Genesis of the Myofibroblast in Lung Injury and Fibrosis

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Tissue injury incites a repair response with a key mesenchymal component that provides the essential connective tissue for subsequent regeneration or pathological fibrosis. The fibroblast is the major mesenchymal cell type to be implicated in this connective tissue response, and it is in its activated or differentiated form that it participates in the repair process. The myofibroblast represents such an activated mesenchymal cell and is a key source of extracellular matrix and inflammatory/fibrogenic cytokines as well as participating in wound contraction. Although successful healing results in gradual disappearance of myofibroblasts, their persistence is associated with chronic and progressive fibrosis. Thus, elucidation of the mechanism involved in the genesis of the myofibroblast should provide insight into both pathogenesis of chronic fibrotic diseases and therapeutic strategies for their management and control. Although the fibroblast is a well-documented progenitor cell for the myofibroblast, recent studies have suggested additional precursor cells that have the potential to give rise to the myofibroblast. Many of the studies focused on mechanisms and factors that regulate induction of α -smooth muscle actin expression, a key and commonly used marker of the myofibroblast. These reveal complex and multifactorial mechanisms involving transcriptional and epigenetic regulation and implicating diverse cell-signaling pathways, including those activated by the potent fibrogenic cytokine transforming growth factor β . Despite these extensive studies, many aspects remain poorly understood, with the suggestion that additional novel mechanisms remain to be discovered. Future studies with the help of newly developed technical advancements should expedite discovery in this direction.

Keywords: cell differentiation; transcriptional regulation; epigenetic regulation

Lung injury can result in progressive chronic fibrosis and in certain circumstances, such as in idiopathic pulmonary fibrosis, lead to respiratory failure. In the airways, injury can also result in localized remodeling or fibrosis of the airway wall. Progression of injury to chronic fibrosis is associated with the persistence of myofibroblasts, which appear *de novo* in response to tissue injury but gradually disappear when the injury fully resolves in successful wound healing (1). The disappearance, presumably by apoptosis, may be aborted or prevented in progressive fibrosis resulting in their persistence. Given their distinct phenotype of induced expression of α -smooth muscle actin (α -SMA), these myofibroblasts have been most amenable for analysis as the prototype of the activated fibroblast or mesenchymal cell in fibrotic disease. Although the involvement of other activated fibroblast populations cannot be completely excluded, there is evidence to indicate that myofibroblasts are a key player in the tissue remodeling process by their high expression of extracellular matrix and fibrogenic mediators (2). Thus, much attention has been focused on the origin of the myofibroblast, the

mechanism of its genesis, and its fate in the context of chronic fibrotic disease. A benefit of a more complete understanding of these aspects of myofibroblast biology is the provision of novel therapeutic targets that could be designed for management of chronic fibrotic diseases.

ORIGIN OF THE MYOFIBROBLAST

Myofibroblasts arise *de novo* during the repair process and are not normally present in adult tissues except for specialized regions (1). Based on these observations, they are originally believed to arise from local or adjacent cells that normally do not express α -SMA, or perhaps migrate from adjacent areas populated by α -SMA-expressing cells, such as smooth muscle cells. However, fibrotic lesions often are found at tissue sites quite distant from smooth muscle, thus favoring the possibility of induced expression of α -SMA in cells that do not express this gene in the absence of injury. The fibroblast is ubiquitously present in most normal tissues, albeit in relatively small numbers, and thus is a candidate progenitor for the myofibroblast. However, subsequent studies indicate additional possibilities including, more recently, from distal sites (Figure 1).

Fibroblasts as Myofibroblast Precursors

The ability of fibroblasts to express α -SMA *de novo* on appropriate treatment, such as with transforming growth factor β (TGF- β), is well-documented, thus providing *in vitro* evidence and proof that fibroblasts can indeed serve as precursors for myofibroblasts (1). Additionally, *in vivo* initial induction of α -SMA expression in a model of pulmonary fibrosis is found to localize to the adventitia of blood vessels, suggesting adventitial fibroblasts as the precursors for myofibroblasts (3). This perivascular location has also suggested the pericyte as another potential myofibroblast precursor cell, and indeed recent evidence suggests such a possibility in renal fibrosis (4).

