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Host Responses in Tissue Repair and Fibrosis

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Abstract

Myofibroblasts accumulate in the spaces between organ structures and produce extracellular matrix (ECM) proteins, including collagen I. They are the primary "effector" cells in tissue remodeling and fibrosis. Previously, leukocyte progenitors termed fibrocytes and myofibroblasts generated from epithelial cells through epithelial-to-mesenchymal transition (EMT) were considered the primary sources of ECM-producing myofibroblasts in injured tissues. However, genetic fate mapping experiments suggest that mesenchyme-derived cells, known as resident fibroblasts, and pericytes are the primary precursors of scar-forming myofibroblasts, whereas epithelial cells, endothelial cells, and myeloid leukocytes contribute to fibrogenesis predominantly by producing key fibrogenic cytokines and by promoting cell-to-cell communication. Numerous cytokines derived from T cells, macrophages, and other myeloid cell populations are important drivers of myofibroblast differentiation. Monocyte-derived cell populations are key regulators of the fibrotic process: They act as a brake on the processes driving fibrogenesis, and they dismantle and degrade established fibrosis. We discuss the origins, modes of activation, and fate of myofibroblasts in various important fibrotic diseases and describe how manipulation of macrophage activation could help ameliorate fibrosis.

Keywords

myofibroblast; pericyte; fibrotic disease; fibrocyte

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DISCLOSURE STATEMENT

V.J.T. holds a patent with the University of Michigan on treatment of fibrotic diseases with RNAi approaches targeting NOX4. T.A.W. holds patents related to the targeting of IL-13 and IL-21. J.S.D. holds patents related to the targeting of DKK proteins and bone marrow progenitor cells in the treatment of fibrotic diseases. J.S.D. is on the scientific advisory board of Regulus Therapeutics and Promedior, Inc. M.L. holds patents related to the use of PTX-2 therapeutics and is an employee of Promedior, Inc.

INTRODUCTION

Fibrosis results when tissues are damaged and normal wound-healing responses persist or become dysregulated, usually in response to some type of repetitive injury. Despite having distinct etiological and clinical features, most chronic fibrotic disorders have in common a persistent irritant that sustains the production of growth factors, proteolytic enzymes, angiogenic factors, and fibrogenic cytokines, which stimulate the deposition of extracellular matrix (ECM) components (e.g., hyaluronic acid, fibronectin, and interstitial collagens) that progressively remodel tissues. Although mechanical injuries and structural changes can contribute to pathological fibrosis (1), most fibrotic diseases result from chronic exposure to toxins, irritants, immune complexes, or persistent infections. There is also emerging evidence that commensal bacteria living on our skin and mucosal tissues contribute to progressive fibrotic disease by persistently activating the innate immune response (2). Inherited genetic disorders, chronic autoimmune reactions, minor human leukocyte antigen mismatches in transplants, cardiovascular disease, high serum cholesterol levels, obesity, poorly controlled diabetes, and hypertension are also important triggers of fibrosis. In many fibrotic diseases, such as liver cirrhosis, cardiovascular fibrosis, systemic sclerosis, chronic kidney disease, and idiopathic pulmonary fibrosis, pathological tissue remodeling can progress to a stage in which organ failure and death are an inevitable outcome.

When injuries occur, dead or dying structural cells (e.g., epithelial and endothelial cells) release inflammatory mediators that initiate an antifibrinolytic coagulation cascade, which triggers platelet aggregation, clot formation, and development of a provisional ECM. Platelet degranulation also promotes vasodilation, increased blood vessel permeability, and production of enzymes known as matrix metalloproteinases (MMPs), which temporarily disrupt the basement membrane, allowing the efficient recruitment of inflammatory cells to the site of injury. Epithelial and endothelial cells also secrete growth factors, cytokines, and chemokines, which promote the recruitment and activation of leukocytes that participate in wound repair. During this initial inflammatory phase, macrophages and neutrophils debride the wound. They also produce soluble mediators that amplify the wound-healing response by recruiting T cells and other inflammatory cells. These cells, in turn, secrete various wound-healing/profibrotic cytokines, including platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β1, and interleukin (IL)-13 (3). These mediators transform local and recruited fibroblasts into α–smooth muscle actin (α-SMA)-expressing and ECM-producing myofibroblasts. Myofibroblasts are derived primarily from an extensive network of organ resident mesenchymal cells, which include cells known as fibroblasts and those known as pericytes due to their close relationship with the capillary wall. Once activated, myofibroblasts promote wound contraction, a process in which the edges of the wound are physically pulled toward the center. They also secrete factors that are mitogenic and chemotactic for epithelial and endothelial cells, which grow inward, forming new ECM and blood vessels as they migrate toward the center of the wound. In situations wherein the injury is repetitive, this normal wound-healing program becomes dysregulated, causing an excess deposition of ECM components (scar tissue) that progressively distorts normal tissue architecture.

