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Ligand-independent transforming growth factor- β type I receptor signaling mediates type I collagen-induced epithelial-mesenchymal transition

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Abstract

Evidence suggests epithelial-mesenchymal transition (EMT) as one potential source of fibroblasts in idiopathic pulmonary fibrosis. To assess the contribution of alveolar epithelial cell (AEC) EMT to fibroblast accumulation *in vivo* following lung injury and the influence of extracellular matrix on AEC phenotype *in vitro*, Nkx2.1-Cre;mT/mG mice were generated in which AEC permanently express green fluorescent protein (GFP). On days 17-21 following intratracheal bleomycin administration, ~4% of GFP-positive epithelial-derived cells expressed vimentin or α -smooth muscle actin (α -SMA). Primary AEC from Nkx2.1-Cre;mT/mG mice cultured on laminin-5 or fibronectin maintained an epithelial phenotype. In contrast, on type I collagen, cells of epithelial origin displayed nuclear localization of Smad3, acquired spindle-shaped morphology, expressed α -smooth muscle actin (α -SMA) and phospho-Smad3, consistent with activation of the transforming growth factor- β (TGF β) signaling pathway and EMT. α -SMA induction and Smad3 nuclear localization were blocked by the TGF β type I receptor (T β RI, otherwise known as Alk5) inhibitor SB43154, while AEC derived from Nkx2.1-Cre;Alk5^{fllox/KO} mice did not undergo EMT on collagen, consistent with a requirement for signaling via Alk5 in collagen-induced EMT. Inability of a pan-specific TGF β neutralizing antibody to inhibit effects of collagen together with absence of active TGF β in culture supernatants is consistent with TGF β ligand-independent activation of Smad signaling. These results support the notion that AEC can acquire a mesenchymal phenotype following injury *in vivo* and implicate type I collagen as a key regulator of EMT in AEC through signaling via Alk5, likely in a TGF β ligand-independent manner.

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No conflicts of interest are declared.

Author contributions

LD, PF, AB and ZB conceived and designed the experiments. LD, STB, MSK, MD, AB, BZ and YX performed the experiments. LD, PF, CE, PM, BZ, EDC and ZB analyzed the data. LD, EDC and ZB drafted the manuscript.

Keywords

pulmonary fibrosis; epithelial-mesenchymal transition (EMT); TGF β type I receptor (Alk5); alveolar epithelial cells

Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive disease characterized by fibroblast/myofibroblast accumulation and excessive deposition of extracellular matrix (ECM) proteins leading to destruction of alveolocapillary units and impaired gas exchange [1]. The origin of lung fibroblasts/myofibroblasts in IPF is not well-defined, but possible sources include resident lung fibroblasts, marrow-derived circulating progenitors and alveolar epithelial cells (AEC) via epithelial-mesenchymal transition (EMT) [2]. EMT is central to cancer development and progression [3-4] and has been increasingly suggested to contribute to organ fibrosis in kidney [5-6], liver [7] and, more recently, lung [4, 8]. Mice permanently expressing β -galactosidase (β -gal) or green fluorescent protein (GFP) in AEC were used to demonstrate EMT in lung fibrosis following administration of transforming growth factor- β 1 (TGF β 1) or bleomycin [9-10]. However, a role for EMT during lung fibrosis and fibrosis in other organs remains controversial, in part because of technical limitations associated with co-localization using the β -gal reporter [11] and lack of specificity of some mesenchymal markers that have been evaluated [8, 12].

Despite alterations in expression of ECM components in IPF, most studies of disease pathogenesis have focused on effects of growth factors or cytokines (e.g., TGF β 1) on proliferation of fibroblasts and/or EMT, rather than on a potential causal role of ECM components themselves. Basement membranes are thin layers of highly organized ECM comprised primarily of type IV collagen, laminin 332 (or laminin-5), entactin and heparin sulfate proteoglycans that provide a dynamic supporting structure for epithelial cells and are known to influence cellular behaviors such as differentiation and proliferation [13, 14]. Several studies suggest that degradation of basement membrane proteins by matrix metalloproteinases (MMPs) [15-17] is altered in IPF [16] and may contribute to disease pathogenesis. In contrast to the complex ECM suprastructures comprising the basement membrane, interstitial matrix is a loosely organized form of ECM predominantly composed of fibrillar type I and III collagens, elastic fibers and heparin sulfate proteoglycans [14, 18]. Denudation of basement membrane exposes AEC to interstitial fibrillar collagens and/or degraded or monomeric collagens (type I, III, and/or IV), allowing direct interactions with integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$, the two major epithelial receptors for collagens. A role for monomeric type I collagen has been implicated in the induction of EMT in lung [21], breast [22] and pancreatic [23-26] carcinoma cell lines. However, little is known regarding the influence of ECM on EMT in primary epithelial cells or the downstream signaling pathways that may mediate this process.

We generated a novel transgenic mouse line, Nkx2.1-Cre;mT/mG, in which AEC permanently express green fluorescent protein (GFP) in order to evaluate the influence of ECM proteins on the capacity of AEC to undergo EMT *in vitro* and to contribute to

fibroblast/myofibroblast accumulation in the course of pulmonary fibrosis *in vivo*. Our results demonstrate that AEC undergo EMT *in vitro* and *in vivo* and implicate type I collagen as a key regulator of EMT in AEC through signaling via Alk5, likely in a TGF β ligand-independent manner.

Methods

Animals

Nkx2.1-Cre mice [28] (Stewart A. Anderson (Weill Medical College of Cornell University, New York, NY)) were crossed to mT/mG reporter mice (Jackson Laboratory, Bar Harbor, ME) to generate double heterozygous animals. Expression of the GFP reporter gene from the mT/mG knockin allele is dependent on Cre/loxP recombination, where deletion of a loxP-flanked stop sequence results in activation of GFP and simultaneous deletion of Tomato. Mice containing a heterozygous deletion of exon 3 in the Alk5 gene and a floxed allele of this exon, Alk5^{flox/KO}, were generated as previously described [29]. Mice deficient for Alk5 in AEC were generated by crossing Alk5^{flox/KO} to Nkx2.1-Cre driver mice. Mice were raised in a pathogen-free environment and given food and water *ad libitum*. All animal experiments were reviewed and approved by the Institutional Animal Care and Utilization Committee at the USC Keck School of Medicine.

Bleomycin-induced lung injury

Mice 8–10 weeks old were injected intratracheally with bleomycin (1 U/kg; Bristol-Myers Squibb, Princeton, NJ) or saline in a volume of 50 μ L after sedation with ketamine/xylazine. Mice were analyzed on days 17–21 following bleomycin administration.

