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TGF- β 1 Signaling and Tissue Fibrosis

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Activation of TGF- β 1 initiates a program of temporary collagen accumulation important to wound repair in many organs. However, the outcome of temporary extracellular matrix strengthening all too frequently morphs into progressive fibrosis, contributing to morbidity and mortality worldwide. To avoid this maladaptive outcome, TGF- β 1 signaling is regulated at numerous levels and intimately connected to feedback signals that limit accumulation. Here, we examine the current understanding of the core functions of TGF- β 1 in promoting collagen accumulation, parallel pathways that promote physiological repair, and pathological triggers that tip the balance toward progressive fibrosis. Implicit in better understanding of these processes is the identification of therapeutic opportunities that will need to be further advanced to limit or reverse organ fibrosis.

Commonly affecting many organs including lung, heart, kidney, and liver, tissue fibrosis is a leading cause of morbidity and mortality worldwide (Friedman et al. 2013). Yet the capacity for rapid wound repair with collagen accumulation facilitates restoration of tissue strength as well as tissue integrity in many injury situations. For example, early cardiac fibrosis after myocardial infarction is important for protection against fatal cardiac rupture (Hofmann et al. 2012). In broader terms, the capacity of cells to secrete and adhere to a collagenous extracellular matrix (ECM) must be a strong evolutionary force for advantage because virtually all metazoans express genes for fibrillar collagens (Hynes 2012). In mammals, the potential role of transforming growth factor (TGF)- β 1 in physiological repair and collagen accumulation

was recognized soon after the discovery of TGF- β 1 expression in cancer cell lines (Moses et al. 2016). TGF- β 1 was found to promote fibronectin and collagen production by both epithelial and mesenchymal cells in culture, and to do so by transcriptional activation of the relevant genes (Ignatz and Massagué 1986). Additionally, subcutaneous injection of TGF- β 1 was seen to strongly promote collagen accumulation and a fibrotic tissue response (Roberts et al. 1986). These observations and several subsequent lines of evidence emerged solidifying the role of TGF- β 1 as a master regulator of ECM accumulation and consequently a potential key driver of fibrosis (Bartram and Speer 2004). TGF- β 1 is also intricately involved in regulating inflammation (Shull et al. 1992; Kulkarni et al. 1993). Deletion of the *Tgfb1* gene in mice results



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in rapid demise after birth because of overwhelming, systemic inflammation (Shull et al. 1992). Based on this and many other observations (Travis and Sheppard 2014), TGF- β 1 is generally considered as a suppressor of excessive inflammation. Regulated inflammation itself is an important determinant of tissue repair, as resolution of provisional fibronectin-rich ECMs and elaboration of mediators that limit collagen accumulation after injury limit fibrosis. Inflammatory cells, especially macrophages, are important initiators of inflammation and mediators of inflammation resolution (Vannella and Wynn 2016; Minutti et al. 2017). This review will examine our current understanding of the core functions of TGF- β 1 in promoting collagen accumulation and parallel pathways that help determine whether there is physiological repair or progressive fibrosis. Although increased levels of TGF- β 2 and TGF- β 3 have been observed in fibrotic disease (Burke et al. 2016), there is little evidence implicating either TGF- β 2 or TGF- β 3 with progressive tissue fibrosis, and therefore these TGF- β isoforms will not be discussed further here.

INTERPLAY BETWEEN INTEGRINS AND ECM STIFFNESS IN TGF- β 1 ACTIVATION AND STROMAL EXPANSION

Newly translated TGF- β is processed by cleavage of an amino-terminal fragment, called the latency-associated peptide (LAP), from the polypeptide that homodimerizes to form the mature cytokine (Munger et al. 1997; Moses et al. 2016; Robertson and Rifkin 2016). The cleaved LAP also homodimerizes and associates noncovalently with mature TGF- β in a complex that prevents access of TGF- β to its receptors. In most cells, this “small latent complex” is disulfide linked to either a member of a family of latent TGF- β binding proteins (LTBPs) and stored in the ECM or to the transmembrane protein glycoprotein A repetitions predominant (GARP) for presentation on the cell surface (Stockis et al. 2009). Most organs of healthy adult mammals contain substantially more latent TGF- β than would be required, when activated, to cause extensive tissue fibrosis.

Therefore, much of the regulation of TGF- β function in fibrotic diseases depends on the regulation of TGF- β activation rather than synthesis or secretion (Robertson and Rifkin 2016).

In vitro it is relatively easy to induce conformational change in latent TGF- β that leads to release of the active cytokine (i.e., TGF- β activation). For example, purified latent TGF- β can be activated by acidic or basic pH, heat denaturation, and oxidation by reactive oxygen species (ROS), incubation with thrombospondin 1, or a wide variety of proteases (Munger et al. 1997; Crawford et al. 1998). However, with the exception of thrombospondin 1, for which gene inactivation has identified some phenotypic features consistent with loss of TGF- β activation, and ROS in the setting of mammary gland irradiation (Barcellos-Hoff et al. 1994), the roles of these activation pathways in vivo have not been established. The most intensely studied activation mechanism with demonstrated relevance in vivo has been activation by interaction with a subset of integrins (Munger et al. 1999; Annes et al. 2002; Mu et al. 2002; Jaramillo et al. 2003; Henderson et al. 2013; Hinck et al. 2016).

The first indication that integrins could participate in the activation of TGF- β came from observations of the phenotype of mice lacking the β_6 subunit of the epithelially restricted $\alpha_v\beta_6$ integrin (Munger et al. 1999). These mice show exaggerated inflammatory responses to normally trivial injurious stimuli in multiple epithelial organs (Huang et al. 1996). Despite these exaggerated responses, *Itgb6*^{-/-} mice are dramatically protected from tissue fibrosis (Munger et al. 1999; Hahm et al. 2007). *Tgfb1*^{-/-} mice have even more exaggerated tissue inflammation, reflecting the more limited tissue expression of *Itgb6* relative to *Tgfb1* (Kulkarni et al. 1993). Biochemical evidence has shown direct binding of the $\alpha_v\beta_6$ integrin to an arginine-glycine-aspartic acid (RGD) tripeptide within TGF- β 1 LAP (Munger et al. 1999), and cell culture assays have shown that epithelial cells can use this integrin to bind and activate TGF- β 1 and - β 3 (Munger et al. 1999; Annes et al. 2002). Since these initial observations, several studies have shown reduced TGF- β activation in epithelial organs of *Itgb6*^{-/-} mice,

by cells lacking the $\alpha_v\beta_6$ integrin, and in response to blocking antibodies targeting this integrin (Munger et al. 1999; Weinreb et al. 2004; Jenkins et al. 2006; Aluwihare et al. 2009; Giacomini et al. 2012).

Because the inflammatory phenotype of mice lacking even one of the three isoforms of TGF- β (e.g., TGF- β 1) is much more severe than the phenotype of mice lacking the $\alpha_v\beta_6$ integrin, additional mechanisms must regulate activation of TGF- β 1 and - β 3 in vivo. It is now clear that additional RGD-binding integrins are important contributors to this process. Cell culture studies with epithelial cells showed that the closely related integrin, $\alpha_v\beta_8$, can also potently activate TGF- β (Mu et al. 2002). Mice that lack integrin β_8 also recapitulate some features of mice lacking TGF- β 1, such as defects in vascular development, or TGF- β 3 (e.g., cleft palate) (Zhu et al. 2002). When *Itgb8*^{-/-} mice are crossed to an outbred genetic background that allows some mice to survive into early adulthood, treatment of these mice at birth with blocking antibody to the $\alpha_v\beta_6$ integrin results in a complete phenocopy of all developmental effects of loss of TGF- β 1 and TGF- β 3 (Aluwihare et al. 2009), suggesting that $\alpha_v\beta_6$ and $\alpha_v\beta_8$ together contribute to activation of all of the TGF- β 1 and TGF- β 3 required for normal development.

Additional RGD-binding integrins may play important roles in activating TGF- β to drive tissue pathology in adults. For example, Cre recombinase-mediated, tissue-restricted deletion of the RGD-binding integrins that share the α_v subunit in cells expressing platelet-derived growth factor β (PDGF β) protects mice from pathologic fibrosis in the lungs, liver and kidney (Henderson et al. 2013). Fibroblasts derived from α_v -null mice following *Pdgfb*-Cre-mediated gene deletion have an impaired ability to bind and activate TGF- β 1, and these mice show reduced evidence of TGF- β signaling in models of pathologic tissue fibrosis. The cells targeted by *Pdgfb*-Cre do not express the $\alpha_v\beta_6$ integrin, but express $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_v\beta_8$. However, global deletion of $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$, by specific deletion of their β chains, has no effect on induced tissue fibrosis, and

conditional deletion of $\alpha_v\beta_8$ in the same cells has no effect on liver fibrosis. These results suggest that one or more α_v integrins, in addition to $\alpha_v\beta_8$ and $\alpha_v\beta_6$, can contribute to pathologic TGF- β activation in vivo. Accordingly, a small molecule inhibitor of the $\alpha_v\beta_1$ integrin that has minimal effects on other RGD-binding integrins was shown to inhibit TGF- β activation by primary lung and liver fibroblasts (Reed et al. 2015). Continuous subcutaneous administration of this inhibitor during the late phases of bleomycin-induced pulmonary fibrosis or carbon tetrachloride (CCl₄)-induced hepatic fibrosis results in protection that is similar to that by a small molecule inhibitor of all α_v integrins (Henderson et al. 2013). These data suggest that the fibroblast integrin responsible for TGF- β activation and progression of lung and liver fibrosis is $\alpha_v\beta_1$.

ROLE OF MECHANICAL FORCE IN INTEGRIN-MEDIATED TGF- β ACTIVATION

In the original description of $\alpha_v\beta_6$ -mediated TGF- β activation, it was shown that sequences within the β_6 subunit cytoplasmic domain required to link the integrin to the actin cytoskeleton were essential for TGF- β activation (Munger et al. 1999). Activation was also completely inhibited by cytochalasin D, an inhibitor of actin polymerization. These observations provided the first evidence that integrin-mediated TGF- β activation requires linkage to and active re-organization of the actin cytoskeleton. Subsequently, cell culture studies showed that contractile fibroblasts can use integrins to activate latent TGF- β , and that this process depends on actin-myosin interaction and generation of mechanical force (Wipff et al. 2007). Similar requirements were shown for $\alpha_v\beta_6$ -mediated TGF- β activation by epithelial cells (Giacomini et al. 2012), although the role, if any, of mechanical force in TGF- β activation by $\alpha_v\beta_8$ is less clear (Mu et al. 2002). $\alpha_v\beta_6$ -mediated TGF- β 1 activation was also shown to require covalent disulfide linkage of LTBP-1 to TGF- β 1 LAP (Annes et al. 2004). The effect of deletion of *Ltbp1* could be rescued by expression of a short fusion protein com-

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posed of the region containing the cysteine residues responsible for disulfide linkage of LTBP-1 to LAP and the region required for direct binding to fibronectin in the ECM. These observations suggest that the requirement for LTBP-1 is explained by its role in physically tethering latent TGF- β .

The important role of mechanical force in integrin-mediated TGF- β activation fits very well with the crystal structures of latent TGF- β 1 and of cocrystals of the $\alpha_v\beta_6$ integrin with TGF- β 1 LAP (Shi et al. 2011; Dong et al. 2014, 2017; Hinck et al. 2016). The latent TGF- β 1 structure showed a 180° axis between the exposed loop in LAP containing the integrin binding RGD sequence and the cysteine residues that form the disulfide linkage to LTBP-1, identifying how the force of cellular contraction could be transmitted through $\alpha_v\beta_6$ to deform physically constrained latent TGF- β . Furthermore, a single unstructured loop, called the latency lasso, was shown to make all of the critical contacts with active TGF- β within the latent complex and to be the likely weakest link that would be unfolded by mechanical force, thereby releasing free TGF- β from the complex. The $\alpha_v\beta_6$ structure identified critical residues, especially a hydrophobic pocket that provide a docking site for hydrophobic residues adjacent to the RGD sequence in TGF- β 1 and - β 3 LAP that help to explain the unusually high affinity of the interaction between $\alpha_v\beta_6$ and LAP.