Because myofibroblasts are the key pathogenic cells in all fibrotic diseases, research on the mechanisms of fibrosis has focused on identifying the cellular origins of myofibroblasts, the factors that activate myofibroblasts, and the mechanisms that contribute to myofibroblast deactivation and apoptosis. Recent studies have also identified critical roles for various macrophage subpopulations in the initiation, maintenance, and resolution phases of fibrosis (4). Macrophages are derived either from resident tissue populations or from bone marrow immigrants. In this review, we discuss the roles of the various myofibroblast and

macrophage populations in fibrogenesis and briefly describe how this information might be used to generate the first generation of highly effective antifibrotic drugs.

MYOFIBROBLASTS AS EFFECTOR CELLS IN TISSUE REPAIR AND FIBROSIS

Myofibroblasts, originally described in skin wounds in 1972, are defined biochemically by their expression of actin-myosin proteins shared by smooth muscle cells (SMCs), morphologically by the formation of stress fibers, and physiologically by their ability to contract tissues (5, 6). Myofibroblasts are connective tissue fiber–forming cells, that is, cells that generate and deposit pathological ECM proteins and proteoglycans, known as fibrosis, that mature and organize into scar tissue. They accumulate in the virtual spaces between organ structures and produce ECM proteins (7), including collagen I and EDA-containing fibronectin (8). They also produce soluble mediators and reactive oxygen species (ROS). By virtue of these and other salient properties, myofibroblasts are strongly implicated as the primary “effector” cells in tissue remodeling and fibrosis. Myofibroblast activation may be sufficient to explain the cardinal morphologic features of fibrosis in diverse organ systems that are characterized by organ contraction (resulting in reduced size), loss of tissue architecture and cellular homeostasis, deposition of highly cross-linked ECM, and aberrant phenotypes of neighboring cells.

Myofibroblasts as Contractile Cells

Despite the lack of a unique set of biomarkers that define myofibroblasts, the functional capacity to contract tissues is an essential feature. Indeed, the commonly used marker α-SMA, although not specific, is essential for generating maximal myofibroblast contractile activity (9). Although both SMCs and myofibroblasts express α-SMA, their contractile activities are distinct. Whereas SMC contractions are reversible and short lived, myofibroblasts generate isometric contractions that are irreversible and long lived (10). Maintenance of isometric contraction by myofibroblasts is accomplished by a so-called lockstep mechanism that requires continual synthesis and remodeling of the ECM to sustain pericellular tension (10). Indeed, there is growing recognition that biomechanical signaling, via tissue “stiffness,” is a pivotal mechanism for promoting and sustaining the differentiated, contractile myofibroblast phenotype (11, 12). Recent studies indicate that increased matrix stiffness may be sufficient to activate latent TGF-β1 (13), which supports a positive feedback cycle that may perpetuate fibrogenesis. Relaxin, an endogenous peptide hormone, modulates fibrogenic responses to lung injury by inhibiting myofibroblast contractility (14).

Myofibroblasts in Matrix Deposition and Remodeling

Newly secreted matrix undergoes active remodeling by secreted MMPs and tissue inhibitors of metalloproteinases. Myofibroblasts “sense” the biochemical and biophysical properties of the ECM, primarily via cell-surface integrins. Matrix properties are also modified by the action of cross-linking enzymes such as lysyl oxidase (15) and transglutaminases (16). ECM cross-linking may also be mediated by oxidant-dependent mechanisms involving ROS-generating enzymes and extracellular peroxidases (17). Thus, dynamic changes in ECM deposition and remodeling “feed back” on the myofibroblast to regulate its activities, while also influencing the function and fate of neighboring cells.

ORIGINS OF MYOFIBROBLASTS

Historical Context

In historical studies dating back to 1867, principally in studies of skin wounding, myofibroblasts were referred to as wound fibroblasts (18) and, until the 1980s, were studied

and defined by electron microscopy (EM). In 1970, a set of elegant experiments using parabiotic rats (rats surgically joined together so as to give them a common circulation) showed that wound fibroblasts do not arise from hematogenous precursors but rather are of local origin (18). Thus, the wound fibroblast, defined by EM in skin, derives from local mesenchyme cells. The term myofibroblast, coined in 1972 by Gabbiani & Majno (6) at the University of Geneva, Switzerland, from EM studies of skin, is perhaps now a misnomer because it implies a separate lineage of cells that appear in a diseased tissue; this implication has been refuted by recent fate mapping studies (see later in this section) (19–25). This terminology is, however, firmly embedded in the study of fibrosis and will persist. The distinction between wound fibroblasts and myofibroblasts is made by EM: Wound fibroblasts have dense rough endoplasmic reticulum (ER) and lack lysosomal vacuoles, whereas myofibroblasts also have modestly developed myofilaments with visible stress fibers (Figure 1) (6). In normal internal organs, activated myofibroblasts are believed to be absent.