Mouse AT2 cell isolation and culture

AT2 cells were isolated from adult mice of both genders using dispase (BD Biosciences, San Jose, CA) digestion/agar instillation as previously described [30] (see Supporting Information). To prepare 2-dimensional monomeric ECM, Transwell polycarbonate filters (0.4 μ m pore size, 1.1 cm²; Corning Costar, Corning, NY) were coated with 100 μ g/mL type I collagen (Advanced BioMatrix, San Diego, CA), 1 μ g/mL laminin-5 (Chemicon, Billerica, MA) or 10 μ g/mL fibronectin (Sigma) for 1 h at room temperature [31]. TGF β isoforms in type I collagen solution were not detected by silver staining of SDS-PAGE gels and would have been degraded by pepsin enzymatic digestion according to the manufacturer's specifications. AT2 cells isolated from Nkx2.1-Cre;mT/mG, Nkx2.1-Cre;Alk5^{flox/KO} or Alk5^{flox/KO} mice were cultured in complete mouse medium (CMM) plus 2% newborn bovine serum (NBS; Omega Scientific, Tarzana, CA) and plated at 7.5x10⁵ cells/cm². Medium was replaced with serum-free CMM on day 2 post-plating and every other day thereafter. The TGF β type I receptor inhibitor SB431542 (Sigma), pan-specific TGF β neutralizing antibody (AB-100-NA; R&D Systems, Minneapolis, MN) or TGF β 1 (R&D Systems) were added on day 2 post-plating at 10 μ M, 2.5 μ g/mL and 2.5 ng/mL, respectively. Transepithelial resistance (R_T) and potential difference (PD) were measured using a MilliCell-ERS device equipped with silver/silver chloride electrodes (Millipore, Billerica, MA). Equivalent short-circuit current (I_{EQ} , μ A/cm²) was calculated from Ohm's law ($I_{EQ} = PD/R_T$). Only monolayers that achieved R_T values ≥ 1 k Ω ·cm² were studied.

Flow cytometry

Crude single-cell suspensions were prepared from saline- and bleomycin-injured mouse lungs using the dispase digestion/agar instillation isolation method without use of negative selection antibodies. Cells were analyzed and sorted for GFP expression using a FACS Aria II cell sorter (BD Biosciences).

Fluorescence microscopy

GFP and Tomato fluorescence were directly observed in lung cryosections (5–7 μm thickness), cytopins of isolated lung cells and mouse AEC monolayers (MAECM) derived from Nkx2.1-Cre;mT/mG mice. Immunofluorescence was used to evaluate expression and localization of epithelial and mesenchymal markers and Smad3 (see Supporting Information). Nuclear staining was accomplished with 4',6-diamidino-2-phenylindole (DAPI) Vectashield mount (Vector Laboratories, Burlingame, CA). Images were acquired with a Zeiss confocal microscope 510 Meta NLO CLSM imaging system (Jena, Germany) equipped with argon and red/green HeNe lasers or a Qimaging Retiga 2000R digital camera (Qimaging, Surrey, BC) attached to a Nikon Eclipse 80i (Nikon, Melville, NY).

Western analysis of FACS-sorted GFP-expressing cells

All cells (expressing either Tomato or GFP) comprising MAECM grown on type I collagen or laminin-5 were trypsinized (0.25% in 5 mM EDTA) after 8 or 14 days in primary culture. GFP-positive cells were purified using flow cytometry and protein from only the GFP-positive cell population was harvested using 2% sodium dodecyl sulfate (SDS) sample buffer. Western blots were performed as previously described [30] (see Supporting Information). Antigen-antibody complexes were visualized by enhanced chemiluminescence (Pierce, Rockford, IL) and analyzed with an Alpha Ease RFC Imaging System (Alpha Innotech, San Leandro, CA).

Detection of TGF β 1 in culture medium

TGF β 1 protein was quantified using an ELISA kit (R&D Systems). Neutral serum-free CMM was used to determine active TGF β 1 concentrations. Serum-free CMM that was acidified and subsequently neutralized according to the manufacturer's instructions was used to determine total TGF β 1 concentrations.

Statistical analysis

Data are presented as mean \pm standard error. Significance ($P < 0.05$) was determined by one-way analysis of variance followed by post-hoc procedures based on modified Student-Newman-Keuls tests. For ratiometric data, we used z-tests to determine differences from control. $P < 0.05$ was considered significant.

Results

Analysis of reporter expression in distal lung of Nkx2.1-Cre;mT/mG mice

Nkx2.1 (or thyroid transcription factor-1 (TTF-1)) is expressed in all pulmonary epithelial cells during lung development and becomes restricted to AT2 and Clara cells towards the

end of gestation and postnatally [32]. To confirm AEC-specific expression of GFP, frozen lung sections from Nkx2.1-Cre;mT/mG mice were immunostained for pro-SPC (Figure 1A-B). GFP-positive cells were immunoreactive for pro-SPC, confirming expression in AT2 cells. All GFP-positive AT2 cells isolated from Nkx2.1-Cre;mT/mG mice were immunoreactive for pro-SPC (Figure 1C-D), confirming GFP reporter expression in AT2 cells. A subset of pro-SPC-positive AT2 cells (<20%) did not express GFP, suggesting that the efficiency of Cre/LoxP-mediated recombination of GFP was not 100%, consistent with prior reports of recombination efficiency in the mT/mG reporter line [27, 33]. Nevertheless, recombination efficiency >80% in AT2 cells demonstrates feasibility of a cell fate mapping strategy based on AEC-specific expression of GFP in this newly derived mouse line.

AEC undergo EMT *in vivo* in response to bleomycin instillation in Nkx2.1-Cre;mT/mG mice

To evaluate EMT *in vivo*, double transgenic Nkx2.1-Cre;mT/mG mice received intratracheal instillation of saline ± bleomycin sulfate (1 U/kg). After 21 days, immunostaining of lung cryosections with vimentin (Figure 2A-C), type I collagen (Figure 2D-F) or α-SMA (Figure 2G-I) demonstrates co-localization with the GFP reporter, indicating that a subset of AEC-derived cells express mesenchymal markers after bleomycin treatment. The GFP reporter did not co-localize with mesenchymal markers *in situ* in saline-treated lungs (see Supporting Information, Figure S1).

To precisely quantify the contribution of alveolar EMT to accumulation of fibroblasts/myofibroblasts, crude single-cell suspensions were prepared from saline- and bleomycin-injured lungs of AEC reporter mice and purified by FACS followed by immunostaining for α-SMA and vimentin. ~10% of crude lung cell populations from reporter mice expressed GFP (Figure 3B); lung cells isolated from single transgenic mT/mG mice did not express GFP after bleomycin injury (Figure 3A). Expression of α-SMA or vimentin was observed in $4.1 \pm 1.3\%$ and $3.9 \pm 1.0\%$ of AEC-derived GFP-expressing cells, respectively, on days 17-21 post-administration of bleomycin (Figure 3C-H), but not in saline-treated mice. Co-localization of GFP with vimentin or α-SMA in frozen lung sections and single cell suspensions prepared from bleomycin-injured reporter mice indicates that AEC express mesenchymal markers *in vivo* in response to bleomycin.

Characterization of MAECM grown on various extracellular matrices in the absence or presence of TGFβ1

. We compared effects of monomeric type I collagen, monomeric laminin-5 and fibronectin on AEC phenotype. AT2 cells from Nkx2.1-Cre;mT/mG mice grown on laminin-5 formed confluent monolayers with characteristic cobblestone-like morphology after 8 days in culture (Figure 4A-C). Immunoreactivity for ZO-1 and E-cadherin was localized to cell-cell contacts in the majority of cells (Figure 4D-E), including occasional cells which did not express the GFP reporter. Reactivity for α-SMA was absent (Figure 4F). Mouse AEC grown on fibronectin exhibited similar morphology and localization of cell-cell junctional and adherens proteins, ZO-1 and E-cadherin, as seen in AEC grown on laminin-5 (see Supporting Information, Figure S2). In contrast, GFP-positive cells grown on type I collagen after 8 days in culture exhibited an irregular morphology (Figure 4G-I), with less intense localization of ZO-1 and E-cadherin at cell-cell contacts (Figure 4J-K). α-SMA co-localized

with GFP in epithelial-derived cells (Figure 4L), demonstrating that primary AEC undergo EMT when grown on type I collagen.