Epithelial and Endothelial Origin of Myofibroblasts

The importance of epithelial-mesenchymal transition (EMT) in development has been extended to metastasis in carcinomas and more recently to the genesis of myofibroblasts in tissue fibrosis (5–7). A similar process with derivation from endothelial cells has also been reported in development and fibrosis (8, 9). These processes are commonly monitored by loss of differentiated epithelial or endothelial cell marker gene expression accompanied by expression of mesenchymal cell markers, including α -SMA. Expression of certain EMT-associated transcription factors, such as Snail, Slug, and Twist, has also been used to demonstrate EMT (10). Transitional cell types with both epithelial and mesenchymal markers have been reported and presented as evidence for occurrence of EMT (6, 7). With respect to genesis of the myofibroblast, it remains unclear if EMT or endothelial-mesenchymal transition first has to assume a fibroblastic phenotype before myofibroblast differentiation in a two-step process (Figure 1). However, despite extensive studies, the importance and extent of derivation of myofibroblasts from epithelial cells in tissue repair or fibrosis remain an unsettled issue due to the conflicting published evidence, even from

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Figure 1. Myofibroblast precursors. Bone marrow–derived precursors for the fibrocyte and endothelial cell are presumably the hematopoietic stem cell and endothelial progenitor cell, respectively. Origination of epithelial cells from bone marrow precursors is controversial and indicated with a question mark. It is unclear at this time whether endothelial (endoMT) and epithelial–mesenchymal transition (EMT) could bypass the fibroblast stage and directly assume the myofibroblast phenotype. Other local mesenchymal progenitor cells (MPC) are also shown for completeness, but their specific identity is unclear and poorly defined.

studies using the same animal model. Thus, in studies of renal, hepatic, and pulmonary fibrosis, divergent results were obtained showing both a significant and undetectable derivation of mesenchymal cells or myofibroblasts from epithelial precursors (8, 11–14). The basis for these divergent and conflicting results is not entirely clear, but methodological and analytical differences are notable between the conflicting studies. The extent of derivation from endothelial cells is also unclear and remains to be fully investigated.

Bone Marrow as a Source of Myofibroblasts

Fibrocytes are rare circulating cells that express mesenchymal markers, such as vimentin and collagens I and III, but also are positive for CD34 and CD45 (15, 16). They originate from the bone marrow, and in view of their positivity for CD34 (bone marrow stem cell marker, e.g., endothelial progenitors), CD45 (hematopoietic stem cell marker), and derivation from CD14 (monocyte/macrophage marker)-expressing cells, presumably arise from hematopoietic stem cells. These cells are found to migrate to sites of tissue injury and represent a source of fibroblasts and myofibroblasts in wound healing (17). Tracking of bone marrow–derived cells in green fluorescence protein bone marrow chimera mice supports this concept of bone marrow–derived fibroblast-like cells, such as in studies of fibrosis in a variety of tissues, including skin and lung (18, 19). However, evaluation of these cells at tissue sites does not consistently show uniform expression of all known fibrocyte markers, which was suggested as being due to loss of certain markers on activation or differentiation of these cells at the site of tissue remodeling or fibrosis (20). Although there is good agreement with respect to influx of bone marrow–derived type I collagen–expressing cells, there is some uncertainty as to precise identity of these cells. In particular, their ability to differentiate to myofibroblasts as reported in some studies (17, 21, 22) cannot be demonstrated in others, which show instead that myofibroblasts are mostly of local tissue origin, with minimal or no contribution from the bone marrow (18, 19, 23, 24). Hence, the significance of the bone marrow derivation of myofibroblasts in fibrotic lesions remains unclear. Despite this uncertainty, there is evidence that the recruited type I collagen–expressing cells from the bone marrow can promote fibrosis at the injured distal tissue sites due to their ability to promote local myofibroblast differentiation by production of TGF- β (25, 26). Thus, irrespective of their ability to undergo myofibroblast differentiation, these bone marrow–derived cells appear to have a profibrotic effect in the tissues to which they are recruited.