To exert physical force on tethered latent TGF- β , cells must themselves be firmly attached to a relatively stiff substrate. Indeed, one of the principal methods used to show a requirement for physical force in integrin-mediated TGF- β activation has been plating cells on polyacrylamide gels of varying stiffness, and showing that cells plated on more deformable substrates have progressively impaired ability to activate TGF- β as the substrate deformability increases (Wipff et al. 2007; Giacomini et al. 2012). Integrins are one of the main families of receptors cells used to adhere tightly to components of the ECM, so it seems likely that inhibitors targeting integrins could inhibit TGF- β activation, even if the inhibited integrins do not directly bind tightly to TGF- β LAP. For example, a blocking antibody

targeting the $\alpha_v\beta_3$ integrin can inhibit TGF- β activation by cultured renal fibroblasts but has no effect on adhesion of these cells to LAP. Rather, this inhibitory effect is completely dependent on the substrate on which these cells are plated, with marked inhibition when the cells are plated on the $\alpha_v\beta_3$ ligand or vitronectin, but no inhibition when the cells are plated on an irrelevant ligand, collagen 1 (Chang et al. 2017). Thus, integrin interactions with the ECM are intricately involved in how the ECM impacts TGF- β 1 activation.

TGF- β 1 SIGNALING AS A DRIVER OF COLLAGEN ACCUMULATION

TGF- β 1 signals through its specific heterotetrameric receptor complex comprised of a pair of T β RI receptors that phosphorylate the receptor activated-Smads (R-Smads), termed Smad2 and Smad3, and a pair of T β RII, which contain the initial binding site for TGF- β 1. These receptors are expressed on most cells in culture and their roles in TGF- β signaling have been discussed elsewhere (Feng and Derynck 2005; Heldin and Moustakas 2016). Cell-specific gene deletion by Cre-mediated recombination of either *Tgfb1* or *Tgfb2* revealed that TGF- β 1 signaling, rather than TGF- β 1 expression, in both epithelial cells and mesenchymal cells is required for bleomycin-induced lung fibrosis (Huang et al. 2002).

These findings indicate that TGF- β 1-mediated signaling in both epithelial and fibroblastic cells is a common and critical feature of fibrogenesis. These findings also support the concept that TGF- β 1 mediates fibrosis through multiple cell types and multiple interacting signaling pathways. The latter include not only coregulators of R-Smad-dependent transcription of TGF- β 1 target genes such as *AP-1* and *Egr-1*, specific negative regulators of R-Smad function such as Smad7, but also early response genes such as *Nox4* and *Tgfb1* itself further promote TGF- β 1 signaling (Derynck and Zhang 2003; Feng and Derynck 2005). An altered profile of microRNAs (miRNAs) induced or suppressed in response to TGF- β 1 also acts to stabilize translation of mesenchymal genes important

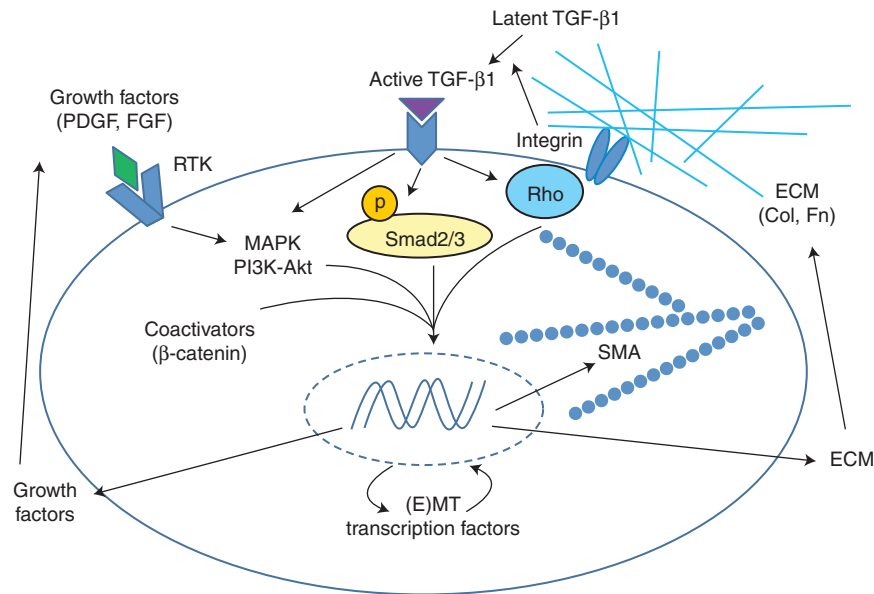


Figure 1. Epithelial and mesenchymal cell fibrogenic activation by TGF- β 1: costimulation by other signaling inputs and opportunities for feed-forward activation. Latent TGF- β is activated by α_v integrins. Active TGF- β binds to its receptors leading to activation of canonical Smad signaling and Smad-independent signaling pathways, including MAPK pathways and small GTPases such as RhoA. Inputs from other signaling pathways converge on these pathways to regulate the TGF- β response. These are activated by other growth factors, such as PDGF, which generally signal through RTKs and through MAPK, integrin-mediated mechanotransduction mediated by Rho family GTPases, and coactivators such as β -catenin. These signaling factors lead to expression of profibrotic genes such as those encoding α -SMA, ECM proteins, and secreted cytokines and growth factors, which further modify the fibrogenic effector cell response. The expression of transcription factors involved in epithelial or mesenchymal cell transition into an activated state is also induced. PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; RTK, receptor tyrosine kinase; MAPK, mitogen-activated protein kinase; (E)MT, (epithelial-) mesenchymal transition; Col, collagen; Fn, fibronectin; SMA, α -smooth muscle actin; ECM, extracellular matrix.

to fibrosis and minimize antifibrotic pathways (Bowen et al. 2013). Finally, numerous other signaling pathways provide input that allows integration of Smad and non-Smad signaling immediately downstream from TGF- β receptor activation with contextual cues from the micro-environment that favor expansion of the ECM (Fig. 1). Integrins, in addition to mediating latent TGF- β 1 activation, activate prosurvival and adhesive signals as a function of ECM composition and stiffness. Receptor tyrosine kinases respond to ligands after injury and promote proliferation and survival of fibroblasts. Hypoxia, acting through hypoxia inducible factors (HIFs), promotes epithelial-mesenchymal transition (EMT) and/or mesenchymal expan-

sion with enhanced collagen expression (Falanga et al. 2002; Zhou et al. 2009; Chapman 2010; Kottmann et al. 2012). Several morphogen pathways, especially those induced by Wnt and Notch ligands, are activated in response to injury and have been implicated in promoting tissue fibrosis in multiple organs. The elaborate cellular response of stromal cells within injured tissues, directed in part by the influx of inflammatory cells, appears heavily oriented toward transient ECM accumulation accompanied by proliferation and migration of epithelial and stromal elements, followed by stabilization of the collagenous components of the ECM, and finally fibroblast apoptosis and removal of excess collagen to restore normal architecture

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(Beers and Morrisey 2011). TGF- β 1 is a key cytokine participating in virtually all elements of the early tissue response to injury.

Collagens are comprised of three polypeptide chains highly organized into a triple helical conformation. Collagen monomers are not very soluble and, as discussed below, numerous post-translational modifications are necessary for proper procollagen folding and secretion. There are 28 collagen proteins in mammals, the most abundant being collagen I, a major component of bone, most musculoskeletal structures, eye, lung, and the vasculature (Hulmes 2008). Collagen I belongs to the group of fibrillar collagens, including collagens I, II, III, and VI, that are largely triple helical structures and thus become organized into fibrils with very defined “packing” dependent on extensive covalent cross-linking of carboxyl terminus to amino terminus of adjacent collagen monomers. This elaborate organization results in high tensile strength for the collagen fibrils. This is in contrast to the much less extensively organized nonfibrillar collagens, such as collagen IV that is the major basement membrane collagen. After injury, collagens I and III are the dominant collagens whose expression is induced to restore tensile strength and tissue integrity. TGF- β 1 is a required cytokine for the induction of these collagens after tissue injury.

The intricate nature of TGF- β 1 signaling in fibrogenesis is highlighted by the mechanisms engaged in expression and tissue accumulation of type I collagen. Type I procollagen is comprised of two polypeptide chains, pro- α 1 and pro- α 2, assembled in a 2:1 ratio (2 α 1/ α 2) leading to a heterotrimeric three-dimensional helical structure (Fig. 2). Following activation of latent TGF- β 1 and TGF- β receptor engagement, initial transcription of *COL1A2* gene (encoding type I collagen α 2) is dependent on the nuclear translocation of Smad3 and Smad4, the DNA-binding transcription factors Sp1 and AP-1 (c-Jun/c-Fos), as well as CREB-binding protein (CBP) or p300 that act as coactivators and histone acetylases to facilitate chromatin relaxation and access of the transcription factors to the *COL1A2* promoter (Zhang et al. 1998; Ghosh et al. 2000). The appearance of AP-1 in

the nucleus in turn depends on activation of Smad-independent Erk or c-Jun amino-terminal kinase (JNK) mitogen-activated protein kinase (MAPK) signaling pathways by TGF- β 1. Similar mechanisms operate to induce an early response of *Egr-1* that has been shown to be critical for collagen I expression by fibroblasts in response to TGF- β 1 (Chen et al. 2006). Numerous other factors coordinate with Smad2/3 signaling in the nucleus to promote *COL1A1* and *COL1A2* transcription. Once transcribed, the translation of collagen I mRNAs is also heavily regulated, in no small part by TGF- β 1-induced repression of the expression of miRNAs known to target collagen mRNAs or mRNA for proteins required for collagen expression (Bowen et al. 2013). miR-29, for example, is known to repress the expression of heat shock protein 47 (HSP47) and lysyl oxidase like-2 (LOXL2), which both participate in the highly organized process of stable collagen accumulation in the ECM (discussed below). The expression of the miR-29 family is down-regulated in response to TGF- β 1 in fibroblasts, and this repression corresponds to increased fibroblast collagen expression. Mice treated with miR-29 mimics have attenuated lung fibrotic responses to bleomycin (Yang et al. 2013). miR-96 and miR-130b also attenuate collagen accumulation and their expression is repressed in response to TGF- β 1. Similarly, miR-326 has been shown to attenuate TGF- β 1 translation (Das et al. 2014). Levels of miR-326 were found to be low in the bleomycin mouse model and in lungs of patients with idiopathic pulmonary fibrosis (IPF). Additionally, mice treated intranasally with a miR-326 mimic have attenuated fibrosis in this model. Collectively, the changes in miRNA expression by TGF- β 1 signaling act to stabilize collagen protein expression, secretion, and stabilization in the ECM (Fig. 2).