TGF β has been shown to mediate EMT *in vitro* [35] and *in vivo* [36]. In the present study, GFP-positive cells grown on laminin-5 and exposed to TGF β 1 (2.5 ng/mL) on days 2–8 in culture exhibited fibroblast-like morphology after 8 days (see Supporting Information, Figure S3A–C) compared to AEC grown on laminin-5 in the absence of TGF β 1 (Figure 4A–C). Furthermore, immunoreactivity for ZO-1 was decreased at cell borders (Figure S3D). Localization of E-cadherin was also less concentrated at cell-cell contacts after exposure to TGF β 1 (Figure S3E) compared to its localization in AEC not exposed to TGF β 1 (Figure 4E). Immunoreactivity for α -SMA co-localized with the GFP reporter in a subset of cells (Figure S3F). In the presence of TGF β 1, the majority of GFP-expressing cells grown on type I collagen expressed α -SMA (Figure S3I and L), indicating TGF β 1 augments effects of collagen on EMT. Mouse AEC grown on fibronectin in the presence of TGF β 1 exhibited similar morphologic changes and alterations in localization of ZO-1 and E-cadherin as seen in mouse AEC grown on type I collagen in the absence of TGF β 1 or laminin-5 in the presence of TGF β 1 (Figure S2).

In the absence of TGF β 1, a minor subset of cells expressing the GFP reporter (<3%) was immunoreactive for α -SMA when cultured on laminin-5 or fibronectin, whereas on type I collagen α -SMA was expressed in ~55% of GFP-labeled cells (Figure 4M). Addition of exogenous TGF β 1 caused ~50%, ~40% and ~80% of GFP-labeled cells to undergo EMT when grown on laminin-5, fibronectin or type I collagen, respectively. These data indicate that monomeric type I collagen induces EMT which can be augmented by addition of TGF β 1. After 8 days in culture, MAECM grown on laminin-5 developed $R_T > 2.0 \text{ k}\Omega\text{-cm}^2$ (Figure 4N), consistent with the presence of a relatively pure epithelial cell population in culture and our previous report of MAECM barrier properties [30]. Monolayers grown on fibronectin attain $R_T \sim 50\%$ of that attained on laminin-5, whereas on type I collagen or any of the matrices in the presence of TGF β 1, R_T was near zero after 8 days in culture, consistent with observed loss of epithelial phenotype.

Induction of EMT by type I collagen requires signaling via TGF β type I receptor (Alk5)

Given the established role of TGF β as a central mediator of EMT, a potential role for TGF β signaling in the induction of EMT by type I collagen was further investigated. The Alk5 inhibitor SB431542 blocked collagen-induced expression of α -SMA in GFP-positive cells as shown by immunocytochemistry (Figure 5A–D) and Western blot (Figure 5I). GFP-positive cells showed no expression of α -SMA when grown on laminin-5 with or without the Alk5 inhibitor (Figure 5E–H). Cellular localization of Smad3 was determined in AEC grown on laminin-5 and type I collagen via immunocytochemistry. On day 8 in primary culture, localization of Smad3 was cytoplasmic in MAECM grown on laminin-5 (Figure 6A–D) and nuclear on type I collagen (Figure 6E–H). In MAECM grown on type I collagen, SB431542 prevented nuclear localization (Figure 6I–L) and phosphorylation of Smad3 (Figure 6M), suggesting that induction of EMT by type I collagen requires activation of the canonical TGF β receptor-Smad pathway and signaling via Alk5.

To further validate the role of signaling via the TGF β receptor complex, AEC deficient for Alk5 from Nkx2.1-Cre;Alk5^{flox/KO} mice or control Alk5^{flox/KO} mice carrying only a heterozygous deletion of exon 3 of the T β RI gene (see [29] and Supporting Information, Figure S4) were plated on filters coated with laminin-5 or type I collagen. After 8 days in culture, monolayers derived from both Alk5-deficient and control mice exhibited ZO-1 reactivity at cell-cell contacts and lacked α -SMA reactivity when grown on laminin-5 (Figure 7A-D). On type I collagen, MAECM derived from Alk5^{flox/KO} control mice expressed α -SMA and showed discontinuous ZO-1 reactivity at cell borders (Figure 7G-H) and nuclear localization of Smad3 (Figure 7K-L and 7O-P), whereas in MAECM deficient for Alk5, expression of α -SMA was absent, localization of ZO-1 was continuous at cell-cell contacts (Figure 7E-F) and localization of Smad3 was cytoplasmic (Figure 7I-J and 7M-N). These results confirm that induction of EMT in AEC by type I collagen requires signaling via Alk5.

Secreted TGF β ligands are not required for type I collagen-induced EMT in AEC

AT2 cells were grown on collagen in the presence or absence of neutralizing antibody against TGF β (2.5 μ g/mL). The pan-specific neutralizing antibody did not inhibit type I collagen-induced expression of α -SMA in GFP-positive cells as shown by immunocytochemistry (Figure 8A-D) and Western blot (Figure 8E-F). Furthermore, Smad3 remained nuclear in the presence of blocking antibody (data not shown). Neutralizing antibody blocked induction of α -SMA (Figure 9A-H) and reduction in R_T (Figure 9I) by exogenous TGF β 1 (2.5 ng/mL) in MAECM generated from wild-type mice grown on laminin-5, confirming efficacy of the antibody. In addition, total and active levels of TGF β 1 in culture fluid from MAECM grown on collagen and laminin-5 on days 5-8 post-plating were not significantly different from each other (see Supporting information, Figure S5). Taken together, these data suggest that induction of EMT by type I collagen is likely independent of secreted TGF β ligand and does not involve autocrine TGF β production.

Discussion

Evidence suggests a role for EMT in the pathogenesis of IPF by serving as a mechanism for generation of fibroblasts/myofibroblasts, key effector cells in extracellular matrix deposition. Willis and colleagues [35] identified cells co-expressing epithelial and mesenchymal markers in IPF lung, suggesting that AEC might serve as progenitors for (myo)fibroblasts. Fate mapping studies evaluating EMT following instillation of bleomycin or overexpression of active TGF β 1 using different mesenchymal markers and reporter mice have yielded variable results with regard to the contribution of EMT to the myofibroblast population [10, 36]. Kim and colleagues [9, 36] demonstrated in transgenic mouse reporter systems in which AEC permanently expressed β -gal or GFP that, following over-expression of active TGF β 1 and bleomycin instillation, approximately 5% and 10% of reporter-positive cells, respectively, expressed α -SMA and vimentin. Using alveolar epithelial-specific β -gal reporter mice, Tanjore and colleagues [10] reported that \sim 1/3 of FSP1/S100A4-positive fibroblasts were derived from alveolar epithelium post-bleomycin instillation while α -SMA-expressing myofibroblasts were rarely derived from EMT. Using a novel Nkx2.1-Cre;mT/mG reporter line, we detected approximately equal numbers (\sim 4%) of α -SMA- and

vimentin-expressing GFP-positive cells by FACS following intratracheal bleomycin, providing additional evidence supporting the ability of AEC to undergo EMT following injury. The discrepancy between our results and those of Tanjore et al. [10] regarding overall frequency of EMT-derived cells may be due to differences in the mesenchymal marker evaluated as well as difficulties in interpreting β -gal expression using immunohistochemical approaches, although the reasons for a lower number of α -SMA-positive epithelial-derived cells in their study remain uncertain [11]. Given the difficulties with interpretation of β -gal activity in many mammalian tissues and especially lung [38], direct fluorescent reporters such as GFP may be better suited to evaluate EMT in cell fate reporter mice.