The Quest for the Myofibroblast Precursor

In the late 1970s to 1980s, investigators found that antibodies that bind specifically to the smooth muscle marker α-SMA specifically label myofibroblasts in the skin. Subsequently, many common and rare diseases of internal organs were discovered to be characterized by fibrosis and the presence of cells equivalent to the skin myofibroblasts (Figure 1) (26–29). Other antibodies against quiescent fibroblasts in vitro, including the calcium binding S100A4 protein, fibroblast-specific protein 1 (FSP1), did not detect a resident population in the internal organs (30). Because of the apparent absence of mesenchymal progenitors in most internal organs, the quest for the myofibroblast progenitor has identified an extensive number of candidates: circulating cells derived from a stromal precursor possibly of bone marrow origin, circulating cells derived from a hematopoietic precursor, epithelial cells, and endothelial cells. Through this quest, investigators have gained many insights into the function, injury responses, and potential plasticity of the respective candidate progenitors. Until very recently, it was widely accepted that in several internal organs (e.g., liver, kidney, and lung), epithelial cells functioned as a primary source of myofibroblasts through a dedifferentiation process known as epithelial-to-mesenchymal transition (EMT), a process that was previously well recognized in embryogenesis (31). Also widely reported is evidence for leukocyte progenitors of solid organ myofibroblasts, which are now known as circulating fibrocytes (32–34). Less well established, but reported to occur in several organs including the heart, is the transition of endothelial cells to myofibroblasts (EndoMT) (35). However, the original studies that initially established the candidacy of these cell types as precursors for myofibroblasts were performed on cells cultured in vitro. In retrospect, these cells appear to have suffered from artifacts of cell culture: Almost all cells, when grown in plastic dishes, can be stimulated to activate α-SMA or FSP1 (S100A4) (22, 36–38), but that does not mean they are pathological fibrillar matrix–forming myofibroblasts in vivo. Moreover, FSP1 (S100A4), now widely used as a surrogate marker of EMT or EndoMT, is in fact highly expressed in subpopulations of activated monocyte–derived macrophages (not myofibroblasts) in vivo, both in liver and in kidney; this observation confounds interpretation of many studies (21, 22, 39). In addition, fate mapping studies that used Tie-1 or Tie-2 promoters to drive Cre recombinase suffered from the well-described activation of Tie-1 and Tie-2 in myeloid lineage cells, which led to an obvious source of artifact without the concurrent use of bone marrow chimerism (40, 41). These potential problems, among others, have been reported in several prominent review articles (42, 43).

Although these cellular sources may be of merit and are difficult to completely discount, there is an extensive network of embryonic mesenchyme–derived cells throughout all of our internal organs, and these cells become activated in response to tissue injury (Figure 2).

Recent advances in immunodetection, fixation methods, and novel genetic methods to study cells in mice have rendered this mesenchymal network much more visible to investigators. More importantly, state-of-the-art genetic fate mapping experiments in several organs, including kidney, lung, central nervous system, skin, liver, and skeletal muscle, now indicate that these mesenchyme-derived cells are the precursors of scar-forming myofibroblasts (19–25, 44–47), and many parallel genetic fate mapping studies show little or no evidence of direct differentiation of epithelial cells or leukocytes, or even endothelial cells, into myofibroblasts (Figure 3) (19–22, 42, 47–54).

As of early 2012, there has been a sea change in our thinking about the origin of myofibroblast progenitors in the solid organs. Almost all myofibroblast progenitors probably derive from activation of the embryonic mesenchyme–derived cells within each of our organs. What remains unclear is what contribution, if any, myeloid leukocytes make to the pool of myofibroblasts. There are conflicting reports from studies on the extent of myeloid cells in injured organs that directly lay down pathological matrix, compared with the extent of myeloid cells that drive fibrosis by indirect mechanisms. Perhaps the most compelling studies are those using bone marrow chimera mice that express collagen Iα1–transgenic reporter in bone marrow–derived cells to show myeloid cells that actually make pathological collagen I protein. These studies in kidney, skin, and lung confirm that a rare (<0.1%) myeloid leukocyte subset that expresses CD45 and CD34, known as circulating fibrocytes, can generate low-level transcripts for the collagen I protein in kidney disease, but these cells do not become myofibroblasts (i.e., do not express α-SMA) and probably do not deposit fibrillar matrix (21, 54). In models of liver disease, however, such fibrocytes made up 5% of all collagen-producing cells, and a minority expressed the myofibroblast marker α-SMA (54). There may be organ-to-organ variation in myeloid cell differentiation that makes myeloid leukocytes more likely to directly lay down filamentous matrix. Regardless of such nuances, current data indicate that endothelial cells, epithelial cells, and myeloid leukocytes contribute to fibrogenesis predominantly by indirect mechanisms that involve cell-to-cell signaling, rather than differentiation into a new and distinct cell type.