MECHANISM OF MYOFIBROBLAST DIFFERENTIATION

Genesis of the myofibroblast from its precursor cell *in vitro* can be triggered by several factors, including TGF- β , in particular. Such a soluble factor in conjunction with some degree of mechanical stress is required for complete differentiation to the myofibroblast (1). Based on studies of fibroblasts undergoing myofibroblast differentiation, the entire process is found to be highly complex, involving well-defined changes in gene expression with the participation of select cell-signaling pathways, transcription factors, and epigenetic alterations.

Cell Signaling

Because TGF- β is a prototypical factor for induction of myofibroblast differentiation, it has been used extensively in the study of this process, thus resulting in more information being available for pathways triggered by this cytokine. In this regard, the involvement of TGF- β –induced signaling pathways has been reported, including the importance of Smads and relevant kinase pathways (27–30). The importance of Smad signaling in pulmonary fibrosis is demonstrated by studies using Smad3-null mice (31, 32), which may be mediated by reduction in myofibroblast differentiation in the absence of Smad3 (33, 34). Interestingly, lysophosphatidic acid–induced myofibroblast differentiation is dependent on autocrine TGF- β production and associated Smad signaling (35). In addition to TGF- β , other factors have also been shown to activate myofibroblast differentiation in fibroblast cultures and likely to be mediated by their respective downstream signaling pathways. However, these have not been as well studied as the TGF- β –associated signaling pathways. Additional signaling pathways that have been implicated have been reported for resistin-like molecule α (RELM α or FIZZ1, found in inflammatory zone 1), wherein Notch signaling is found to be activated and required for myofibroblast differentiation *in vitro*, as well as for optimal pulmonary fibrosis *in vivo* (36). The mechanism involves FIZZ1 induction of the Notch1 ligand, Jagged1, with consequent downstream activation of the α -SMA gene, a marker of myofibroblast differentiation. This requirement for Notch signaling is not unprecedented, having been previously implicated in EMT and endothelial–mesenchymal transition via activation of the Notch downstream effector CSL (37, 38). The Wnt signaling pathway has been implicated also in EMT (39) and directly in myofibroblast differentiation (40). Recent studies, mostly in cancer-associated EMT and myofibroblast differentiation, reveal the importance of Hedgehog signaling (41). It appears that virtually all the major signaling pathways may be involved in myofibroblast differentiation, depending on the nature of the inducing stimulus and/or the precursor cell type. Some of these pathways are reminiscent of those that are critical for development, which have given rise to the concept that some element of recapitulation of development may be involved in pathogenesis of chronic fibrotic disease in the adult (42). Finally, oxidative stress is also implicated in myofibroblast differentiation, but the precise mechanism has not been elucidated, although recent reports indicate the importance of NADPH oxidase-4 (NOX4) in this process (43). This role in genesis of the myofibroblast may be responsible for the *in vivo* importance of NOX4 in pulmonary fibrosis (44).

Transcriptional Regulation

Expression of multiple genes is altered in cell differentiation, and most studies are focused on the smaller set of genes that is responsible for the differentiated cell genotype and phenotype. In the case of the myofibroblast, *de novo* expression or induction of

α -SMA with incorporation to stress fibers is a hallmark of its phenotype. It is also an essential part of the empirical definition for this cell. Expression of other genes is also altered in this differentiation process, but their specificity as a marker of differentiation requires further elucidation.

There is ample evidence to indicate that α -SMA gene expression is transcriptionally regulated both in myofibroblast differentiation as well as in bona fide smooth muscle cells (1, 2). The list of transcription factors that mediate this regulation has been expanding since the discovery that SRF is a key regulator of α -SMA gene expression via direct binding to the CARG elements present in the gene promoter (2, 33). Especially in the case of myofibroblast differentiation, additional factors and their cognate elements are involved in modulating transcription of the α -SMA gene. In addition to Smad3 (33, 34), additional factors, such as Sp1, Sp3, RTEF-1, and Krüppel-like factor (KLF) 5, have been identified as transcriptional regulators for this gene via binding to several potential target elements found in upstream regulatory regions of the α -SMA gene (1, 2). In the case of Smad3, its potential importance to fibrosis *in vivo* is demonstrated by the reduced fibrosis in Smad3-deficient mice (31, 32).