Degradation of basement membrane and interstitial matrix is a critical upstream event in initiation of pulmonary fibrosis [15-17]. Tissue remodeling and proteolysis produces monomeric ECM and/or neoepitopes from ECM macromolecules that may act as “soluble” peptides in the pericellular microenvironment [39-42], which may exert effects on epithelial phenotype that differ from those of fibrillar or polymerized ECM as previously shown for fibroblasts [43-46]. Furthermore, integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$, the two major receptors for collagens, bind more strongly to monomeric collagen than to fibrillar collagen [47]. Our current results showing that mouse AEC grown on monomeric type I collagen undergo EMT, whereas AT2 cells grown on laminin-5 or fibronectin maintain epithelial phenotype, suggest that interstitial collagen, to which cells would be exposed following basement membrane disruption and matrix degradation, plays a causal role in EMT of AEC which may contribute to progression of fibrosis. Investigation of effects of collagen on EMT has almost exclusively been conducted in cancer cell lines in a process that primarily involves interaction with $\alpha_2\beta_1$ integrin [48-49], with limited evidence for collagen-induced EMT in primary cells. Mechanisms implicated in collagen I-induced EMT in malignant cells include integrin-mediated Src activation, inhibition of PTEN [50], integrin-like kinase (ILK)-dependent activation of NF- κ B and LEF-1 [25] as well as autocrine induction of TGF β 3 signaling [21]. We demonstrate that type I collagen is able to efficiently induce EMT in primary cells, perhaps involving mechanisms distinct from those previously described in malignant cells undergoing EMT. In contrast to the study by Shintani et al. [21], we found that despite activation of TGF β signaling, observed effects of collagen appear to be independent of autocrine TGF β induction. Given that the major integrins (i.e., $\alpha_1\beta_1$ and $\alpha_2\beta_1$) engaged by type I collagen are not known to modulate interactions of TGF β with latency-associated peptide and its activation, these results suggest a novel mechanism whereby induction of EMT by type I collagen involves signaling via Alk5 independent of secretion/activation of TGF β ligands. Soluble collagen peptides have recently been shown to activate Smad signaling in the absence of native TGF β ligands through a mechanism involving crosstalk between $\alpha_2\beta_1$ integrin collagen receptors and a membrane signaling complex that includes Alk5, T β RII, integrins α_2 and β_1 , cellular Src and FAK [41, 51]. Results of the current study suggest that a similar mechanism may be implicated in collagen I-induced EMT. We recognize that the possibility that secreted ligand could immediately bind to the autologous receptor in a manner that is not susceptible to neutralization by exogenous antibody has not been completely excluded, requiring further investigation [52].

Our results differ from those reported by Kim and colleagues [36], in which fibronectin was shown to induce EMT in primary AEC in the absence of exogenous TGF β through integrin α v β 6-dependent activation of latent TGF β 1, but are similar to results of others in which type I collagen but not fibronectin induced EMT in cancer cell lines [21, 26]. These somewhat discrepant findings may be due to differences between model systems. Kim and colleagues [36] plated AT2 cells on chamber slides, a condition in which cells may remain subconfluent without direct cell-cell contact. Based on studies in renal tubular epithelial cells, it is likely that the influence of ECM proteins on AEC phenotype also depends on cell confluence and integrity of cell-cell contacts [53].

Although EMT has now been demonstrated using several different Cre drivers and reporters in at least two mouse models of pulmonary fibrosis [36], and epithelial and mesenchymal markers have been co-localized to a majority of AEC in IPF, the ability of AEC to undergo EMT *in vivo* remains controversial while the significance of a small number of EMT-derived cells has been questioned. Recent studies have challenged the prevalence of EMT *in vivo* in other tissues and raised concerns about its significance in chronic fibroproliferative diseases [54, 55]. However, it should be noted that although only a small number of GFP-positive cells of epithelial origin express α -SMA or vimentin after bleomycin injury, the absolute number of EMT-derived fibroblasts may not accurately reflect their overall contribution to the fibrogenic process and it is possible that a minority population of fibroblasts/myofibroblasts derived from EMT stimulates proliferation and activation of resident fibroblasts through aberrant epithelial-fibroblast crosstalk [9]. In addition, the protocol used for isolation of GFP-expressing cells from lungs of injured animals in the current study was similar to that used for isolation of AT2 cells and may be less efficient for isolation of fibroblasts, likely underestimating the number of cells that had undergone complete phenotypic transition. Nevertheless, we recognize that further investigations (including studies to inhibit EMT) are needed to address functional contributions of EMT-derived cells to the fibrotic process. Our results indicate a causal role for monomeric type I collagen in the development of EMT *in vitro*, implicate degraded ECM proteins as potentially important facilitators of EMT in IPF and support an expanded role for ECM in direct regulation of the TGF β pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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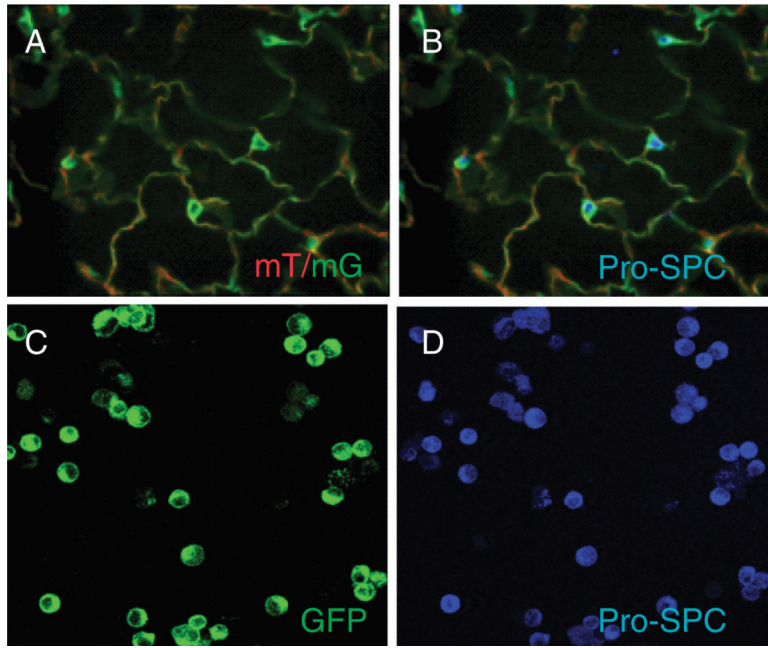


Figure 1.