During embryogenesis, mesenchyme-derived cells form an important and large cellular component of the forming organs. Investigators had long assumed that these cells, known in embryonic development as stromal cells (which are embedded in loose connective tissue framework), essentially disappear in adult fully formed organs. However, recent studies indicate that these cells persist in large numbers; although they silence numerous gene markers, rather than become inactive or defunct, they probably play critical roles in organ homeostasis that are currently poorly or only partially understood (21–23, 42, 47, 55–60). In the quiescent state, these mesenchyme-derived cells are extremely discrete; they have extensive, extremely fine cell processes that extend over many cell lengths (Figure 4). During development, embryonic mesenchyme–derived stromal cells play critical roles in organogenesis, angiogenesis, and cell maturation. Strikingly, during embryonic development, these stromal cells express the same markers as myofibroblasts (including collagen transcripts and α-SMA) observed in adult organ disease, yet they do not make scar tissue; rather, one of their roles is to synthesize loose connective tissue or stroma (21). Therefore, it appears that (*a*) in development, the mesenchymal stromal cells are activated and migratory; (*b*) in the healthy mature organ, they are less activated and not migratory; but (*c*) in response to disease, they reactivate and become migratory again. The deposition of pathological filamentous matrix is only one of their functions.

Adult Mesenchymal Cells

The mature organ mesenchyme–derived cell populations (also known as stromal cells) bear remarkable similarities across organs but also have distinct differences. All these cell populations have long processes, which are characteristic of neurons and neuronally

associated cells (Figure 4). They express many neuronal markers (Table 1), and although the stroma of each organ is widely believed to be derived from the mesenchyme that is local to that organ (and therefore is thought to be imprinted), there is tantalizing evidence that early neural crest precursors may migrate to and contribute to the mesenchymal precursors throughout the body (47, 61). Whereas the lymphoid subpopulations are well characterized, mesenchymal characterization is still in its infancy, held back by the failure over many years to adequately identify these cells and by the lack of a broad array of antibodies against cell-surface markers.

Pericytes

Pericytes, first described nearly 140 years ago (62), are mesenchymal cells that are defined electron micrographically by the direct communication between pericyte processes and endothelial cells. Pericytes are sheathed with basement membrane (Figure 4) (64–66), a duplication of capillary basement membrane (CBM). Almost all microvasculature has a discontinuous coating of mesenchymal cells known as mural cells or pericytes (Figure 4) (64, 65). Pericytes contribute to the synthesis and maintenance of the CBM (69). The CBM coverage between pericytes and endothelial cells is frequently incomplete, which allows for direct cell-cell contact (64–66), and invaginations in either cell type known as peg-and-socket junctions may contain tight, gap, and adherens junctions and may be sites of signaling (Figure 4). Although pericyte processes are always covered by the CBM, this coverage may be incomplete in the area of the cell body (62, 67, 68). Due to a lack of well-characterized markers, pericytes remain defined histologically by their relationship to the CBM. Given that most studies of resident fibroblasts do not visualize the CBM, many mesenchymal cells in solid organs that are reported as resident fibroblasts may also have pericyte functions.

Pericyte Functions

Over the past 20 years, studies of pericytes, derived predominantly from brain and eye, but more recently described in cancer biology, have established a broad range of functions for these cells that relate mainly to the vasculature. A key property of pericytes is that they migrate to and bind to capillaries (Figure 4). However, they are critical for building new blood vessels, stabilizing them, and actively regulating their functions (Table 2)

Pericytes as Myofibroblast Precursors

Although pericytes were described in the kidney 30 years ago (22, 67) and have been little studied until recently, it was studies in the kidney that first uncovered the pericyte origin of myofibroblasts (21, 22). Through the use of a transgenic mouse expressing green fluorescent protein (GFP) under the regulation of the collagen I promoter (Coll-GFP), numerous pericytes and fibroblasts that were closely associated with the vasculature were identified in normal kidney cortex and medulla. The pericytes were attached by multiple long processes to peritubular capillaries embedded in the CBM (Figure 4) (21, 70). In response to kidney injury, adult kidney pericytes detached from capillaries, rapidly upregulated collagen Iα1 expression, migrated from the endothelium, and reactivated expression of the pericyte markers P75 nerve growth factor receptor and α-SMA within a few hours of the onset of injury (Figure 4) (21). Four days after injury, the population of active, collagen Iα1–expressing interstitial pericyte cells had expanded markedly, and the vast majority of these cells activated the expression of α-SMA, thereby defining them as myofibroblasts. A mathematical kinetic analysis of the expansion of Coll-GFP-expressing pericytes with time after injury strongly suggested that the appearance of Coll-GFP-expressing myofibroblasts could be explained by detachment, migration, and expansion of the original population of mesenchyme-derived pericytes (21).