Actual assembly and cellular trafficking of collagen I polypeptides through the cell secretory machinery is problematic because of the large size and tenuous solubility of the collagen chains. Extensive posttranslational modifications are required for the proper assembly of procollagens into very well ordered helical structures early in the secretory process (Fig. 2)

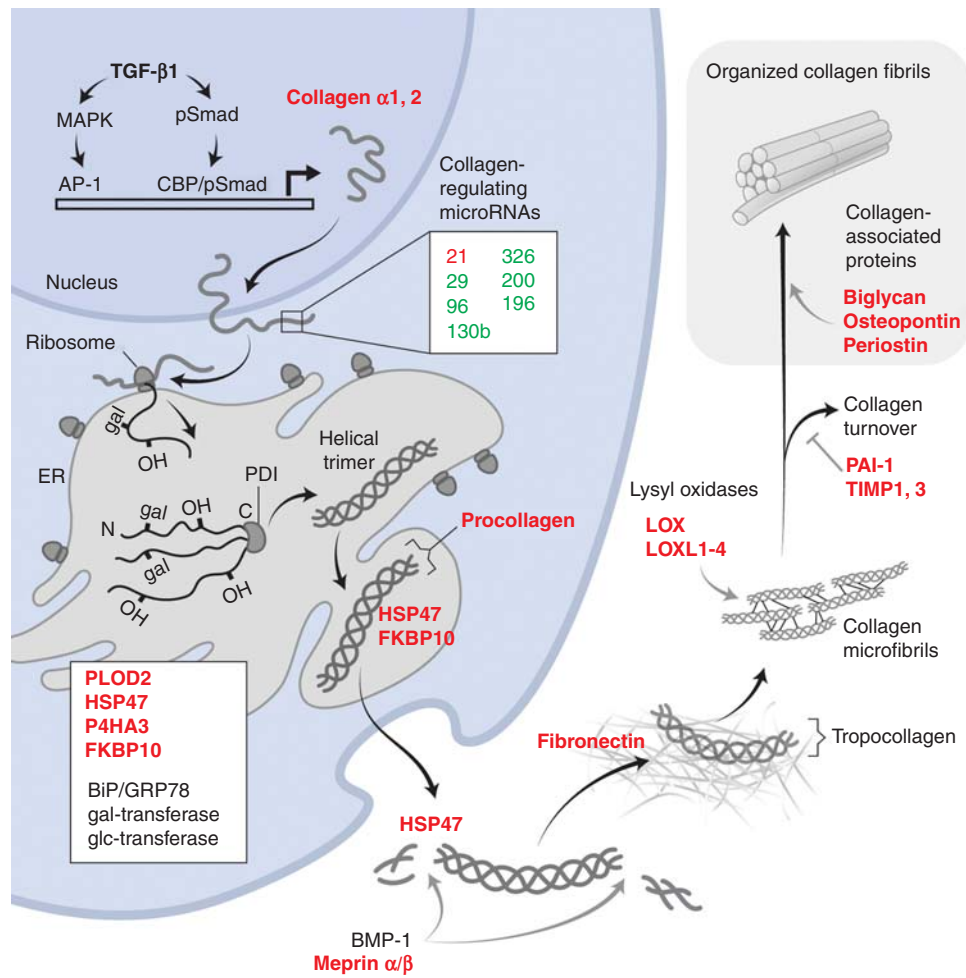


Figure 2. TGF- β 1-induced collagen expression program. The schematic illustrates the proteins and microRNAs that are coordinately regulated by TGF- β 1 to effect tissue collagen accumulation. Proteins whose expression is induced by TGF- β 1 are highlighted in red, indicating that the expression of more than a dozen proteins is induced along with that of collagen and is required for substantial collagen expression and tissue accumulation. These proteins act at nearly every stage of collagen processing, from posttranslational proline and lysine hydroxylations by procollagen-lysine 2-oxoglutarate 5-deoxygenase (PLOD2) and prolyl-4-hydroxylase (P4HA3), and glycosylation in the endoplasmic reticulum (ER), to required chaperones (HSP47 and FKBP10) to sustain the trimeric collagen structure and prevent premature fibril formation during passage through the secretory machinery. Additionally, constitutively expressed proteins, such as protein disulfide isomerase (PDI), and quality control sensors, such as BiP/GRP78, are also important for collagen folding and subsequent expression. Once secreted, procollagen is proteolytically processed to generate tropocollagen that then spontaneously begins to self-associate into microfibrils. The final physical form and extent of stable, matrix fibrillar collagens depend heavily on additional TGF- β 1-induced proteins such as fibronectin, the lysyl oxidases, and inhibitors of collagen turnover, plasminogen activator inhibitor 1 (PAI-1) and tissue inhibitor of metalloproteinases 1 and 3 (TIMP1, TIMP3). Finally, proteins such as biglycan and periostin associate with mature fibrils and control packing and organization. Collectively, this reprogramming of cells can be considered as the TGF- β 1-induced collagen expression program.

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(Hulmes et al. 2002; Bourhis et al. 2012; Ishikawa and Bachinger 2013). The primary amino acid sequences of fibrillar collagens underlie the propensity of these collagens to form a helical structure, with approximately 300 repeats of Gly-X-Y motifs providing sufficient glycines for tight chain folding. HSP47, whose expression is strongly induced in response to TGF- β 1 in nearly all cells that express collagen I, acts as a key chaperone in collagen assembly, binding to collagen and promoting stability of both the unfolded collagen chains in the endoplasmic reticulum (ER) and the more assembled structures trafficking through the Golgi. Assembly into a properly folded procollagen also requires many distinct modifications. Immediately after synthesis in the rough ER, and possibly bound to its inner membrane, the unfolded collagen chains begin to undergo lysine and proline hydroxylations. The two key enzymes in this process are procollagen-lysine 2-oxoglutarate 5-deoxygenase (PLOD2 or LH2) and prolyl-4-hydroxylase (P4HA3). The expression of both enzymes is strongly induced in response to TGF- β 1. A variable fraction of the hydroxy-lysines are then glycosylated, and the remainder is further processed to very stable collagen cross-links once collagen is secreted. Like glycines, the hydroxyprolines are required for tight helical folding as the three collagen chains begin to assemble. The modified collagen chains are also substrate for a constitutively expressed protein disulfide isomerase (PDI) that mediates disulfide formation in the carboxy-terminal region. This is thought to place the chains in “register” for subsequent helical folding, beginning at the carboxyl terminus and proceeding toward the amino terminus, and full assembly of the procollagen molecule. Once the ordered, helical procollagen molecule assembles, there is a critical need for additional proteins to chaperone the trafficking of procollagen through the Golgi and secretory process without denaturation or premature fibril formation. HSP47 and a FK506 binding protein 10 (FKBP10) serve these functions. Their expression is strongly induced by TGF- β 1 in fibroblasts (Ishida and Nagata 2011; Staab-Weijnitz et al. 2015).

Collagen is finally secreted as a procollagen with the extended carboxy- and amino-termi-

nal nonhelical peptide chains (carboxy- and amino-terminal telopeptide extensions) that are then removed by proteolysis to generate the helical structure, tropocollagen (Fig. 2). A number of proteases appear to be capable of amino- and carboxy-terminal processing of procollagen and it is unclear if these are induced by TGF- β . The earliest described carboxy-terminal processing protease is bone morphogenetic protein-1 (BMP-1), a metalloproteinase that is critical to collagen maturation in bone (Li et al. 1996; Prockop et al. 1998; Asharani et al. 2012). Recently, meprin α and β metalloproteinases were shown to cleave both the amino- and carboxy-terminal peptides of procollagen, leading to fibril formation (Prox et al. 2015). These meprin levels are high at sites of collagen accumulation and fibrosis (Broder et al. 2013). Meprin expression is induced by pathways that lead to activation of nuclear AP-1, and thus may be induced by TGF- β 1 (Biasin et al. 2014). Tropocollagen is thought to spontaneously form a polymeric collagen fibril, but collagen stabilization as a fibril (containing hundreds of tropocollagen molecules) requires lysyl (or hydroxyl-lysyl) oxidation to an aldehyde that is both necessary and sufficient for irreversible collagen cross-linking. Lysyl oxidases, a set of key enzymes that mediate collagen cross-linking, are all strongly induced by both TGF- β 1 and HIF1 α (Erler et al. 2006; Blaauboer et al. 2013). In addition to supporting fibril assembly, TGF- β 1 concurrently induces the expression of protease inhibitors such as plasminogen activator inhibitor 1 (PAI-1) and tissue inhibitor of metalloproteinase 3 (TIMP3) that attenuate the breakdown of newly deposited tropocollagens that are vulnerable to proteases. The pattern of deposition of collagen is strikingly similar, and follows temporally that of fibronectin secretion, a prominent mesenchymal protein that is induced by TGF- β 1 signaling (Hernnas et al. 1992; Muro et al. 2008). Extracellular fibronectin assembly, itself a complicated and integrin-dependent process, provides the suitable extracellular fibrillar substrate for organized deposition of collagen fibrils. The further organization of fibrils, depending perhaps on the needed tensile strength, is promot-

ed by yet additional TGF- β 1 target genes that encode collagen-interacting proteins such as osteopontin, periostin, and the proteoglycan biglycan (Oku et al. 2008; Farhat et al. 2012). Transglutaminase and nonenzymatic interactions among these proteins are thought to further enhance tissue stiffness over time (Cox and Erler 2011).

The evolutionary expansion of collagens from early metazoans to mammals has been accompanied by expansion of the family of monoamine oxidases, lysyl oxidases, needed to cross-link and stabilize ECM collagen (Kagan and Trackman 1991). The parent enzyme, LOX, is critically important to normal musculoskeletal development as deletion of LOX in mice is perinatal lethal with aortic aneurysms, defective skeletal structures, and defective airway development (Trackman 2005). In addition to LOX, there are four LOX-like enzymes (LOXL1–4) that share highly conserved catalytic domains but also are comprised of additional protein interaction domains. LOX and LOXL1 are closely related and expressed in a similar pattern. LOXL2, 3, and 4 have similar structures and, among these, LOXL2 is the most studied and widely expressed, although primary lung fibroblasts in culture express all of the LOX-like enzymes at variable levels. The expression of LOXL2 is strongly induced by TGF- β 1 and hypoxia, and is reported to not only function extracellularly in collagen cross-linking but also intracellularly to promote Snail1 protein accumulation. Because of the strong positive impact of Snail1 on the expression of fibrillar collagens, the induction of LOXL2 expression in response to TGF- β 1 can be viewed as a positive feedback loop to support ECM expansion after injury. Thus, LOXL2 levels are strongly linked to stable collagen accumulation, ECM stiffness, and fibrosis progression (Barry-Hamilton et al. 2010; Adamali and Maher 2012).

As might be predicted from the elaborate requirement for multiple posttranslational modifications to support fibrillar collagen folding and solubility in the ER, high-level expression of collagen renders the cell susceptible to ER stress from misfolded protein. Accordingly, TGF- β 1 induces the expression of proteins

that are linked to protection from the unfolded protein response (UPR) (e.g., BiP/GRP78, and HSP47) in parallel to expression of fibrillar collagens and modifiers of its primary structure (Fig. 2). Nonetheless, it has been reported that inositol-requiring enzyme-1 α (IRE-1 α), a key mediator of the UPR, is activated after TGF- β 1-induced collagen expression in fibroblasts and in fibroblasts from patients with fibrotic disease (Heindryckx et al. 2016). The investigators implicated both increased X-box binding protein 1 (XBP-1) signaling, resulting in ER expansion and cleavage of miR-150 by the RNAase activity of activated IRE-1 in altering collagen expression, and fibrogenesis. Elevated miR-150 levels have been linked to protection from fibrosis (Honda et al. 2013). Hence, somewhat paradoxically, the activation of IRE-1, indicative of an activated UPR, promotes rather than suppresses collagen expression. Inhibition of IRE-1 in cell culture and in vivo attenuates the fibrotic response to TGF- β 1 in liver and skin fibrosis models. These findings underscore the potential pathological operation of the UPR in the context of ER stress with a negative impact on tissue integrity (Fig. 3). This conclusion is supported by the findings that mutations in surfactant protein (SP) A2, a cause of familial pulmonary fibrosis, leads to type II alveolar epithelial cell ER stress accompanied by increased TGF- β 1 secretion (Wang et al. 2009).