GFP-positive AEC from lungs of Nkx2.1-Cre;mT/mG mice express pro-SPC. A and B: Lung cryosection from an Nkx2.1-Cre;mT/mG mouse expressing GFP and Tomato (A; green and red) was immunostained for pro-SPC (B; blue). GFP-positive cells were immunoreactive for pro-SPC, indicating that GFP is expressed in AT2 cells. Magnification x40. C and D: Freshly isolated AT2 cells from Nkx2.1-Cre;mT/mG mice expressing GFP (C) were immunostained for pro-SPC (D; blue). All GFP-positive cells were reactive for pro-SPC. A subset of cells (~16%) were pro-SPC positive but did not express GFP, indicating that Cre/LoxP-mediated recombination of mT/mG occurs in >80% of AT2 cells. Results are representative of $n = 4$ independent experiments. Original magnification x40.

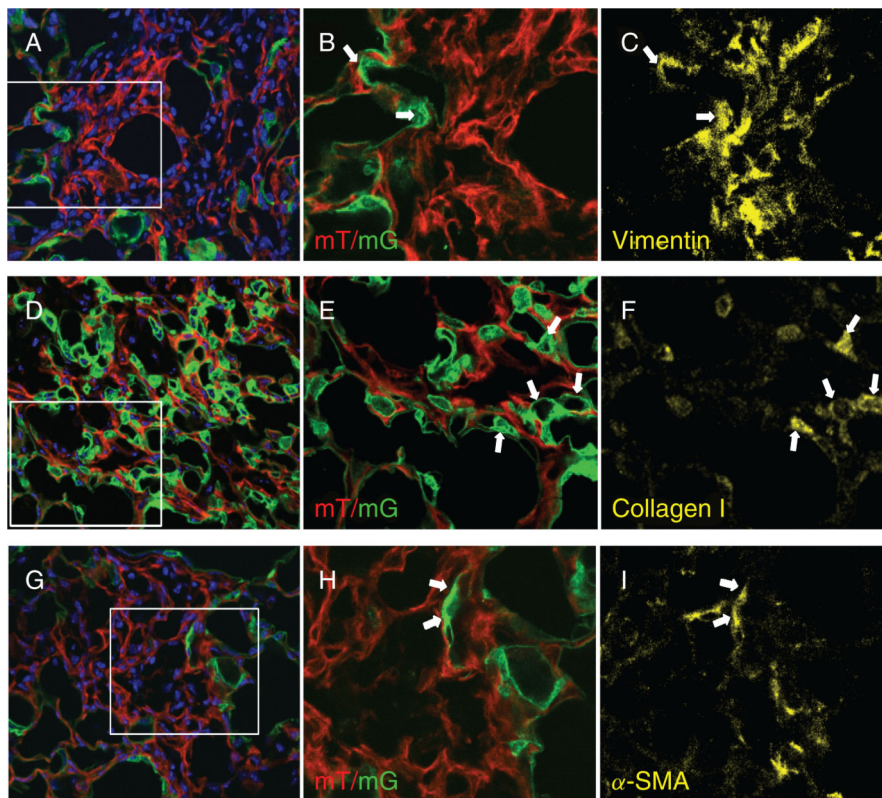


Figure 2.

Detection of epithelial-mesenchymal transition (EMT) in lung sections of Nkx2.1-Cre;mT/mG reporter mice 21 days post-bleomycin instillation. Confocal images demonstrate co-localization of membrane-associated GFP with mesenchymal markers vimentin (A–C), type I collagen (D–F), and α -SMA (G–I). Panels A, D and G show direct detection of Tomato and GFP (red and green) in sections counterstained with DAPI (blue). Original magnification x40. Panels B, E and H show magnified views of the rectangles shown in panels A, D and G, respectively, after cropping and re-sizing using Adobe Photoshop. Lung sections were immunostained for vimentin (C), type I collagen (F) and α -SMA (I) as shown in yellow. Arrows point to representative cells which co-express GFP and mesenchymal markers. Thresholds of staining intensity for the three primary antibodies were established using reference sections treated with only secondary antibodies.

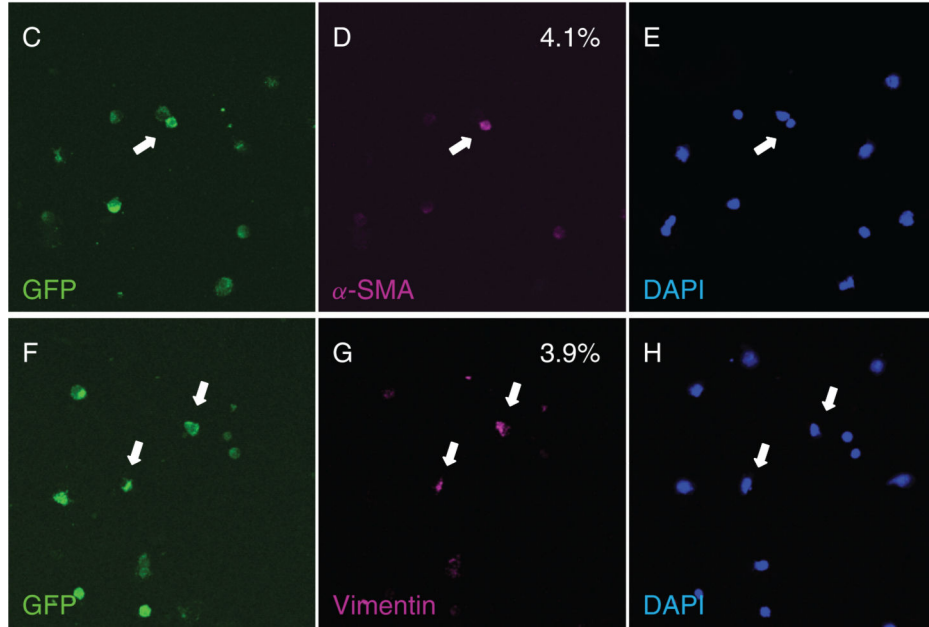
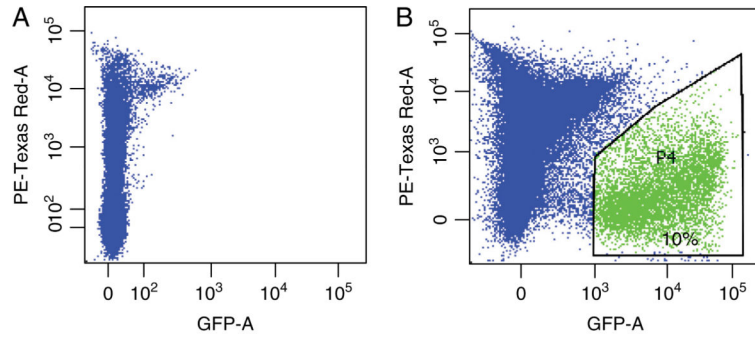
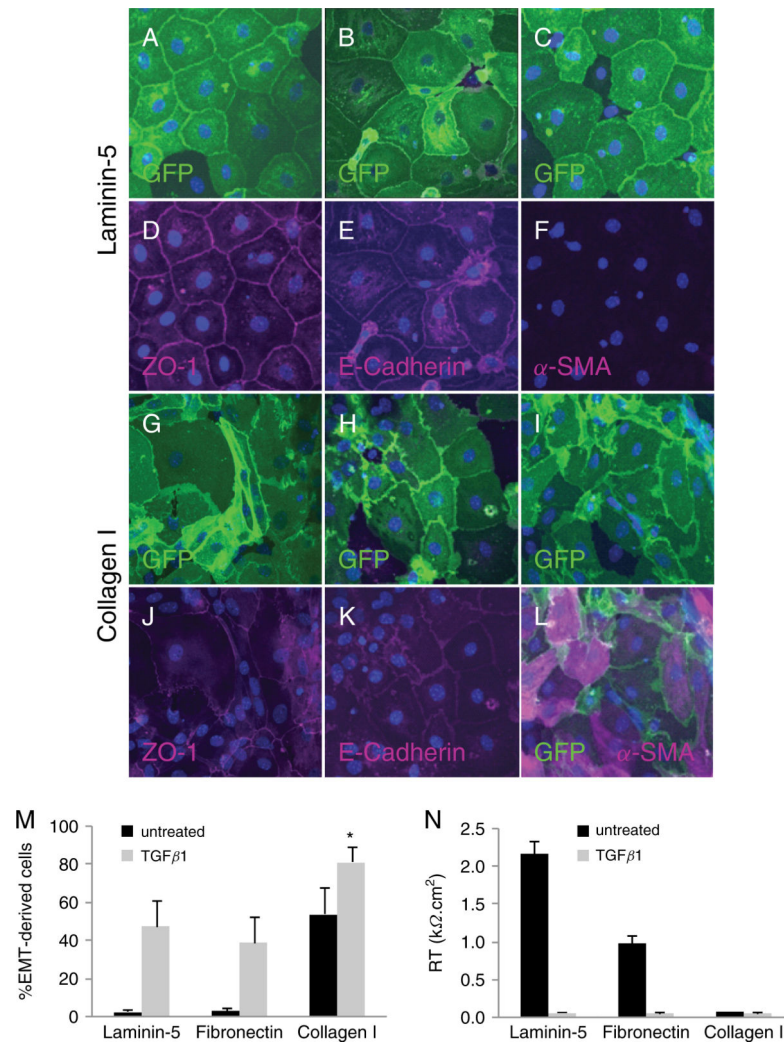