To characterize the fate of kidney pericytes more definitively, Humphreys et al. (22) adopted a genetic fate mapping approach. These authors generated mice expressing the DNA recombinase enzyme Cre at the loci of transcription factors expressed by discrete subpopulations of embryonic kidney precursors (stem cells) in metanephric mesenchyme (22). The Foxd1 transcription factor gene locus was selected because Foxd1 progenitors become kidney pericytes as well as mesangial cells and vascular SMCs (22, 36, 71–73). Expression of the *Cre* transgene under regulation of the Foxd1 locus in Rosa26 reporter mice allowed all pericytes, vascular SMCs, and mesangial cells of the kidney to be labeled (Figure 3) (22). In response to kidney injury (ischemia reperfusion injury, ureteral obstruction, or nephrotoxic serum nephritis), over 2 to 3 weeks there was a 15-fold increase in the Foxd1 reporter–labeled progeny, and all these cells activated the myofibroblast marker α-SMA (Figure 3) (22)—a fate that is identical to that of Coll-GFP cells in the *Coll-GFP* mouse (21). These findings strongly suggest that pericytes and perivascular fibroblasts are the predominant source of myofibroblasts in mouse kidney injury. The fate mapping findings are further supported by definitive cohort labeling obtained through the conditional, tamoxifen-sensitive, CreER recombinase at the Foxd1 locus (22).

Another research group identified the same cells by using a different reporter system, and these investigators drew similar conclusions: All myofibroblasts in kidney disease come from activation of the PDGF receptor (PDGFR)-β^+ $CD73^+$ mesenchymal cells found in the normal kidney. Not long after these experiments were performed, investigators studying spinal cord, lung, skin and skeletal muscle, and intestine also identified pericytes as the major population of myofibroblast precursors (21–23, 42, 47, 55–60, 74).

A role for the hepatic stellate cell (HSC) of liver in fibrogenesis has long been suspected (75). However only recently has the fate of HSCs as the major source of myofibroblasts been definitively established (19, 39, 48, 50, 76). In parallel with studies in the kidney, fate mapping studies have comprehensively demonstrated that epithelial cells are not a source of myofibroblasts but that, in most liver diseases, myofibroblasts derive almost exclusively from HSCs and SMCs of the larger vessels (19, 39, 48, 50, 76). These studies used Cre/Lox and inducible Cre systems to map epithelial cells, HSCs, or portal fibroblasts. Studies of biliary tract disease show that portal fibroblasts also contribute to the total population of myofibroblasts, although in this disease, HSCs are nevertheless major contributors to the total myofibroblast population. HSCs express PDGFR-β and glial fibrillary acidic protein but have a different lineage from that of Foxd1 progenitors in the kidney (19, 39, 48, 50, 76). HSCs lie in apposition with sinusoidal endothelial cells, and their long cell processes lie within a very loose CBM. They play an important homeostatic role in the maintenance of sinusoidal endothelial cell fenestrations by delivering vascular endothelial growth factor (VEGF); when they become myofibroblasts, this function is lost, which causes capillarization of the sinusoid (77). HSCs have direct angiogenic functions similar to those of pericytes (78, 79). HSCs are, therefore, a modified form of pericytes. Although much work remains to be done, it is likely that pericytes throughout the body are major sources of myofibroblast precursors.

RESIDENT FIBROBLASTS AND OTHER CELLS

In many tissues, including skin, heart, and lung, the presence of poorly defined resident fibroblasts has been well described, and these cells have been relatively easy to culture and study in vitro. However, until very recently the precise nature of resident fibroblasts [cells embedded in connective tissue (stroma) that produce collagen and other fibers] was poorly understood (70, 74). EM studies have revealed that many of these cells have close relationships with epithelial or endothelial cells. In lung development, the critical role of stromal cells in epithelial differentiation has been well described. Recent studies that have

enabled easy visualization of these cells indicate that they are much more extensively distributed than previously thought and that they may have critical functions in homeostasis, including pericyte functions. Many resident fibroblasts also have close associations with epithelial cells and may function as epithelial pericytes.

Surrounding the arterioles are perivascular fibroblasts or fibrocytes (21, 70, 74). These cells may be termed adventitial cells in other texts, and evidence suggests they have important immunomodulatory functions and that some serve as vessel wall progenitors (80). In some tissues, including skin and liver, some resident adult microvascular wall mesenchymal cells (i.e., pericytes) have progenitor cell functions (81); that is, they can differentiate into mature cells of the tissue, including vascular smooth muscle, white adipocytes, and possibly neurons. It is unclear whether all adult mesenchyme cells have this ability to act as progenitors for other cell types within their organ, or whether there are more restricted subpopulations of perivascular cells that have this capacity.