Collectively, it is clear that TGF- β 1 drives a large, multifaceted reprogramming of both epithelial and mesenchymal cells to favor expression and extracellular accumulation of matrix proteins that provide rapid restoration of tissue integrity in the setting of injury and/or disruption of tissue barriers (Fig. 2). The phylogenetic origin of fibrillar collagen, and its complicated assembly process, dates to the earliest metazoans and has been highly conserved and further amplified for increased structural strength throughout the vertebrate family. The importance of collagen and numerous other ECM proteins in the injury response is highlighted not only by the highly coordinated induction by TGF- β 1 of a large set of ECM structural proteins but also the parallel induction of numerous enzymes and chaperones that promote proper folding, secre-

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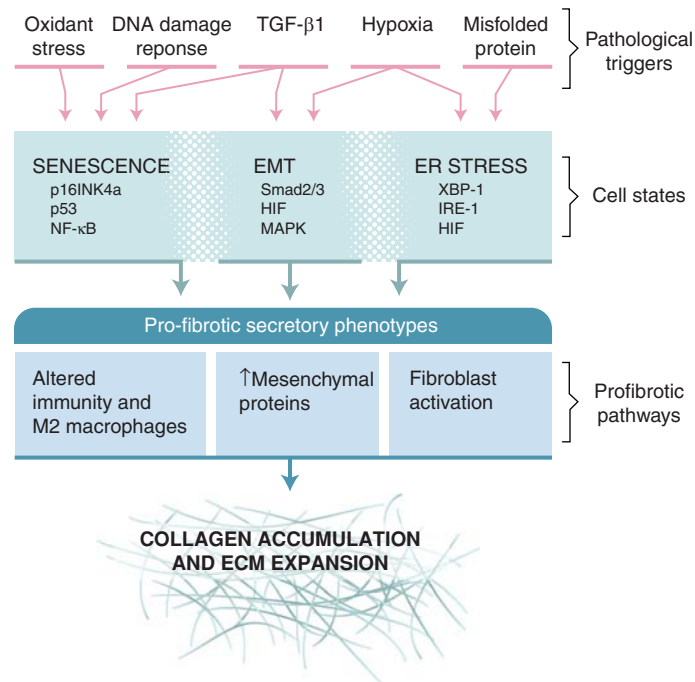


Figure 3. Diverse pathways of epithelial responses to stress converge on profibrotic secretory phenotypes supporting extracellular matrix (ECM) expansion. The figure highlights three major epithelial states resulting from different pathological triggers: epithelial–mesenchymal transition (EMT), senescence, and endoplasmic reticulum (ER) stress with unfolded protein responses (UPRs). Each epithelial state has its characteristic transcriptional drivers. All of these states elicit secretory responses that share the potential to promote collagen accumulation. Distinctions among the secretory responses are still not completely defined and represent opportunities to better understand the connections between epithelial and mesenchymal biology in the context of cell stress. As discussed, TGF- β 1 is a major driver of fibroblast activation and collagen secretion. Like epithelial cells, fibroblasts also respond to many of the same pathological triggers with activation of senescent or UPR signaling pathways, potentially further promoting fibrosis.

tion, and extracellular stabilization of collagen as well as down-regulation of miRNAs that specifically attenuate collagen expression and accumulation. It is hardly surprising then that such a robust and complicated cellular response system, driven after injury largely by a single master regulator, is subject to breakdown and disordered function leading to disease.

CONTRIBUTIONS OF INTEGRINS AND TGF- β 1 TO TISSUE STIFFNESS ON PROGRESSION OF FIBROSIS

It is now abundantly clear that tissue stiffness can exert profound effects on cell differentiation and behavior. For example, mesenchymal stro-

mal cells differentiate into fat cells, muscle cells, or bone cells when they are plated on substrates with stiffness mimicking that normally found in fat, muscle, or bone, respectively (Engler et al. 2006). Stiffness also profoundly affects the behavior of tissue fibroblasts, with increased stiffness favoring increased collagen production and expression of contractile proteins such as α -smooth muscle actin (SMA), and decreased stiffness favoring a more quiescent and noncontractile phenotype (Liu et al. 2010). As a consequence, the process of tissue fibrosis, which locally increases tissue stiffness, participates in a feed-forward loop, where the presence of local fibrosis increases fibroblast collagen production and force generation, further accelerating fibro-

sis. One of the mechanisms underlying this feed-forward loop is integrin-mediated TGF- β activation, because, as noted above, this process depends on cell contraction and is enhanced when cells are tethered to a stiff substrate. However, increased TGF- β activation does not fully explain this feed-forward loop. For example, tissue stiffness increases the sensitivity of cells to already activated TGF- β (Liu et al. 2010).

EPITHELIAL–MESENCHYMAL TRANSITION

An important potential contribution of EMT to tissue fibrosis was first proposed as early as the mid 1990s (Strutz et al. 1995). More insight has emerged with greater usage of fibrotic human tissue samples for biomedical research, the development of novel techniques for genetic modifications to mice, and a greater understanding of some of the prominent EMT signaling pathways (Kalluri and Neilson 2003; Teng et al. 2007; Kalluri and Weinberg 2009; Thiery et al. 2009; Flier et al. 2010; Lamouille et al. 2014). However, rather than establishing a definite role for EMT during fibrosis, these advances have led to increased controversy regarding the role or even the occurrence of EMT during fibrosis. Seemingly solid evidence both supports and refutes EMT as having a major contribution to fibrosis. Some of the controversies regarding EMT point to broader uncertainties and controversies in the fibrosis field at large.

Definition of EMT

Epithelial cells are characterized by a relatively sessile state with a defined shape (e.g., cuboidal, columnar, or squamous) forming tight cell–cell contacts, which establish an epithelial barrier that is important for the function of many organs. Epithelial cells have an apical–basal polarity and often produce factors that are secreted from the apical surface into a luminal space. In contrast, the mesenchymal cell phenotype provides front–rear polarity and enables migratory behavior. The cell–cell contacts are often transient and the cell shape is more dynamic and less defined. EMT can thus be defined as a process in which epithelial cells

lose epithelial cell–cell contacts, undergo cytoskeletal changes and change polarity from apical–basal to anterior–posterior, all favoring acquisition of migration and invasion. These changes are accompanied by changes in gene and protein expression, including proteins that are involved in cell contact (e.g., loss of E-cadherin and gain of N-cadherin) and cytoskeletal proteins (e.g., loss of cytokeratin and gain of vimentin and SMA). There is also loss of proteins that are normally secreted into the luminal space (e.g., SPs, hormones) in favor of proteases and ECM proteins that promote migration and invasion. EMT can therefore be identified and defined by a set of biomarkers, reflecting loss of epithelial and gain of mesenchymal proteins (Zeisberg and Neilson 2009). EMT occurs during and is required for normal embryonic development. Roles for EMT during carcinogenesis and fibrosis have been suggested, but remain subjects of discussion and further research (Cardiff 2005; Tarin et al. 2005; Zeisberg and Duffield 2010). EMT during these different scenarios has overlapping features but also important differences leading some to classify EMT into type I (development and regeneration), type II (fibrosis) and type III (cancer) EMT, dependent on the physiological and pathological context (Kalluri and Weinberg 2009; Zeisberg and Neilson 2009). The transition of endothelial or endodermal cells into a mesenchymal phenotype has been labeled endothelial–mesenchymal transition (EndMT), but is often categorized within EMT, as these processes are similarly regulated and have many overlapping characteristics (Teng et al. 2007; Kokudo et al. 2008; Kovacic et al. 2012). EMT may simply represent a tunable, stereotypical epithelial response to the demand for dynamic cellular changes, as might occur during embryonic development, response to injury, and carcinogenesis. Frequently, the acquisition of such dynamic changes in epithelial behavior is reversible.

EMT Pathways

Features of EMT can be induced by many ligand–cell surface receptor interactions. TGF- β

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is the most extensively studied inducer of EMT. TGF- β binding to its receptors, T β RI and T β RII, activates Smad-dependent and Smad-independent pathways that can directly activate mesenchymal gene and suppress epithelial gene expression (Derynck et al. 2014; Lamouille et al. 2014). TGF- β –Smad signaling can also activate EMT through induction of the expression of several mesenchymal transcription factors, such as Snail1 and Twist, and these are therefore often referred to as EMT transcription factors. The TGF- β pathway intersects with a number of other pathways that may augment or attenuate the EMT response (Derynck et al. 2014). Hepatocyte growth factor (HGF), initially described as a “scatter factor,” and fibroblast growth factor (FGF) were among the earliest examples of a single factor capable of inducing EMT in cell culture (Stoker and Perryman 1985; Valles et al. 1990; Nusrat et al. 1994). Subsequently, many other factors have been shown to induce aspects of EMT through activation of cell surface receptors that ultimately lead to transcriptional changes. Among the better studied ligand–receptor combinations are Wnt factors that act through Frizzled receptors, which leads to stabilization of β -catenin, Sonic Hedgehog (Shh) ligand that acts through Patched receptors, which leads to Gli activation, and the Delta–Notch combination, which leads to cleavage and activation of the intracellular Notch domain. For example, β -catenin has emerged as an important Smad coactivator, so in addition to activating its own target genes through interactions with T-cell factor (TCF) or lymphoid enhancer-binding factor (LEF), β -catenin can regulate the EMT response through cooperation with the Smad complex. Among the most prominent Smad-independent EMT pathways is activation of Rho family GTPases, including RhoA, Rac1, and Cdc42. Rho GTPases are important regulators of cell morphology, adhesion, and movement. TGF- β signaling through the Rho GTPases allows convergence of signaling inputs from the ECM and cytoskeleton to control the EMT response to TGF- β , and allows TGF- β to coordinate transcriptional expression changes with cytoskeletal changes involved in mesenchymal tran-

sition. Activation of these GTPases leads to loss of adherens junction complexes, breakdown of the apical–basal polarity, and cytoskeletal rearrangement. Activation of small GTPases also promotes transcriptional changes through factors such as myocardin-related transcription factor (MRTF). Inhibition of several mediators of the Rho GTPase signaling pathway, including Rho kinase and MRTE, in genetically modified mice or with chemical inhibitors has been shown to attenuate EMT and fibrosis (Theriault et al. 2007; Bendris et al. 2012; Harris et al. 2013; Okada et al. 2015; Sisson et al. 2015). Other prominent pathways activated by TGF- β are the MAP kinase pathways and the phosphoinositide-3 kinase (PI3K)–Akt pathway. These pathways again allow for interaction and input from a number of different factors, such as FGF, epidermal growth factor (EGF), and tumor necrosis factor (TNF)- α , and correspondingly activated receptors. The extensive cross-talk between intracellular pathways directly activated by TGF- β and secondary pathways that modulate impact of proximal TGF- β signals on gene expression is discussed extensively in other reviews (Derynck et al. 2014).

EMT in Development

The role of EMT during development is discussed at length in many other reviews (Thiery et al. 2009), but is briefly mentioned here to provide context to studies exploring the potential role of EMT in fibrosis. Embryonic development has been proposed to involve multiple different EMT events that have been extensively studied (Acloque et al. 2009). EMT occurs early in embryogenesis during gastrulation, as epiblasts migrate to form the primitive streak, move to the interior of the blastocyte, and form the mesodermal germ layer (Nieto et al. 1994; Viebahn 1995; Viebahn et al. 1995; Nieto 2011). Later, a subset of neural tube cells detach and undergo EMT to form the neural crest cells that migrate and eventually give rise to many structures of mesenchymal origin (Duband et al. 1995; Sauka-Spengler and Bronner-Fraser 2008). EMT is also prominent at later stages of embryogenesis during organogenesis. Among



the most studied are cardiac valve formation (Nakajima et al. 2000), palate closure (Fitchett and Hay 1989; Griffith and Hay 1992), and pancreatic development. During organogenesis, EMT is often followed by a reversal of EMT or mesenchymal–epithelial transition (MET), highlighting the plasticity of cells during development (Davies 1996; Li et al. 2014). For example, a subset of cells from the developing pancreatic bud undergo EMT, dissociate, and migrate into the interstitium where they undergo MET to form the islets of Langerhans (Johansson and Grapin-Botton 2002). More durable or permanent EMT also occurs during organogenesis in later embryonic development. Recent studies of lung and gut development suggest that serosal mesothelial cells, which are squamous epithelial cells, are a major source of vascular smooth muscle cells, which are mesenchymal cells (Wilm et al. 2005; Dixit et al. 2013). Notably, there is no common epithelial cell progenitor during embryogenesis, as epithelial cells are derived from all three embryonic primordial germ layers. For example, lung airway and alveolar epithelial cells are derived from the endoderm, renal tubular epithelial cells are derived from mesoderm, and epidermal skin cells are derived from the ectoderm. Similarly, mesenchymal cells can derive from ectoderm (e.g., facial cartilage) and mesoderm (e.g., skeletal muscle) (Acloque et al. 2009; Thiery et al. 2009).