A complex role for CCAAT enhancer binding protein β (C/EBP β) has been identified that depends on a cognate binding element identified in the α -SMA gene promoter (2). Because both long (liver-activating protein or LAP) and short (liver-inhibitory protein or LIP) isoforms are present in the fibroblast, either activation or suppression of gene expression may result from the binding of these factors to the promoter. Although IL-1 β -mediated suppression of α -SMA expression may depend on relative abundance of the LIP isoform, the overall *in vivo* effect of C/EBP β deficiency is the suppression of pulmonary fibrosis and myofibroblast differentiation (2). This would indicate that the primary role of C/EBP β *in vivo* is in the up-regulation of myofibroblast differentiation, presumably via its LAP isoform. Interference with C/EBP β activation and function *in vivo* also results in suppression of fibrosis (45). However, these *in vivo* observations on the importance of C/EBP β have not ruled out the possibility of additional or alternative mechanisms mediated by effects on other C/EBP β target genes.

The importance of Notch signaling in myofibroblast differentiation implicates its downstream effector molecules in transcriptional regulation of the relevant target genes. CSL represents a key downstream effector of Notch signaling, and a binding element for this factor has been identified in the α -SMA gene promoter, which may mediate Notch-dependent activation of myofibroblast differentiation (38). Deficient Notch signaling *in vivo* suppresses pulmonary fibrosis with reduction in myofibroblast differentiation (36). However, while providing further evidence for the importance of Notch signaling in myofibroblast differentiation, this *in vivo* effect is also likely to impact on other Notch target genes that may contribute to overall suppression of fibrosis. Finally, c-Myb and the myocardin-related transcription factors A (MRTF-A) and B (MRTF-B) have also been implicated in transcriptional regulation of myofibroblast differentiation (1, 46). In the case of MRTF-A, it may act via enhancement of SRF activity on gene transcription.

Although transcriptional activation of the α -SMA gene can be demonstrated, the possibility of derepression of this gene in myofibroblast differentiation is suggested by identification of transcriptional repressors of this gene. The list of known repressors includes KLF4, PPAR γ , YB-1, and Nkx2.5 (1, 2, 47). KLF4 appears to repress gene expression by competing with the transcriptional activator KLF5 for binding to the same element (TGF- β control element) in the α -SMA promoter and by interacting with the MH2 domain of Smad3 to interfere with

its binding to the Smad binding element located upstream from the TGF- β control element. Nkx2.5 mediates repression by distinct consensus binding sequences present in the α -SMA promoter (47). Induction of myofibroblast differentiation is accompanied by reduction in Nkx2.5 expression, which is also observed *in vivo* in pulmonary fibrosis. Conversely, inhibition of differentiation, such as by treatment with FGF2, is accompanied with heightened expression of Nkx2.5. These observations provide some support for the concept that myofibroblast differentiation may be a derepression phenomenon, or at least a contributory factor to the transcriptional activation mechanism.

Epigenetic Regulation

In conjunction with transcriptional regulation, epigenetic regulation is an additional level of control in cell differentiation. This can be broken down to several interacting mechanisms that include DNA methylation, modification of histones and their interaction with DNA, and microRNA (miRNA)-mediated regulation of gene expression. There is mounting evidence that all these mechanisms are involved in myofibroblast differentiation. For example, the importance of histone acetylation is suggested from studies showing that inhibition of histone deacetylase (HDAC) with trichostatin A results in suppression of differentiation (48). Subsequently, HDAC4, HDAC6, and HDAC8 are found to be the key ones that mediate activation of differentiation, with HDAC4 mediating its effect via activation of Akt (49, 50). Interestingly, HDAC inhibition *in vivo* suppresses fibrosis, suggesting that this may be mediated by suppression of myofibroblast differentiation (51). Evidence is more limited for the role of histone methylation in regulation of myofibroblast differentiation (52). Given that histone acetylation is associated with activation of gene expression, the importance of HDAC in the induction of α -SMA gene expression is likely indirect, perhaps by suppressing the expression of genes that inhibit or repress the α -SMA gene.