Studies over the past several years have investigated some of the signaling pathways involved in pericyte-to-myofibroblast transition. Although this area is rapidly evolving, it appears that the same signaling pathways that regulate angiogenesis in cross talk between endothelial cells and pericytes are critical in pericyte and fibroblast activation. Those pathways include TGF-β, PDGFR-β, PDGFR-α, and VEGF receptor 2 (VEGFR2). Indeed, loss-of-function studies of these pathways have shown them to be critical in the early events of pericyte activation, the appearance of myofibroblasts, and fibrogenesis. Other developmental pathways that are important in building the vasculature may also be significant in the development of a myofibroblast. These pathways include angiopoietin signaling; sphingosine kinase signaling; and the developmental pathways WNT, Hedgehog, and Notch. Current evidence suggests that in development these signaling pathways are carefully regulated but that in disease they are markedly dysregulated, resulting in an overactivated phenotype relative to development. It may be desirable to “dial down” these over-activated developmental signaling pathways to counteract the appearance of myofibroblasts in tissues. Also, extracellular regulators of the VEGFR signaling pathway and metalloproteinases play important roles in the activation of pericyte function. Pericytes must detach from capillaries, spread, and migrate and must withdraw from the CBM, which requires proteolytic activity. One such factor that is activated strongly and early after injury in kidney pericytes is the metalloproteinase ADAMTS1 (87). In different organs, distinct metalloproteinases probably play important roles in pericyte acquisition of the myofibroblast phenotype, and these metalloproteinases may prove to be useful targets in reversal of phenotype.

EPITHELIAL-TO-MESENCHYMAL TRANSITION VERSUS EPITHELIAL SIGNALING IN FIBROGENESIS

Although recent comprehensive studies (of up to 3 months’ duration) in animal disease models indicate that epithelial cells do not become scar-forming myofibroblasts in solid organs (20), ample data suggest that activation or injury to epithelial cells alone is sufficient to drive fibrogenesis in many organs, including liver, lung, and kidney (82). Injured epithelial cells undoubtedly undergo profound phenotypic changes, which include the acquisition of a migratory phenotype (83). However, in the relevant animal models, this activated migratory phenotype is restricted to migration to areas of denuded or damaged epithelial basement membrane, where repair and restitution occur. This activated migratory phenotype in response to injury may be termed EMT, given that injured epithelial cells (*a*) activate a program of gene expression, which includes the transcription factor SNAIL and the intermediate filament vimentin, and (*b*) acquire a more mesenchymal appearance and migrate. To many investigators, EMT has become synonymous with the appearance of

myofibroblasts in the interstitial space. Furthermore, injured epithelial cells do not express many of the genes that characterize the embryonic mesenchyme, and they do activate many genes that are not developmentally active.

Injured epithelial cells share only a few features with the embryonic mesenchyme. Increasing examples of epithelial stress, including ER stress, dysregulation of energy metabolism, and arrest in G_2 of the cell cycle, are sufficient to activate intracellular signaling programs that confer a profibrotic phenotype on the epithelial cell without necessarily activating the developmental EMT genes (84). Although there is no consensus, perhaps the term EMT should be restricted to situations wherein the EMT transcriptional program is selectively activated, rather than describing all activated, injured epithelial cells that are profibrotic. Only malignant epithelial cells migrate outside the confines of the epithelial structure. In the absence of epithelial cell differentiation into the cells that actually deposit pathological fibrillar matrix between cell structures, the epithelium must signal to the interstitial and perivascular spaces to activate stromal cells to become myofibroblasts.

Thanks to many years of EMT research, we know of several candidate molecules that drive fibrogenesis and may act as soluble factors that activate the pericyte and fibroblast populations (Table 3) (Figure 5). One of these soluble factors is TGF-β. Indeed, antibodies against TGF-β and antibodies against the TGF-β-activating $\alpha_v\beta_6$ integrin are in clinical trials as antifibrotics. Injured epithelial cells are a major source of both cytokines and activating integrin receptors. TGF-β acts as an important factor in vascular development through the signaling of endothelial cells to pericytes (85, 86). In fact, TGF-β mutant mice and TGF-β-activated kinase mutant mice die during fetal development due to vascular defects (87–89). Many other epithelium-derived factors probably play a role in epithelial cell signaling to the perivascular and interstitial spaces.

Recent studies indicate that active TGF-β is an important cytokine in pericyte- and fibroblast-to-myofibroblast transition, but across different organ settings it does not stimulate proliferation (90). Another epithelial factor that can signal to pericytes or fibroblasts is PDGF. PDGF signaling from the endothelium to pericytes is also critical in vascular development, particularly through PDGFR-β (91, 92). Injured or activated epithelial cells are a potent source of PDGFs, and the PDGFR signaling pathways are important in the initiation and progression of fibrogenesis in response to tissue injury (70, 90, 93). Several studies have implicated connective tissue growth factor (CTGF) and CyR61, two related ECM proteoglycans in fibrogenesis, and these factors are upregulated in injured epithelial cells across several organs. Other potential factors that are currently under scrutiny are the developmental signaling pathways Notch, WNT, and Hedgehog, as well as the nerve growth factor signaling pathway (94–96). Clearly, injured epithelium is a source of ligands for each of these pathways, and they may be important factors in driving fibrogenesis. In addition to developmental signaling pathways, epithelial cells are significant sources of proinflammatory cytokines and chemokines, which contribute directly to fibrogenesis in several organs (97).