EMT in Fibrosis

The rationale for considering EMT during fibrosis arose from similarities in prominent EMT and fibrotic signaling pathways. TGF- β is the best defined profibrotic factor and, as mentioned above, a prominent inducer of EMT (Bartram and Speer 2004). Identification of aberrant Wnt/ β -catenin signaling in IPF lung samples prompted interest in the possibility of EMT in lung fibrosis (Chilosi et al. 2003). Subsequently, almost every cytokine or intracellular signaling pathway that has been implicated in fibrosis or fibroblast activation has been shown to activate EMT in cell culture. Thus, the cytokine milieu in fibrotic tissue suggests a

pro-EMT stimulus. Given the abundance of epithelial cells in tissues like the lung and kidney, EMT of even a small fraction of epithelial cells could potentially account for a significant number of activated fibroblasts. Further, the proposed myofibroblast function may promote further activation of other fibroblasts through the production of profibrotic factors and induction of mechanical forces. Thus, even transient EMT could initiate a process that leads to more sustained and robust fibroblast responses. As with cancer, the identification of cells that have undergone EMT in human disease in vivo has been challenging. Many studies have relied on co-immunostaining for epithelial and mesenchymal markers. Identification of costaining cells is suggestive of EMT, but has inherent limitations (Kakugawa et al. 2005; Willis et al. 2005; Kim et al. 2006). The co-immunostaining approach is at best descriptive, potentially identifying expression of two or more markers within a cell but lacking an assessment of the functional contribution of that cell. Even if an epithelial cell expresses pro-collagen I or SMA, to what extent is it contributing to fibrogenesis? The co-immunostaining approach likely underrepresents EMT because most studies of EMT show simultaneous decrease in epithelial markers and up-regulation of mesenchymal markers. EMT cells in early transition may have weak expression of mesenchymal markers, and EMT cells in late transition may have weak expression of epithelial markers. Co-immunostaining is also beset by difficulties in achieving specificity of antibodies and by the potential for artifactual staining of overlapping cells giving the appearance of a costained cell. Microscopic approaches, such as confocal or deconvolution microscopy, have been used to overcome this limitation (Willis et al. 2005; Rock et al. 2011). To partially address these issues, several groups have isolated cells by flow cytometry to unequivocally show expression of mesenchymal proteins in epithelial cells and expression of epithelial cell proteins in fibroblasts (Larsson et al. 2008; Marmai et al. 2011). To more directly track EMT during fibrogenesis, the alternative approach of fate mapping individual cells has emerged.

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Cell Fate Mapping

The Cre-recombinase/lox P sequence system in mice is a powerful way to map and trace the fate of specific cell types in vivo (Akagi et al. 1997). The system involves a reporter gene (e.g., green fluorescent protein [GFP] or β -galactosidase) regulated by a strong constitutively active promoter but its expression is disrupted by a short sequence containing a stop codon. This sequence is flanked by loxP sites, enabling it to be permanently removed by Cre recombinase. A second transgene encodes the Cre recombinase under the control of a cell type-specific promoter, enabling cell type-specific removal of the stop codon sequence, and labeling those cells by expression of the reporter. Importantly, the labeling occurs at the level of the genomic DNA and is thus permanent. Thus, the Cre/lox system has been proposed to enable cell type-specific and permanent labeling of cells, making it an attractive way to map the fate of cells in response to injury, and to identify the origin of cells of interest, such as a myofibroblasts in fibrosis. A limitation of this approach is the potential for off-target expression of a constitutive Cre recombinase allele, either transiently during organ development or in response to new signals appearing after tissue injury. For example, a promoter specific to an adult epithelial cell may have been transiently expressed in a mesenchymal cell during organ develop, or only after tissue injury, resulting in spurious conclusions on the extent or even presence of EMT. To obviate this limitation, Cre recombinase alleles have been constructed that enable temporal as well as spatial control of Cre activity. Most early versions of this methodology used cell type-specific expression of a reverse tetracycline transactivator (rtTA) to regulate expression of Cre recombinase by a tetracyclin-response element (TRE), but this suffered from the requirement of three transgenes for a fully operational system and the problem that the rtTA function was not completely doxycycline-dependent. More recently, Cre recombinase activity has been temporally regulated through the use of a fusion protein comprised of Cre recombinase and a modified estrogen

receptor “knocked in” to a cell-specific promoter. The estrogen receptor has been further modified such that it is much more sensitive to tamoxifen than estrogen (e.g., ERT2) (Feil et al. 2009, 2014). Virtually all expressed Cre recombinase remains in the cytoplasm unless tamoxifen is present to empower nuclear translocation and DNA recombination. Various Cre-dependent strategies have been used extensively to map the fate of epithelial cells in fibrosis, but with conflicting outcomes. In lung fibrosis, at least four different groups have identified significant numbers of lung epithelial-derived cells that coexpress mesenchymal markers in models of fibrosis (Table 1) (Kim et al. 2006, 2009; Tanjore et al. 2009; DeMaio et al. 2012; Balli et al. 2013). These groups used different promoters to drive expression of Cre (Nkx2.1 and surfactant protein C [SPC]), different reporter genes (GFP/LacZ), different techniques to quantify colabeled cells (immunohistochemistry, single cell suspension with cell sorting, cytopsin staining, RNA quantification, and immunoblotting), and several models of fibrosis (bleomycin, overexpression of TGF- β , and radiation) (Kim et al. 2006, 2009; Balli et al. 2013). However, one report, also using the bleomycin model of fibrosis, found no evidence of EMT in mice using *Sftpc* promoter-driven CreERT2 expression to fate-map type II alveolar epithelial cells (Rock et al. 2011). Contradictory results on EMT cell fate mapping are not limited to lung fibrosis. Similar cell fate mapping studies in models of kidney and liver fibrosis found substantial evidence or lack of evidence of EMT. Contradictory results in cell fate mapping are not limited to EMT. Pericytes and fibrocytes, which have been proposed as sources of activated myofibroblasts, have cell fate mapping studies in favor of or against them serving as significant sources of myofibroblasts. For pericytes, the conflict is even more stark. For example, in the bleomycin model of lung fibrosis, one report concludes that more than two thirds of the myofibroblasts are derived from pericytes (Hung et al. 2013). In contrast, that same report concluded that there is no evidence of EMT and excludes pericytes and two epithelial cell populations at the origin of myofibroblasts

Table 1. Reports on fate mapping during fibrosis

Report	Fate mapping system	Model	EMT
Lung fibrosis			
Kim et al. 2006	<i>Sftpc</i> -rtTA/tetO-Cre/RosaLacZ	AdTGF- β	Yes
Kim et al. 2009	<i>Sftpc</i> -rtTA/tetO-Cre/ZEG	Bleo	Yes
Tanjore et al. 2009	<i>Sftpc</i> -Cre/RosaLacZ	Bleo	Yes
Hashimoto et al. 2010	<i>Tek</i> -Cre/RosaLacZ	Bleo	Yes
DeMaio et al. 2012	<i>Nkx2-1</i> -Cre/RosaLacZ	Bleo	Yes
Rock et al. 2011	<i>Sftpc</i> -CreER/dTomato	Bleo	No
Balli et al. 2013	<i>Sftpc</i> -rtTA/tetO-Cre/RosaLacZ	XRT	Yes
Kidney fibrosis			
Iwano et al. 2002	<i>Ggt1</i> -Cre/RosaLacZ	UUO	Yes
Zeisberg et al. 2008	<i>Tek</i> -Cre/RosaYFP	UUO	Yes
Humphreys et al. 2010	<i>Six2</i> -Cre/RosaLacZ	UUO	No
LeBleu et al. 2013	<i>Ggt1</i> -Cre/RosaLacZ	UUO	Yes
	<i>Cdh5</i> -Cre/RosaYFP	UUO	Yes
Gastrointestinal and liver fibrosis			
Zeisberg et al. 2007b	<i>Alb</i> -Cre/RosaLacZ	CCl ₄	Yes
Flier et al. 2010	<i>Vill1</i> -Cre/RosaLacZ	TNBS	Yes
Taura et al. 2010	<i>Alb</i> -Cre/RosaLacZ	CCl ₄	No
Chu et al. 2011	<i>Alb-Afp</i> -Cre/RosaYFP	CCl ₄	No
Cardiac infarct			
Zhou and Pu 2011	<i>Wt1</i> -CreER/tmGFP	Art ligation	Yes
Zeisberg et al. 2007a	<i>Tie1</i> -Cre/Rosa26	Ao banding	Yes
Pericyte \rightarrow Myofib			
Lung fibrosis			
Rock et al. 2011	<i>Cspg4</i> -CreER/fgGFP	Bleo	No
Hung et al. 2013	<i>Foxd1</i> -Cre/dTomato	Bleo	Yes
Kidney fibrosis			
Humphreys et al. 2010	<i>Foxd1</i> -Cre/Rosa26	UUO	Yes
LeBleu et al. 2013	<i>Cspg4</i> -Cre/RosaYFP	UUO	No
	<i>Pdgfrb</i> -Cre/RosaYFP	UUO	No
BM \rightarrow Myofib			
Lung fibrosis			
Hashimoto et al. 2004	GFP BMT	Bleo	No
Schmidt et al. 2003	ex vivo labeling	OVA	Yes
Madala et al. 2014	GFP BMT	TGF- α	No
Kidney Fibrosis			
Iwano et al. 2002	<i>S100a4</i> -GFP BMT	UUO	Yes
LeBleu et al. 2013	<i>Acta2</i> -RFP BMT	UUO	Yes
Liver fibrosis			
Higashiyama et al. 2009	<i>Col1a2</i> -GFP BMT	CCl ₄	No
Kisseleva et al. 2006	<i>Col1a1</i> -GFP BMT	BDL	No

EMT, epithelial–mesenchymal transition; AdTGF- β , adenovirus encoding active TGF- β 1; bleo, bleomycin; XRT, X-ray treatment; OVA, ovalbumin; UUO, unilateral ureteral obstruction; TNBS, trinitrobenzene sulfonic acid; Pericyte \rightarrow myofib, pericyte to myofibroblast transition; BM \rightarrow myofib, bone marrow–derived cell to myofibroblast transition; art, arterial; Ao, aortic; BMT, bone marrow transplant; CCl₄, carbon tetrachloride; BDL, bile duct ligation; GFP, green fluorescent protein.



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(Rock et al. 2011). Thus, those favoring or opposing the possibility of EMT can point to cell fate-mapping publications supporting their conclusion.

Despite the promise of cell fate mapping, this approach has failed to resolve the controversy regarding the origin of activated myofibroblasts during fibrosis. This may result from some of the same limitations as referred to for the analysis of costained tissues. In addition, the Cre recombinase system has particular limitations. Cre-mediated recombination may be overly sensitive because of weak and transient activation of the cell-specific promoter in off-target cells leading to sufficient expression of Cre recombinase to permanently activate reporter gene expression, thus potentially confusing the cellular origin of the labeled cell (Vaughan et al. 2015). Additionally, tamoxifen, which is used to activate estrogen receptor fusion proteins, can persist in tissues or cells for much longer than previously appreciated (Reinert et al. 2012). Typical tamoxifen dosing and “chase” periods intended to allow loss of tamoxifen before injury may fail to eliminate functional tamoxifen levels during experimental injury and this may also result in spurious conclusions on the cells of origin of expanded populations after injury (Vaughan et al. 2015). Ultimately, new methods such as DNA barcoding will have to be applied to studies of cell fate to resolve these uncertainties. Even extremely accurate cell fate mapping may not reveal the functional contributions of subpopulations of epithelial or mesenchymal cells to fibrosis.