Figure 3.

GFP-sorted cells from bleomycin-injured lung of Nkx2.1-Cre;mT/mG mice express vimentin and α -SMA. Representative GFP-FACS profiles of lung cells from a single transgenic mT/mG (A) and double transgenic Nkx2.1-Cre;mT/mG mouse (B) 21 days after intratracheal administration of bleomycin. Percentage of GFP-positive cells is indicated in B. GFP-expressing cells of epithelial origin express mesenchymal markers on days 17-21 post-instillation of bleomycin (C–H). GFP-sorted cells were immunostained for α -SMA (D; magenta) and vimentin (G; magenta). Nuclei were counterstained with DAPI (E and H; blue). Approximately 4% of cells of epithelial origin expressed α -SMA or vimentin in bleomycin-injured mice. None of the GFP-positive cells expressed mesenchymal markers in saline-treated mice. $n = 5$ bleomycin- and saline-treated mice. Arrows point to GFP-positive cells which express α -SMA or vimentin. Original magnification $\times 20$.

**Figure 4.**

Characterization of MAECM grown on laminin-5 and type I collagen. Mouse AT2 cells from *Nkx2.1-Cre;mT/mG* mice plated on laminin-5-coated filters form confluent monolayers with a cobblestone-like morphology after 8 days in culture (A–C). GFP was directly observed in the majority of AEC within the monolayers. A subset of cells did not express GFP, consistent with the observation that *Cre/loxP* recombination driven by *Nkx2.1* is not 100% efficient. Reactivity for ZO-1 and E-cadherin was localized to cell-cell contacts in the majority of cells (D and E; magenta), indicating a relatively pure epithelial cell population. Reactivity for α -SMA was absent (F). AT2 cells plated on collagen I-coated filters exhibit a disorganized and irregular morphology after 8 days in culture as shown by direct GFP expression (G–I). ZO-1 was redistributed from cell borders to the cytoplasm in a subset of cells (J; magenta). Localization of E-cadherin at cell borders was reduced (K; magenta). Reactivity of α -SMA was observed in the majority of GFP-labeled AEC (L; magenta). Nuclei were counterstained with DAPI (blue). Photographs are representative of 4 separate experiments. Original magnification $\times 40$.

M: Semi-quantitative analysis of the percentage of GFP reporter-positive cells which expressed α -SMA on various ECM proteins in the presence or absence of TGF β 1; mean of 4 low-power fields ($\times 20$) per monolayer \pm SEM ($n = 1$ monolayer per ECM condition per preparation from 3 different cell preparations). **N:** Effect of ECM proteins on R_T of MAECM after 8 days in culture in the presence and absence of TGF β 1 (2.5 ng/mL). Values are mean \pm SE ($n = 1$ monolayer per ECM component per preparation from 4 different cell preparations). * $P < 0.05$ vs all other conditions.

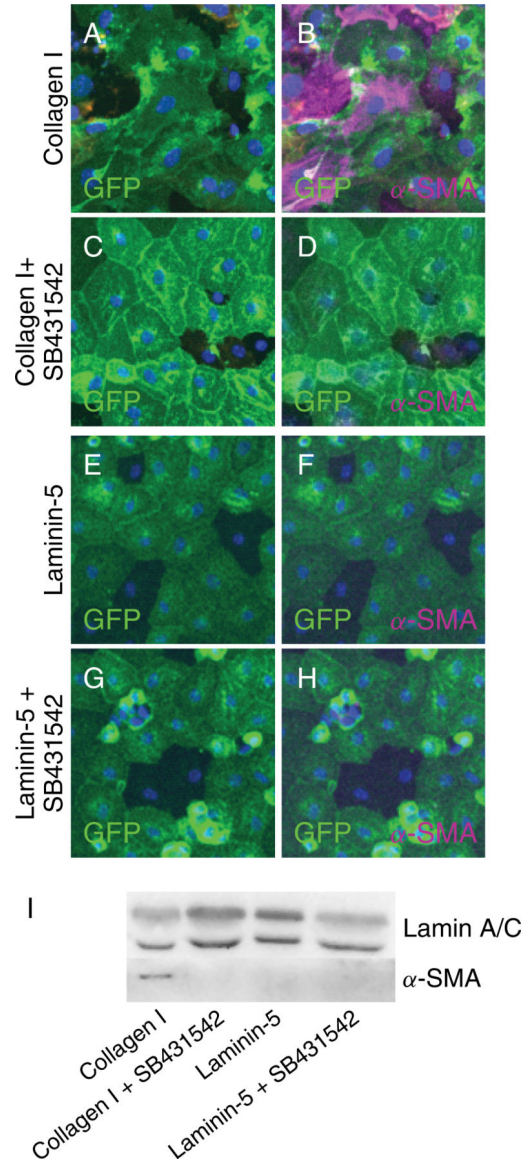


Figure 5.