EVIDENCE FOR INNATE IMMUNE RESPONSES IN MYOFIBROBLASTS AND THEIR STROMAL PROGENITORS

New lines of investigation have highlighted an important but previously poorly appreciated function of activated mesenchymal cells: their role as innate immune cells. Myofibroblasts in the liver, which we now know derive either from HSCs or from biliary duct fibroblasts, are highly phagocytic, and the glomerular myofibroblast precursor in the kidney, the mesangial cell, is also highly phagocytic (98, 99). Although myofibroblasts lack the specialized machinery of macrophages, this immune function is an important component of

their activated state. Whether this function reflects simply a clearance pathway for bacteria and tissue debris, or whether activated mesenchymal cells have antigen presentation roles, remains to be determined.

In recent gain-of-function studies using mutant, constitutively active PDGFR-β, Olson & Soriano (81) showed that PDGFR-β-activated brain pericytes in newborn mice generated many immune response factors. The most enriched factors were antigen presentation genes, pattern-recognition receptor (PRR) genes, interferon (IFN) signaling, chemokines, and the complement pathway (Table 4). This pattern of expression was associated with marked recruitment of leukocytes to the perivascular area. These observations are complemented by studies of PDGFR-β blockade in adult kidney injury. PDGFR-β blockade prevents pericyte activation in the injured kidney and prevents myofibroblast appearance, fibrosis, and microvascular rarefaction. However, blockade of this single receptor, which is restricted to mesenchymal cells in kidney, is potently anti-inflammatory, causing a 70% reduction in leukocyte recruitment to the injured kidney (70, 90).

Similar to the developmental studies, a more recent transcriptional array of adult kidney pericytes in vivo has shown that activated pericytes generate a wide range of inflammatory response genes (Table 4) (87). Together with results from recent studies of skin pericytes or skin myofibroblasts (100), these data indicate that activated pericytes and activated fibroblasts have critical immune response functions that had previously been unappreciated. Strikingly, chemokine receptor blockade in liver fibrosis exerts anti-inflammatory effects and antifibrotic effects through blockade of chemokine signaling in liver myofibroblasts (97). Anti-inflammatory strategies that have been directed at leukocytes may confer unexpected benefits by additionally targeting inappropriately activated mesenchymal cells.

THE FATE OF MYOFIBROBLASTS: KEY DETERMINANT BETWEEN NORMAL REPAIR AND FIBROSIS

The activation of tissue myofibroblasts is a highly conserved and stereotypic response to injury and normal wound healing (7, 101). Resolution of the reparative phase of wound healing requires apoptotic clearance of myofibroblasts (102). The mechanisms that cause myofibroblast apoptosis in physiologic wound repair, however, remain unclear; similarly, the mechanisms underlying an apparently apoptosis-resistant myofibroblast phenotype in progressive fibrotic disorders are not well defined (103).

In addition to inducing myofibroblast differentiation, TGF-β1 itself promotes myofibroblast survival. TGF-β1 activates two prosurvival signaling pathways, focal adhesion kinase and PKB/AKT, by mechanisms that involve cell adhesion and release of soluble growth factors, respectively (104, 105), and contribute combinatorially to myofibroblast survival (106). Importantly, the administration of a protein kinase inhibitor that modulates the activities of these prosurvival pathways attenuates fibrosis in a model of bleomycin-induced lung fibrosis (107). Therapeutic approaches that induce apoptosis of myofibroblasts and/or interfere with myofibroblast contractility and tissue stiffness may prove effective in the treatment of progressive fibrotic disorders.

Myofibroblasts are highly synthetic and secretory cells that elaborate a wide range of soluble lipid mediators and ROS, as well as cytokines and chemokines. This property has led to the characterization of myofibroblasts as inflammatory cells (108). Myofibroblasts may directly participate in innate immunity by responding to bacterial components via Toll-like receptors and activating inflammatory responses (109). Myofibroblasts may acquire an immune-privileged phenotype by expressing Fas ligand (FasL), killing Fas^+ lymphocytes, and resisting Fas-induced apoptosis (110). Paracrine effects of FasL-expressing myofibroblasts

may also induce apoptosis of adjacent epithelial cells (111); additional myofibroblast-secreted factors implicated in epithelial cell death include angiotensin peptides (112), TGF-β1 (113), and hydrogen peroxide (H_2O_2) (114).

NOX ENZYMES AND REACTIVE OXYGEN SPECIES IN INNATE IMMUNITY AND FIBROSIS

NADPH oxidase (NOX) enzymes and ROS play essential roles in innate immunity and host defense against pathogens across the plant and animal kingdoms. In plants, the generation of NOX-dependent ROS at the site of pathogen contact elicits both a localized hypersensitivity response and a systemic immune response (115, 116); this localized response includes ECM cross-linking, akin to a fibrotic response, to limit the spread of the pathogen. In fish, wounding triggers activation of a NOX family enzyme, DUOX, in epithelial cells; DUOX releases H_2O_2, thereby serving as both a local microbicidal agent and a neutrophil chemoattractant (117).