Definition of EMT Revisited

Much of the controversy regarding EMT in cancer, fibrosis, and even development stems from differences in the definition of EMT, and some have suggested the need to broaden beyond the original narrow definition of EMT (Nieto et al. 2016). On one extreme, epithelial cells might be defined, in part, as being derived from a parent epithelial cell, and mesenchymal cells as being derived from a parent mesenchymal cell. EMT is then defined as a process in which a fully differentiated epithelial cell loses all epithelial

cell characteristics and gains all mesenchymal characteristics. This definition virtually excludes the possibility of EMT that is seen in vivo. On the other extreme, EMT might be defined as any deviation from a classic epithelial cell phenotype and protein expression pattern toward any acquisition of mesenchymal cell traits or proteins. By this loose definition, type II alveolar epithelial cell differentiation to type I alveolar epithelial cell, or epithelial cell migration to re-epithelialize after injury might be regarded as EMT. Indeed, in some circumstances EMT transcription factors are activated in re-epithelialization after injury (Savagner et al. 2005) in a way that is reminiscent of developmental organogenesis, in which ductal epithelial cells undergo EMT, migrate and invade, and then revert back to an epithelial cell phenotype through MET. Whether this is EMT or simply a transient gain of migratory behavior with accompanying changes in a few proteins is a focus of debate. There is increasing use of the terms “partial” and “full” EMT. But most, if not all, transcriptional responses and other cellular processes are partial. Furthermore, there is increasing recognition of unique functions attributed to the hybrid epithelial–mesenchymal phenotype (Jolly et al. 2016). Defining EMT during fibrosis has relied on lists of epithelial and mesenchymal markers (Zeisberg and Neilson 2009), but it remains unclear at what threshold loss of epithelial markers or gain of mesenchymal markers would be sufficient to achieve at least partial EMT and what threshold is required for full EMT. An alternative approach is to focus on the function of the cells of different origin. This is a departure from defining epithelial and mesenchymal cells as different lineages, but rather a focus on cell phenotype and function.

Similar to the controversy on defining EMT, there is a controversy on the definition of myofibroblasts. Some have argued that expression of SMA itself is not sufficient or required to define a myofibroblast but rather requires stress fiber formation and a contractile phenotype. Given their heterogeneity, some have suggested that the term myofibroblast may better define a phenotype rather than a specific cell type (Hinze 2013). Furthermore, although myofibroblasts





are important fibrogenic effector cells, fibrosis is clearly a multicellular process. Also, myofibroblasts are an important source of type I collagen during fibrosis, but type I collagen expression is clearly not limited to myofibroblasts (Zhang et al. 1994). Furthermore, cell type-specific expression of a cytotoxic protein that can be activated, such as thymidine kinase or the diphtheria toxin receptor, enables deletion of specific cell types and shows that M2 macrophages and myofibroblasts are necessary for fibrogenesis (Duffield et al. 2005; LeBleu et al. 2013). Further evidence shows a critical role for M2 polarized macrophages during fibrosis (Gibbons et al. 2011; Osterholzer et al. 2013).

Deletion of the TGF- β receptor in fibroblasts, epithelial cells, or bone marrow cells results in abrogated fibrosis (Hoyles et al. 2011; Li et al. 2011; LeBleu et al. 2013). TGF- β clearly elicits a phenotypic response in many cell types that might be viewed as an “activated” state (Katsuno et al. 2013; Derynck et al. 2014; Lamouille et al. 2014). Although the epithelial response to TGF- β might result in profibrotic effects other than EMT, epithelial-specific deletion of a gene encoding an EMT transcription factor leads to attenuated fibrosis. For example, hepatocyte-specific deletion of *Snail* attenuates fibrosis in the CCl₄ model of liver fibrosis, and deletion of the gene encoding Forkhead box M1 (FoxM1) in alveolar epithelial cells blunts lung fibrosis after bleomycin or radiation (Rowe et al. 2011; Balli et al. 2013). In models of kidney fibrosis, epithelial-specific deletion of the genes encoding the EMT transcription factor Snail1 or Twist leads to attenuated fibrosis (Grande et al. 2015; Lovisa et al. 2015). These studies support a complex role of the EMT program beyond a simplistic view of EMT-derived cells contributing to a pool of activated myofibroblasts. Rather, cells in partial EMT may play a unique role necessary for fibrogenesis, through regulation of TGF- β -induced cell-cycle arrest and cross-talk with other cell types.

Some studies show that fibrosis can be attenuated by epithelial cell-specific deletion of secreted proteins that have been attributed to the function of activated fibroblasts. For example, connective tissue growth factor (CTGF) is a pro-

fibrotic extracellular matrix protein, whose expression is induced by TGF- β . CTGF expression has been used as a marker of activated fibrotic fibroblasts, but several studies indicate that epithelial cells may be the major source of CTGF during fibrosis and deletion of CTGF within epithelial cells blocks fibrosis (Leask and Abraham 2003; Makino et al. 2013; Al-Alawi et al. 2014). Finally, production of type I collagen may be the most basic function of fibrogenic effector cells and deletion of the *Colla1* gene within lung epithelial cells leads to significant reduction in bleomycin-induced lung fibrosis (Rhim et al. 2014).

Thus, using a loss-of-function approach, there is strong evidence that epithelial cells contribute to fibrosis in ways that overlap with those of other fibrogenic effector cells. Whether this is sufficient to be labeled EMT depends on the precise definition of EMT. A broader question is whether fibrogenesis requires a differentiation event or whether cells can undergo significant phenotypic activation without necessarily differentiating into another cell type. There is increasing evidence that fibrosis is a multicellular process. The overlapping versus nonredundant functional contributions of these cell types during fibrosis remains unclear. Finally, attention to how activation, proliferation, and apoptosis of different cell types are orchestrated in response to injury or other fibrotic stimulus might reveal novel targets for intervention, with better effects and fewer side effects than targeting single cell types.

TGF- β REGULATION OF CELL PROLIFERATION AND APOPTOSIS

TGF- β activates the expression of a number of proteins involved in cell-cycle control, including regulators of cyclin-dependent kinases, and signaling by the MAP kinase and PI3K–Akt signaling pathways that promote cell proliferation. Depending on the context and cell type, TGF- β can have dramatic effects on cell proliferation, cell-cycle arrest, senescence, or apoptosis. Although these effects have mainly been studied in the context of cancer biology, these changes can have important consequences on the progression of fibrosis.

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Regulation of Fibroblast Proliferation and Apoptosis

Fibrosis is characterized by accumulation of fibroblasts and myofibroblasts. The roles of TGF- β in fibroblast activation to a profibrotic phenotype are well described; however, the role of TGF- β in fibroblast proliferation as a way to account for the abundance of these cells remains unresolved (Bartram and Speer 2004). TGF- β was initially identified as an extracellular growth factor that enables anchorage-independent fibroblast proliferation (Roberts et al. 1980). Many early studies show TGF- β -induced proliferation of fibroblasts from multiple tissues (Hill et al. 1986; Moses et al. 1987, 1994; Schrier et al. 1993). However, other studies have found either no effect on proliferation (Fine and Goldstein 1987) or inhibition of fibroblast proliferation by TGF- β in cell culture and in vivo (Moses et al. 1990; McAnulty et al. 1997; Keerthisingam et al. 2001; Hostettler et al. 2008). Furthermore, immunostaining tissue sections from animal models of fibrosis or from lungs of patients with IPF for proliferation markers, such as Ki67, shows substantial proliferation of epithelial cells but seldom of myofibroblasts or fibroblast foci (El-Zammar et al. 2009; Lomas et al. 2012). These conflicting reports on the ability of TGF- β to induce fibroblast proliferation might be resolved by understanding the costimulatory activation of other signaling pathways that affect the cellular response to TGF- β (Grotendorst et al. 2004). TGF- β can induce the activation of several signaling pathways that promote cell proliferation (e.g., MAPK, PI3K, and Rho signaling). However, the activation of these pathways is not unique to TGF- β , and many other mitogens also promote cell proliferation. Therefore, the microenvironment with the cytokines that stimulate fibroblast proliferation may determine the proliferative response in fibrosis. Importantly, TGF- β can stimulate the expression and secretion of a number of growth factors and cytokines by fibroblasts or other cell types. Many of these TGF- β -induced factors, such as FGF2, PDGF, and CTGF, may secondarily act in autocrine or paracrine fashion to induce fibroblast

proliferation (Kay et al. 1998; Strutz et al. 2001; Bartram and Speer 2004; Leask and Abraham 2004; Leask 2009; Xiao et al. 2012). Some studies argue that the TGF- β responses of fibroblasts toward activation and myofibroblast differentiation versus proliferation are mutually exclusive (Grotendorst et al. 2004).

Finally, there is evidence that fibroblasts from fibrotic tissue are resistant to apoptosis, and that TGF- β may confer resistance to apoptosis by classic death pathways such as the Fas-caspase cascade (Zhang and Phan 1999; Chodon et al. 2000; Tanaka et al. 2002; Thannickal and Horowitz 2006). However, the mechanism for their resistance to apoptosis is poorly characterized. It may be mediated through activation of signaling pathways, such as p38 MAPK and PI3K-Akt (Kulasekaran et al. 2009), and involve regulation of “inhibitor of apoptosis” (IAP) family members (Horowitz et al. 2012; Sisson et al. 2012). TGF- β may also promote fibroblast resistance to apoptosis through cell-cycle regulators such as p14^{ARF} (Cisneros et al. 2012). Inhibition of fibroblast and myofibroblast apoptosis through these pathways may account for some of the accumulation of these cells during fibrosis.

TGF- β Regulation of Cell Senescence and Apoptosis

The response to TGF- β by epithelial and endothelial cells includes inhibition of growth, cell-cycle arrest, senescence, or apoptosis (Tucker et al. 1984; Heimark et al. 1986; Takehara et al. 1987). Various insults can lead to damaged cells or undamaged cells that have become essentially nonfunctional within the context of a damaged tissue, promoting either epithelial cell senescence or apoptosis with resulting initiation of fibrosis (Fig. 3).

Cellular senescence can be broadly defined as a process by which cells irreversibly cease to proliferate and typically acquire altered cell shapes and secretory profiles (Kuilman et al. 2010; Campisi 2013; Munoz-Espin and Serrano 2014) whereas apoptosis results from a process of programmed cell death (Campisi 2003). Senescence and apoptosis are adaptive necessary



processes in response to injury. Senescence is characterized by the absence of markers of proliferation and increased expression of tumor suppressor proteins that function as cyclin-dependent kinase inhibitors including p16^{INK4a} and p21^{Cip1}. Strong mitogenic activators such as activated Ras or growth factor receptor activation can provoke a shut down in the cell cycle with induction of senescence (Blagosklonny 2003; Senturk et al. 2010).

Apoptosis can be initiated through an extrinsic or intrinsic pathway (Kuwano et al. 1998, 2004; Kuwano 2007). The extrinsic pathway involves engagement of an extracellular ligand to a cell surface death receptor. A well-studied interaction in this context is the activation of Fas by Fas ligand (FasL), but other ligands such as TNF- α can also initiate an apoptotic pathway. The intrinsic or mitochondrial pathway is initiated by cell stresses, such as oxidative stress. As noted above, this stress can lead to senescence or apoptosis through activation of pro-apoptotic Bcl-2 family members, or release of cytochrome C. The role of p21^{Cip1} may be particularly important in determining the epithelial cell response to stress toward senescence or apoptosis (Zhang et al. 2005).