Another mechanism for gene silencing is via methylation of the relevant DNA sequences, which is mediated by DNA methyltransferases (Dnmts). Demethylated sequences are usually associated with derepression of the affected genes. Analysis of the α -SMA gene and adjacent sequences revealed a high degree of methylation in three identified CpG islands in lung type II alveolar epithelial cells, which do not normally express this gene (53). In lung fibroblasts, methylation is high only in the CpG island located in the promoter region, which is significantly reduced on induction of myofibroblast differentiation. Moreover, induced overexpression of Dnmts suppresses, whereas Dnmt deficiency enhances myofibroblast differentiation. Thus, DNA methylation is associated with silencing of the α -SMA gene or suppression of myofibroblast differentiation. However, DNA methylation affects the expression of other fibroblast genes whose products may also regulate myofibroblast differentiation (52). The net effect of DNA methylation in all the affected genes will ultimately determine the overall impact on this process and α -SMA gene expression. A complicating factor is the role of the methylated DNA-binding protein, MeCP2, which is recently shown to be essential for pulmonary fibrosis (54).

Regulation by miRNA

An expanding list of miRNAs is shown to regulate myofibroblast differentiation and fibrosis. The list of those that affect myofibroblast differentiation is smaller and includes Let7, miR-132, and miR-21, targeting HMGA2, MeCP2, and Smad7, respectively (52, 55). The effect of these miRNAs on their targets is presumed to be the mechanism by which they subsequently regulate

myofibroblast differentiation and fibrosis *in vivo*. Other miRNAs, such as miR-29 and miR-192, have been implicated in regulation of extracellular matrix genes and fibrosis, but their effect on myofibroblast differentiation itself has not been fully investigated.

SUMMARY AND FUTURE DIRECTIONS

Tissue remodeling or fibrosis in response to injury can progress to chronic fibrotic disease associated with persistence of myofibroblasts. Their continued presence results in the persistent abnormally high level of extracellular matrix production and deposition, fibrogenic cytokine expression, and impaired regeneration. Because of these effects, targeting the myofibroblast represents a logical strategy for treatment of fibrotic diseases. Such a strategy would require elucidation of the genesis of this cell type, whose persistence is postulated to be the basis for chronicity and progression of fibrosis. Currently, there is some controversy regarding the candidate precursors for myofibroblasts, with a list that includes fibroblasts, fibrocytes, pericytes, and epithelial and endothelial cells. However, the evidence for fibroblasts as myofibroblast precursors is undisputed. Extensive studies have revealed the mechanisms underlying the genesis of the myofibroblast from the various precursors. These have implicated virtually all known major signaling pathways, including MAP kinases, Wnt, Notch, and hedgehog, depending on the nature of the differentiating stimulus and the precursor cell type. Consequently, it is not surprising that multiple transcriptional and epigenetic regulatory mechanisms have been reported to mediate myofibroblast differentiation. Thus, complex and interacting mechanisms are involved in the genesis of the myofibroblast, and their further elucidation is essential for progress in designing novel therapies targeting the myofibroblast in chronic fibrotic diseases that are currently without effective treatment.

At the level of the genesis of the myofibroblast, a multiplicity of targets is suggested based on the review of the known underlying mechanisms above. For example, if bone marrow derivation is important, strategies to target their recruitment to the affected injured organ could be devised by targeting the suspected responsible chemokines. Other strategies would abrogate differentiation at the level of the culprit agonists or activator of myofibroblast differentiation, such as TGF- β . Associated signaling pathways can also be targeted and with some success in animal model studies. The results of clinical studies using some of these strategies have not been encouraging, however, and underline the need for novel approaches. The more recent advances implicating additional signaling pathways, epigenetic mechanisms, and the importance of epithelial-mesenchymal crosstalk should open up new avenues for future studies into novel therapeutic strategies. It may also be beneficial to investigate approaches that will promote the selective elimination of the myofibroblast. However, despite the importance of targeting the myofibroblast, it is likely that a multimodal therapeutic strategy may be necessary that will additionally promote regeneration of the parenchymal elements. Sole control of the mesenchymal response as represented by the myofibroblast is likely to be insufficient for successful repair and regeneration of normal tissue architecture and function.

Author disclosures are available with the text of this article at www.atsjournals.org.

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