TGFβ1 receptor inhibitor SB431542 blocks induction of α-SMA by type I collagen. MAECM grown on type I collagen exhibit irregular and disorganized morphology (A) and a subset of GFP-labeled cells express α-SMA (B; magenta). MAECM grown on type I collagen in the presence of SB431542 (10 μM) displayed cobblestone-like morphology and GFP-labeled cells did not express α-SMA (D), indicating prevention of collagen-induced EMT. GFP-labeled cells grown on laminin-5 with or without SB431542 retain a cobblestone-like morphology (E, G) and showed no immunoreactivity for α-SMA (F, H). Nuclei were counterstained with DAPI (blue). Original magnification x20. I: MAECM were trypsinized off type I collagen- and laminin-5-coated polycarbonate filters and GFP-positive cells were purified by FACS. Lysates of GFP-positive cells were subjected to SDS-PAGE and immunoblotted with mouse monoclonal anti-α-SMA antibody. Lamin A/C was used as a loading control. Data shown are representative of 3 separate experiments.

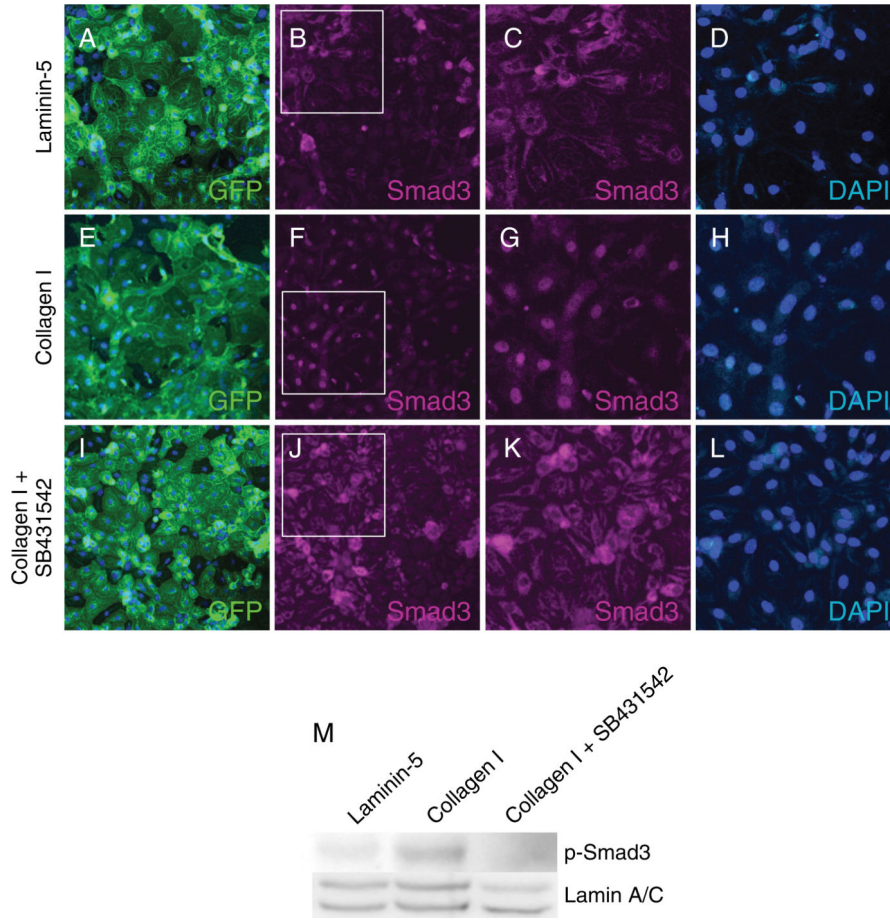


Figure 6.

Type I TGF β receptor inhibitor SB431542 blocks nuclear localization of Smad3 induced by type I collagen. Panels A, E and I show direct detection of GFP in MAECM derived from Nkx2.1-Cre;mT/mG lungs and grown on laminin-5, type I collagen or type I collagen in presence of SB431542 (10 μ M), respectively. Panels B, F and J are the same fields as panels A, E and I immunostained for Smad 3 (magenta). Original magnification x20. Panels C, G and K show magnified views of panels B, F and J, respectively, after cropping and re-sizing using Adobe Photoshop. Panels D, H and L show labeling with DAPI only (blue). These findings indicate that Smad3 nuclear localization by type I collagen is inhibited by SB431542. Western blot (panel M) demonstrates induction of phospho-Smad3 in cells grown on collagen I which is inhibited by SB431542.

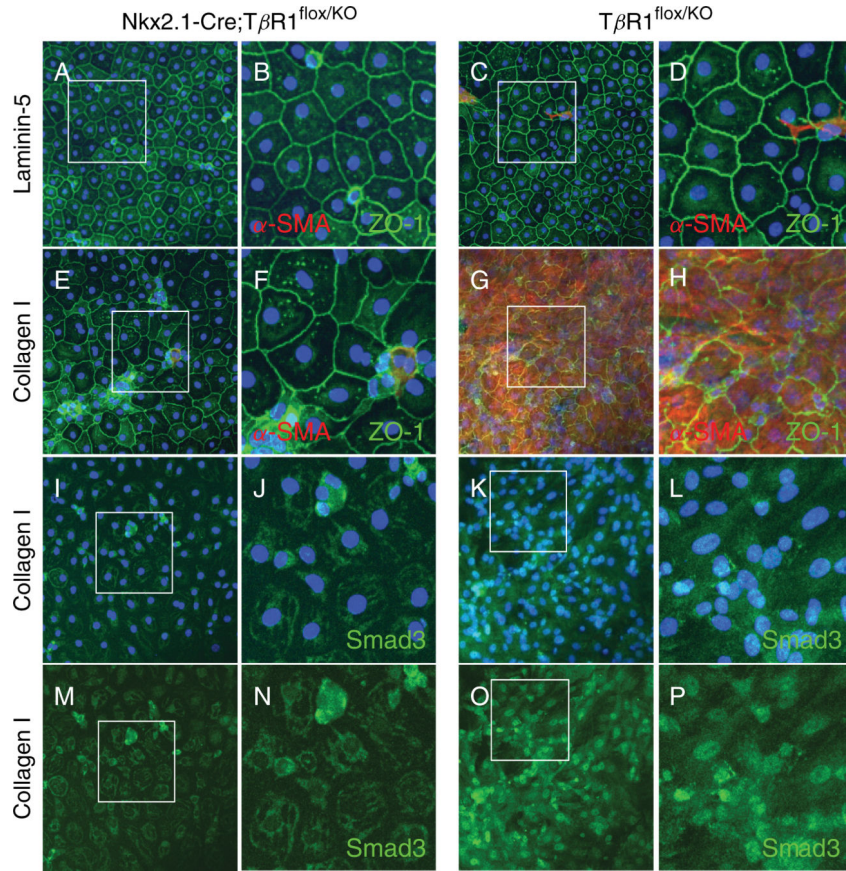


Figure 7.