TGF- β signaling through Smad-dependent and -independent pathways intersects with many senescence and apoptotic pathways (Munoz-Espin et al. 2013; Munoz-Espin and Serrano 2014). Smad complexes can directly activate transcription of p21^{Cip1} (Datto et al. 1995), which, as discussed above, is a critical inducer of cellular senescence (Munoz-Espin et al. 2013). Transcription of the gene encoding p21^{Cip1} is also regulated in response to PI3K and ROS, which are both downstream mediators from TGF- β signaling. ROS generation can also lead to activation of latent TGF- β potentially perpetuating senescence (Yu et al. 2009). In a more delayed fashion, TGF- β can also suppress the expression of transcription factors, such as Id (inhibitor of differentiation or DNA-binding) family members, which have been associated with cell proliferation and inhibition of senescence (Shibanuma et al. 1994; Di et al. 2006). Finally, TGF- β -induced secreted factors could indirectly contribute to epithelial cell

senescence and apoptosis. As mentioned, ROS generation in response to TGF- β can initiate cell-cycle arrest or apoptosis. TGF- β can also induce expression and secretion of pro-apoptotic factors, including FasL and angiotensin (Wang et al. 1999). As with effects on proliferation, concurrent stimulation or activation by other signaling pathways has the potential to tune the degree of this TGF- β response.

Early studies in cell culture indicate that epithelial and endothelial cells undergo apoptosis in response to TGF- β . These observations correlate well with the increased epithelial cell apoptosis in fibrotic tissue and in animal models of fibrosis (Uhal et al. 1998; Barbas-Filho et al. 2001). Indeed, expression of activated TGF- β 1 using transgenic approaches or adenoviral gene transfer is sufficient to induce epithelial cell apoptosis and subsequent fibrosis (Lee et al. 2004, 2006; Ask et al. 2008). Importantly, TGF- β 1-induced fibrosis is attenuated by treating mice with a pan-caspase inhibitor, and in mice with inactivation of BH3-interacting domain death agonist (BID) or BCL-2-associated (BAX) expression, suggesting that apoptosis is critical for fibrosis (Lee et al. 2004; Kang et al. 2007).

The roles of senescence and apoptosis in injury, repair, and fibrosis depend on the context. Transient apoptosis or senescence may result in removal of unwanted cells and cell debris, and facilitate repair to reinstate a homeostatic architecture and function. Conversely, sustained or abnormal senescence or apoptosis could lead to a pathological wound repair response that progresses to fibrosis. Excessive senescence or apoptosis could also lead to delayed restoration of a damaged endothelial or epithelial barrier, thus promoting persistent leak of serum components into the lumen with continued activation of damage response pathways and sustained inflammation. Epithelial cell turnover and impaired proliferation may also delay production of antifibrotic factors, such as prostaglandin E2 (Moore et al. 2003). Activation of senescence leads to dramatic transcriptional changes that are mediated in large part by responses to activation of the NF- κ B and C/EBP- β transcription factors (Campisi 2013; Campisi and Robert 2014). In particular,

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significant changes occur in the expression of many secreted factors, including cytokines, growth factors, and proteases in what has been called a “senescence-associated secretory phenotype” (SASP). The SASP thus allows the senescent cell to become an abundant source of injury and inflammatory mediators with potential important effects on promoting fibrosis (Fig. 3) (Aoshiba et al. 2003; Coppe et al. 2008, 2010; Aoshiba et al. 2013). Thus, the role of TGF- β in regulating fibrosis is mediated by divergent effects on both fibroblast and epithelial cell survival.

ROLE OF TGF- β 1 IN DIVERSE FIBROTIC DISEASES

Tissue fibrosis is a common consequence of many different acute and chronic insults, which can severely impair the function of nearly every organ system. Fibrosis can also occur as a primary progressive process without an apparent inciting event. Collectively, fibrosis is a leading cause of death and morbidity in the developed world, accounting for up to 45% of all deaths by some estimates (Wynn 2007). There is considerable overlap among the different fibrotic conditions, with TGF- β 1 signaling having a prominent role. Although progressive fibrosis, in general, is difficult to reverse or even slow down, understanding the role of TGF- β 1 signaling in this process offers insight into potential novel therapeutic strategies. A review of the history and current thinking of the roles of TGF- β 1 in pulmonary fibrosis serves to highlight this principle.

Evidence Linking TGF- β 1 to Fibrosis in the Lung Parenchyma

As the promoting role of TGF- β 1 in experimental fibrosis was well described soon after discovery of TGF- β 1 (Roberts et al. 1986), the role of TGF- β 1 in pulmonary fibrosis was examined decades ago. TGF- β 1 expression was found to appear early in the bleomycin-induced model of pulmonary fibrosis, preceding collagen deposition, and to localize to sites of subsequent collagen accumulation in both a mouse model and

in humans with fibrotic lung disease (Hoyt DG 1988). Active TGF- β 1, when overexpressed in the lung using a viral vector, was found to be sufficient to cause severe lung fibrosis in rodents, requiring signaling through Smad3 (Sime et al. 1997; Gauldie et al. 1999). Interestingly, in an inducible model of TGF- β 1 expression in lungs, reversal of TGF- β 1 expression led to substantial resolution of the fibrotic process (Lee et al. 2004). TGF- β 1 proved to be both necessary and sufficient for lung fibrosis. As discussed above, activation of endogenous, latent TGF- β 1, via the integrin $\alpha_v\beta_6$, was found to be required for lung fibrosis in both bleomycin- and radiation-induced lung fibrosis models (Munger et al. 1999), as well as models of kidney fibrosis. The critical role of TGF- β 1 in fibrosis was further validated by expression profiling of human fibrotic lungs. Microarray profiles reveal that \sim 80% of the genes activated in fibrotic human lungs are known TGF- β 1 target genes (Kaminski et al. 2000).

Epithelial Activation and Fibrogenesis

Genetic analysis of patients with familial interstitial pneumonitis point to type II alveolar epithelial dysfunction as a consequence of SP gene mutations and as a causal mechanism for pulmonary fibrosis (Nogee et al. 2001; Thomas et al. 2002; Wang et al. 2009; Garcia 2011). Mouse models expressing mutant SPs support this conclusion. This has led to the general concept that epithelial cells are both vulnerable to further injury and “activated” as a driver of fibrosis. There is evidence to support the development of at least three distinct dysfunctional states in the context of chronic injury and/or stress to the epithelium: TGF- β 1-induced mesenchymal transition (EMT as defined above), senescence, and ER stress (Fig. 3). As discussed above, TGF- β 1 may itself contribute to both senescence and ER stress. Each of these dysfunctional states have distinct biological features but all show evidence of enhanced secretory activity, at least some of which consists of profibrotic and/or pro-inflammatory cytokines and mediators. Frequently superimposed on these dysfunctional states are hypoxia, enhanced oxidant



production, and exposure to environmental toxins such as smoke and viruses, further contributing to alveolar dysfunction. Collectively, these stresses reprogram the epithelium toward a secretory phenotype altering the immune milieu and promoting mesenchymal expansion (Fig. 3).

Progressive dysfunction of the alveolar epithelium can lead to apoptosis and alveolar collapse. Collapse of small distal airways (<2 mm diameter) and surrounding alveoli is prominent during parenchymal lung injury and repair (Seeger et al. 1993; Lutz et al. 2015). Active sites of fibrogenesis, termed fibrotic foci, in interstitial pneumonitis have been noted by many investigators to contain basement membrane within the interstitium, suggesting that the focus of fibrogenesis may emanate from hypoxic areas of small airway and alveolar collapse with incorporation of epithelial lining and inflammatory alveolar cells into a mesenchymal compartment (Myers and Katzenstein 1988). This phenomenon has been termed induration fibrosis and may be unique to lung architecture because incorporation of epithelial cells into a mesenchymal compartment has not been observed during either liver or kidney fibrogenesis (Lutz et al. 2015). Airway and alveolar collapse may promote direct epithelial–mesenchymal interactions, including activation and signaling by TGF- β 1. The ultimate fate of such epithelial cells is uncertain but could be either apoptosis and/or persistent EMT, as discussed above. Hypoxia is known to promote collagen expression in fibroblasts and TGF- β 1-dependent EMT in primary epithelial cells and within tumors (Falanga et al. 2002; Tzouveleakis et al. 2007; Zhou et al. 2009; Kottmann et al. 2012). Alveolar collapse also likely contributes to increased tissue stiffness that itself can propagate TGF- β 1 activation as discussed above, raising the possibility that such collapse is an important trigger for disease progression.

TGF- β 1 Signaling in Airway Fibrosis: Integration of Immunity and Stromal Signaling

The major lung airways are a frequent site of injury from environmental exposures. The most common chronic lung diseases, asthma

and chronic obstructive pulmonary disease (COPD), are airway-centric inflammatory processes and in both cases there is repeated epithelial dysfunction and disordered ECM remodeling with collagen accumulation in the submucosa of airways (Aoshiba and Nagai 2004; Davies 2009; Hirota and Martin 2013). The development of fixed airway obstruction in some asthmatic patients over time is thought to result from airway wall remodeling in which airway collagen accumulation is a common and prominent feature. In some studies, high-resolution imaging of asthmatic airway wall thickness correlates with lung function and fixed airflow obstruction, cardinal features of airway disease (de Jong et al. 2005; Bosse et al. 2008). Airway remodeling, however, is not simply fibrotic tissue accumulation in the airway walls. Remodeling involves almost all elements of the airway wall: chronic inflammation involving innate and adaptive immune cells, epithelial goblet cell and subepithelial smooth muscle hyperplasia, as well as submucosal neovascularization in addition to thickening of the subepithelial lamina reticularis, one of the hallmarks of asthma histopathology (Postma and Timens 2006; Hirota and Martin 2013). Lying immediately below the basement membrane, the lamina reticularis is a membranous, collagenous airway structure found in large conducting airways and thickened in asthmatics by the accumulation of several ECM proteins, including the fibrillar collagens type I and type III. Collagen accumulation in the lamina reticularis in asthma is not as well organized into fibrillar bundles as fibrils in fibrotic tissues, but nonetheless collagen fibrils are commonly observed throughout these structures (Saglani et al. 2006). In addition, the airway submucosal collagens surrounding fibroblasts are frequently expanded in both animal models of chronic asthma and asthmatic patients. Similar changes in airway collagen accumulation occur to a somewhat lesser degree in COPD airways (Jeffery 2001; Aoshiba and Nagai 2004; Postma and Timens 2006). Such airway accumulation of fibrillar collagens may restrict airway lumen size, and this has been argued in rodent models. Because of the many elements involved in air-

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way remodeling, it has not been determined to what degree collagen deposition per se contributes to fixed airway obstruction in longstanding active asthma. Nonetheless, airway remodeling involving accumulation of collagen is a prominent feature of asthma, and, as discussed below, accumulating evidence links this pathobiology to TGF- β 1 activation (Makinde et al. 2007).

The dynamic interplay between activated epithelial cells and surrounding airway stromal elements in asthma has led to the concept of an epithelial–mesenchymal “trophic unit,” implying that airway homeostasis is maintained by an ongoing exchange of soluble mediators between the epithelium and surrounding mesenchymal cells (Holgate et al. 2004). In contrast, there is no evidence that activated epithelial cells cross the airway basement membranes to directly contact mesenchymal cells or become mesenchymal cells, in either asthma or COPD. The impact of signaling interactions between the epithelial lining cells and underlying stromal elements has largely been studied in the context of perturbed epithelial and immune cell function in inflammatory airway disease. Most studies point to mediators released by native and adaptive immune cells that activate epithelial cells to release cytokines impacting airway bronchoconstriction and remodeling (Boxall et al. 2006; Doherty et al. 2011; Halwani et al. 2011; Hirota and Martin 2013; Trian et al. 2015). Given the collagen accumulation as a prominent part of airway wall remodeling, it is not surprising that a major target of immune-mediated cytokine signaling is increased TGF- β 1 expression and activation in both the epithelium and the airway walls of asthmatic and COPD patients. Epithelial–mesenchymal cross-talk has been studied in COPD, focusing on the squamous metaplasia commonly observed in large conducting airways of COPD patients (Araya et al. 2007). Squamous epithelial cells in culture, but not the airway basal cells from which they are derived, release cytokines, especially IL-1 β , which then induce TGF- β 1 activation and myofibroblast accumulation. Activated fibroblasts are thought to promote a positive feedback for this process by further releasing TGF- β 1 to impair epithelial proliferation and promote the

squamous phenotype. The squamous morphology of cultured bronchial epithelial cells and the high expression of involucrin and the cyclin-dependent kinase inhibitor p21^{Cip1} are suggestive of a senescent phenotype, a phenotype known to be promoted by stress and accompanied by release of pro-inflammatory cytokines, although this possibility has not been directly addressed in the lung (Rodier and Campisi 2011). Similar pathways are likely activated in asthma. In this case, the production of Th2 cytokines by innate and adaptive immune cells is increased, and their activities on epithelial cells, as well as epithelial injury, lead to the release of cytokines including TGF- β 1, PDGF, and insulin-like growth factors by the epithelial cells. These then promote proliferation of fibroblasts and smooth muscle cells that is characteristic of airway remodeling in asthma (Holgate et al. 2004; Boxall et al. 2006; Davies 2009). As discussed below, a common and important element in altered fibroblast function in all these settings involves TGF- β 1 activation, which is the likely driver of submucosal airway collagen accumulation.

