smad-3 protein content in unstimulated wild-type and TNC-null cells (n = 4).

C: Western blots of Smad-2/3 (whole cell extract) and phospho-Smad-2/3 (nuclear fraction) after TGF-\(^{beta}/\) stimulation. D: Western blots of whole cell extracts showing reduced Smad-3 expression in TNC-null fibroblasts compared with wild type. E and F: quantification of Smad-2 and Smad-3 in nuclear extracts after TGF-\(^{beta}/\) stimulation (mean of 4 replicates). \(*P < 0.05; **P < 0.01; ***P < 0.001 in all panels."

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Third, TNC is known to restrain RhoA-mediated activation of the extracellular regulated kinase (Erk) pathway in fibroblasts (49), and it has recently been shown that Erk signaling opposes Smad-dependent TGF-β signaling in lung epithelial cell lines, potentially regulating both epithelial-mesenchymal and mesenchymal-epithelial transitions (39). While speculative, it is possible that the absence of TNC after ALI leads to excessive Erk signaling which in turn reduce Smad-3 through effects on transcription and proteosomal activity by as yet undescribed mechanisms.

Finally, it is formally possible that reduced Smad-3 expression in TNC-null mice is a direct and unintended consequence of the TNC targeting event. We examined the TNC locus and found that the nearest known participant in the TGF-β signaling pathway is the Tgfrb1 gene that lies 25 Mb from the TNC cap site and is slightly upregulated in TNC-null mice. While we believe this explanation is unlikely, we have not excluded the possibility that the TNC targeting event has altered Smad-3 transcription and/or protein stability in a nonspecific manner.

In summary, we have presented evidence that TNC modulates the host response following bleomycin-induced lung injury, thereby influencing the course of interstitial fibrosis in this disease model. By facilitating the effect of TGF-β on interstitial lung fibroblast transdifferentiation and collagen synthesis, TNC promotes the progression of bleomycin-induced interstitial fibrosis. We hypothesize that the clearance of TNC that normally occurs during the healing phase of ALI is essential to limit TGF-β action and prevent fibrosis, whereas prolonged expression of TNC, as is seen in human pulmonary fibrosis, may contribute to its progression. Presumably, disruption of TNC interactions with the fibroblast cell surface might restrain the profibrotic effects of TNC. Because TNC expression is highly restricted in adult tissues, modulation of its function may allow treatment to be more specifically targeted to the lung than systemic treatment strategies that target TGF-β directly (13, 23, 28).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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