Expression of α -SMA and nuclear localization of Smad3 induced by type I collagen is blocked in MAECM deficient for Alk5. MAECM deficient for Alk5 (A-B) or from control carrying a heterozygous deletion of exon 3 (C-D) exhibited ZO-1 reactivity at the cell-cell contacts and lacked α -SMA reactivity when grown on laminin-5 after 8 days in culture. On type I collagen, MAECM derived from $Alk5^{fllox/KO}$ mice expressed α -SMA and discontinuous ZO-1 reactivity at cell borders (G-H) and showed nuclear localization of Smad3 (K-L and O-P; with and without DAPI), whereas in MAECM completely deficient for Alk5 ($Nkx2.1-Cre;Alk5^{fllox/KO}$), expression of α -SMA was absent, localization of ZO-1 was continuous at the cell-cell contacts (E-F), and localization of Smad3 was cytoplasmic (I-J and M-N; with and without DAPI). Original magnification x20. Panels B, D, F, H, J, L, N and P show magnified views of the rectangles shown in panels A, C, E, G, I, K, M, and O, respectively, after cropping and re-sizing using Adobe Photoshop.

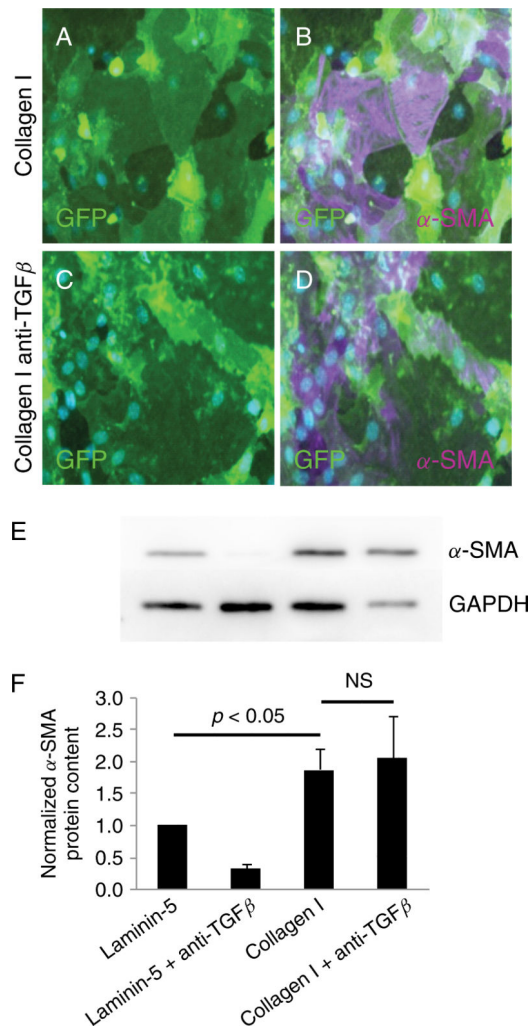


Figure 8.

Pan-specific TGF β neutralizing antibody does not block induction of α -SMA by type I collagen. MAECM grown on type I collagen exhibit irregular and disorganized morphology (A), and a subset of GFP-labeled cells express α -SMA (B; magenta) after 8 days in culture. MAECM grown on type I collagen in the presence of a pan-specific TGF β neutralizing antibody (2.5 μ g/mL) exhibited similar disorganized cell morphology (C) and expression of α -SMA as cells grown on collagen in the absence of neutralizing antibody (D; magenta). Nuclei were counterstained with DAPI (blue). Original magnification x20. E: MAECM treated with and without the pan-specific TGF β neutralizing antibody were trypsinized from collagen I- and laminin-5-coated polycarbonate filters after 8 days in culture and GFP-positive cells were purified via FACS. Total cell lysates of GFP-positive cells were subjected to SDS-PAGE and immunoblotted with mouse monoclonal anti- α -SMA antibody. Lamin A/C was used as a loading control. Representative Western blot demonstrates that the increase in α -SMA in cells grown on type I collagen is not prevented by the TGF β neutralizing antibody. F: Relative densitometric values normalized to α -SMA levels in monolayers grown on laminin-5. Results are an average of 3 independent experiments.

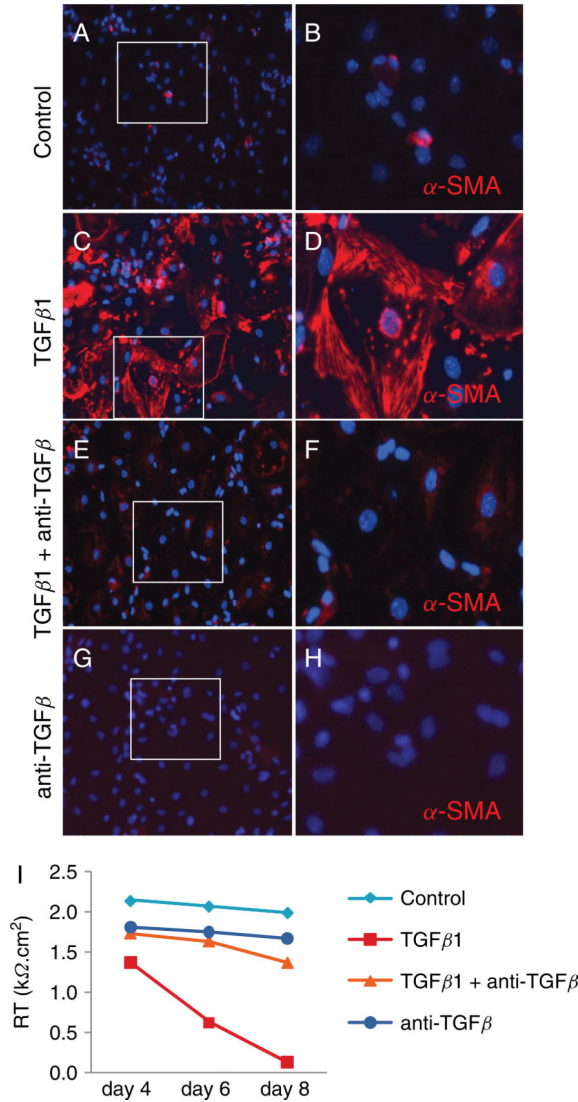


Figure 9

. Pan-specific TGFβ neutralizing antibody blocked induction of α-SMA by exogenous TGFβ1. Wild-type MAECM grown on laminin-5 in the presence of exogenous TGFβ1 (2.5 ng/mL) expressed α-SMA (C-D; red) after 8 days in culture, whereas in the absence of TGFβ1 (A-B), in the presence of TGFβ1 and the TGFβ neutralizing antibody (E-F), or with TGFβ neutralizing antibody alone (G-H), MAECM have minimal or no α-SMA reactivity. Original magnification x20. Panels B, D, F and H show magnified views of the rectangles shown in panels A, C, E and G, respectively, after cropping and re-sizing using Adobe Photoshop. I: Wild-type MAECM grown on laminin-5 develop $R_T > 2 \text{ k}\Omega\cdot\text{cm}^2$ on day 4. Representative experiment demonstrates that exogenous TGFβ1 reduced R_T to near $0 \text{ k}\Omega\cdot\text{cm}^2$ on day 8; the TGFβ neutralizing antibody blocked the TGFβ1-induced reduction in R_T .