Stromal elements involved in remodeling of asthmatic airways show enhanced sensitivity to immune Th2 cytokine signals. Primary human airway fibroblasts in culture respond to IL-13, a prominent Th2 cytokine implicated in asthma pathobiology, with activation of *COL1A2* mRNA expression and collagen protein deposition. This response is greater in primary cells isolated from asthmatic patients when compared with cells of healthy individuals (Firszt et al. 2014). IL-13-induced collagen expression was found to depend on TGF- β 1 activation and signaling by fibroblasts. IL-13 also promotes TGF- β 1 expression and release from asthmatic epithelium. Earlier studies exploring the response of airway fibroblasts to IL-4 report similar findings, suggesting that in some way the ongoing inflammation and stimulation of stromal elements primes these airway fibroblasts for further response (Saito et al. 2003). In addition, smooth muscle cell proliferation has been reported to be hyperactive in asthmatic airways (Trian et al. 2015). Bronchial smooth muscle cells from asthmatic large air-



ways express higher levels of the cysteinyl leukotriene receptor CysLTR1, a receptor for leukotriene C4, and display an increased proliferative response to the epithelial-derived leukotriene C4 compared with smooth muscle cells from normal airways (Triani et al. 2015). These findings underscore the paradigm that the epithelial-derived signals are in part responsive to cytokine stimulation from immune cells, and activate bidirectional signaling that leads to airway remodeling with airway fibrosis in both asthma and COPD.

The effector mechanisms of TGF- β 1-dependent fibrogenesis in airways do not appear different from other sites of fibrosis driven by TGF- β 1, but the mechanisms of latent TGF- β 1 activation are distinct in the conducting airways. Whereas alveolar epithelial TGF- β 1 activation depends on integrin $\alpha_v\beta_6$, large conducting airway epithelial TGF- β 1 activation is mediated by both $\alpha_v\beta_6$ and $\alpha_v\beta_8$ integrins (Munger et al. 1999; Mu et al. 2002). The mechanism of activation of TGF- β 1 by $\alpha_v\beta_8$ requires metalloproteinase (MMP)-mediated cleavage of latent TGF- β 1, in contrast to cytoskeleton-mediated conformational strain on integrins in $\alpha_v\beta_6$ -mediated TGF- β 1 activation. Dendritic cells also require $\alpha_v\beta_8$ to activate surface TGF- β 1 that in turn promotes differentiation of CD4 T cells to T cells that produce IL-17 along with IL-6, and to regulatory T cells (Tregs) (Travis and Sheppard 2014). Expansion of IL-17-producing CD4⁺ T cells enhances airway smooth muscle contraction in response to binding of IL-17 to its receptors that are expressed on smooth muscle cells, extending the well-known role of IL-17 as a pro-inflammatory cytokine (Kudo et al. 2012). Mice that lack expression of the dendritic cell integrin $\alpha_v\beta_8$, as a result of Cre recombinase expression from the *Itgax* promoter and Cre-mediated deletion of β_8 , spontaneously develop an autoimmune colitis at about six months of age, consistent with the well-known role of TGF- β 1 in promoting differentiation of T cells toward an immunosuppressive Treg lineage (Wan and Flavell 2007). The anti-inflammatory and immune regulatory effects of TGF- β 1, mediated in large part by dendritic cells, are prominent in asthma and

are layered on top of the profibrotic pathways initiated in activated epithelial cells and fibroblasts. Hence, it may not be meaningful to attempt to correlate the overall levels of latent TGF- β 1 expression or even TGF- β activity in the lung with the severity or progression of asthma and COPD. For example, inhibition of TGF- β 1 signaling protects mice from airway remodeling in an allergen-induced model, but what affected cell type accounts for this protection is uncertain (McMillan et al. 2005). Mice deficient in periostin expression have enhanced bronchoconstrictor responses and higher allergen-induced IgE expression in an allergy model (Gordon et al. 2012). Periostin interacts with collagen and promotes its organization into fibrils. In the absence of periostin, TGF- β 1 activation is reduced resulting in decreased Treg cell differentiation with an enhanced immune response. Thus, the pathogenic role of TGF- β 1 in asthma pathobiology, and peri-airway fibrosis specifically, is complicated by the high activation level of the mucosal immune system in asthma. Indirect effects through changes in immune cell function rather than directly altered fibroblast collagen production could be a major mechanism for TGF- β 1 effects in asthma and COPD.

THERAPEUTIC IMPLICATIONS

Integrin-mediated TGF- β activation has been shown to substantially contribute to tissue pathology in a number of disease models. For example, mice lacking the $\alpha_v\beta_6$ integrin are protected in murine models of lung, kidney, and biliary fibrosis (Munger et al. 1999; Jaramillo et al. 2003; Hahm et al. 2007). These mice are also protected in models of acute lung injury (Pittet et al. 2001), allergic asthma (Sugimoto et al. 2012; Jolly et al. 2014), and severe influenza infection (Jolly et al. 2014), and in each case, protection appeared to be explained by local inhibition of TGF- β activation. These results, together with evidence that $\alpha_v\beta_6$ integrin expression is dramatically increased in dysfunctional epithelial cells overlying regions of pulmonary fibrosis in humans (Horan et al. 2008), suggest that this integrin could be a promising

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therapeutic target. The development of potent blocking monoclonal antibodies that inhibit $\alpha_v\beta_6$ -mediated TGF- β 1 activation in multiple species has made it possible to evaluate their use in disease models after tissue pathology has already been established. The success of these antibodies in multiple disease models (Horan et al. 2008; Puthawala et al. 2008) has stimulated the development of a humanized monoclonal antibody that targets $\alpha_v\beta_6$ and is currently in a phase II clinical trial for treatment of IPF.

$\alpha_v\beta_8$ -dependent TGF- β 1 activation has also emerged as a promising therapeutic target. Mice lacking this integrin on dendritic cells are dramatically deficient in antigen-specific T helper 17 (Th17) cells, and are protected in experimental autoimmune encephalitis (Melton et al. 2010) and allergic asthma (Kudo et al. 2012). Mice lacking $\alpha_v\beta_8$ -mediated TGF- β 1 activation in fibroblasts are protected in models of airway inflammation, and studies in mice engineered to replace murine β_8 with its human ortholog show that a monoclonal antibody against human $\alpha_v\beta_8$ that blocks TGF- β 1 activation protects against allergic airway inflammation and remodeling induced by exposure to cigarette smoke (Minagawa et al. 2014).

As noted above, recent evidence suggests that the $\alpha_v\beta_1$ integrin on fibroblasts contributes to tissue fibrosis in multiple organs (Lawson et al. 2008, 2011; Tanjore et al. 2011, 2012, 2013; Torres-Gonzalez et al. 2012; Henderson et al. 2013; Reed et al. 2015). Therapeutic intervention with a broadly active α_v integrin inhibitor or a highly specific $\alpha_v\beta_1$ small molecule inhibitor in mice with already established liver or pulmonary fibrosis causes a marked reduction in collagen accumulation, suggesting that $\alpha_v\beta_1$ could also be a therapeutic target for treatment of diseases characterized by excessive tissue fibrosis.

Besides the strong rationale for therapeutic agents directed at integrin-dependent TGF- β 1 activation, elements of TGF- β 1 signaling and other aspects of the pathophysiology of fibrogenesis are also potential therapeutic targets. As noted above, a number of profibrotic cytokines such as CTGF, FGF, and PDGF have been explored as potential targets for antifibrotic

therapy, in large part because of their convergence with the TGF- β pathway. Studies of EMT in fibrosis began largely because of the strong parallels between signaling pathways of fibrosis and pathways of EMT in cell culture. One of the ironies then about the possible roles of EMT in fibrosis is that, from a clinical viewpoint, therapeutic agents directed at either pathways of EMT or pathways of fibroblast expansion and ECM secretion may almost completely overlap, making definitions and cells of origin of little importance. For example, the best-studied EMT transcription factors are Snail1, Slug/Snail2, Twist, ZEB1, and Sip1/ZEB2. Their expression can be induced by TGF- β 1 in both epithelial and mesenchymal cells. Understanding these pathways and the epithelial cell response to TGF- β offers potential for therapeutic intervention in fibrosis, even if many of these pathways are not unique to EMT. In addition, expansion of the ECM impacts fibrogenesis downstream from TGF- β 1 activation by exerting strong pro-survival signals as a result of integrin activation. Activated β_1 integrins promote FAK and Akt activation leading to accumulation of anti-apoptotic factors that could counter the pro-apoptotic signaling in response to TGF- β 1 in the epithelium, ER stress, and senescence (Horowitz et al. 2007; Thannickal et al. 2014). Moreover, recent studies point to ECM stiffness and integrin activation as a TGF- β 1-independent pathway of epithelial Stat3 activation with subsequent further fibroblast activation and ECM accumulation (Prele et al. 2012; Duffield 2016). These studies highlight some additional sites of potential therapeutic intervention downstream from TGF- β 1 activation that are studied in clinical and preclinical trials (Ahluwalia et al. 2014; Blackwell et al. 2014). The current rationales and status of trials directed at TGF- β signaling in fibrosis have recently been well summarized (Ahluwalia et al. 2014).

Emerging evidence indicating the prominence of ER stress and cell senescence in the pathobiology of fibrotic lung disease also offers new therapeutic possibilities (Lawson et al. 2008, 2011; Tanjore et al. 2011, 2012, 2013; Torres-Gonzalez et al. 2012). TGF- β 1 signaling may contribute to this pathophysiology indi-



rectly by the induction of enhanced collagen secretion in cells that are already stressed by the accumulation of misfolded proteins. Indeed, collagen itself is only marginally soluble and its complicated helical structure is prone to misfolding. Whether and how the UPR can be manipulated to attenuate the potentially profibrotic and self-destructive elements in its response to ER stress (Fig. 3) is an important avenue for further investigation. Likewise, the accumulation of senescent cells in the context of chronic and recurring injury, as well as aging itself, may offer new insights (Sanders et al. 2013; Thannickal 2013; Hecker and Thannickal 2016). For example, mice lacking caveolin 1 expression were found to be protected from fibrosis through decreased epithelial senescence after bleomycin-induced injury (Shivshankar et al. 2012). Further studies of telomerase activators have been suggested as a potential point of therapeutic intervention in senescence-initiated fibrosis (Le Saux et al. 2013). Similarly, targeting nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 4 (NOX4), a TGF- β 1-responsive gene, attenuates lung fibrosis at least in part through inhibition of NOX4- or ROS-mediated senescence (Hecker et al. 2009). The potentially profibrotic and pro-inflammatory character of the SASP secretome and regulators of senescence during fibrogenesis need better clarification (Kang et al. 2007). It remains unclear to what degree the SASP and the secretory profile of TGF- β 1-induced EMT and ER stress (Fig. 3) share expression of common profibrotic mediators or exert their effects on fibrosis through distinct pathways. These uncertainties remain important to clarify as attempts to develop new therapeutics center around the early events that lead to epithelial dysfunction and a disordered repair process, not only in the lung but at all sites of fibrogenesis.

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