For several decades, investigators have known that the phagocyte NOX2 enzyme plays a key role in mammalian innate immunity by mediating ROS-dependent pathogen killing (118). More recently, a family of NOX enzymes that participate in expanded roles in innate immunity and other physiological functions was identified (119, 120). To date, seven isoforms of NOX enzymes have been identified in mammals (120).

Fibroblasts may express multiple NOX isoforms; however, the NOX4 isoform has been specifically implicated in myofibroblast differentiation and lung fibrosis (121–123). Transcriptomal analyses (using Affymetrix GeneChips) of human lung fibroblasts treated with TGF-β1 (123) identified *NOX4* as one of the most highly upregulated genes. *NOX4* activation mediates the generation of H_2O_2, myofibroblast differentiation, contractility, and ECM production in human lung fibroblasts (123). In tissues from human idiopathic pulmonary fibrosis (IPF) patients, the expression of NOX4 is localized to myofibroblasts, both within fibroblastic foci and in epithelial cells associated with aberrant bronchiolization (123, 124). Therapeutic delivery of NOX4 small interfering RNA protects against fibrosis in two different animal models of injury-provoked pulmonary fibrosis (123); protection has also been observed in mice with genetic deficiency in NOX4 (124). In addition to its effects on mesenchymal cells, NOX4 may induce apoptosis of lung epithelial cells, thereby contributing to lung fibrosis (124). These observations, in addition to NOX4's potential roles in vascular remodeling and pulmonary hypertension (125, 126), suggest that NOX4 mediates effects on multiple cell types and tissue compartments that contribute to organ fibrosis.

CYTOKINES THAT REGULATE MYOFIBROBLAST ACTIVATION

The proinflammatory cytokines tumor necrosis factor (TNF)-α and IL-1β are important drivers of fibrosis (127). TNF-α was identified as a key player in murine models of silica- and bleomycin-induced pulmonary fibrosis (128, 129). TNF-α may also play a critical role in radiation-induced fibrosis, Crohn's disease–induced intestinal fibrosis, CCL4- and cholestasis-induced liver fibrosis, and nonalcoholic steatohepatitis (NASH) (130–133). A similar role has been established for IL-1β and NALP3/ASC inflammasome signaling (134–136). Pulmonary fibrosis induced by bleomycin and silica, liver fibrosis in hypercholesterolemic mice, renal interstitial fibrosis arising from unilateral ureteric obstruction, and cardiovascular fibrosis following myocardial infarction were reduced in IL-1β-deficient mice (137–141). Like TNF-α, IL-1β is a potent proinflammatory mediator that promotes epithelial changes including EMT and myofibroblast activation through a TGF-β1-dependent mechanism (142, 143). Both cytokines also induce the production of

IL-6, which exhibits autocrine growth factor activity in fibroblasts (144, 145). IL-6 is an important mediator of fibrosis in diffuse systemic sclerosis, liver fibrosis following CCL4 exposure, and fibrosis in chronic cardiac allograft rejection (146–148). Thus, several proinflammatory cytokines appear to participate in the pathogenesis of fibrosis by promoting the differentiation and activation of myofibroblasts. Consequently, clinical trials have been initiated to evaluate whether neutralizing antibodies to TNF-α or other proinflammatory cytokines could be beneficial in the treatment of pulmonary fibrosis and other fibrotic diseases (149).

The $CD4^+$ T helper 17 (Th17) cell subset that expresses the proinflammatory cytokine IL-17A is also emerging as an important initiator of fibrosis. IL-17A expression has been implicated in the pathogenesis of pulmonary fibrosis (141, 150, 151), chronic allograft rejection (152, 153), fibrosis in orthotopic lung transplantation (154), myocardial fibrosis (155), and hepatitis-induced hepatic fibrosis (156). In most cases, IL-17A expression is associated with significant neutrophil recruitment (157, 158), and exaggerated neutrophil responses may contribute to the development of fibrosis by triggering vascular endothelial cell apoptosis (159). Importantly, neutrophilia is a reliable predictor of early mortality in IPF patients (160). Mechanistic studies investigating the IL-17 pathway of fibrosis identified the proinflammatory cytokine IL-1β and the Th17-inducing cytokine IL-23 as important upstream initiators of profibrotic Th17 responses (141, 161). A link between IL-17A expression and the profibrotic cytokine TGF-β1 has also been identified (141, 153, 162). TGF-β1 is important for the development of the Th17 response in mice exposed to bleomycin, whereas the development of IL-17-driven fibrosis depends on TGF-β1. In addition to its role in promoting inflammation, IL-17A directly induces MMP-1 expression in primary human cardiac fibroblasts (163), which suggests that IL-17A promotes fibrosis both by exacerbating the neutrophil-dominated inflammatory response and by regulating the downstream activation of fibroblasts. Together, these data characterize the IL-1β/IL-17A/TGF-β1 cytokine axis as an important pathway driving the development of fibrosis.

Numerous studies have suggested that the type 2 cytokine response can also serve as a key driver of progressive fibrosis (164–169). Th2 responses are defined by the production of IL-4, IL-5, and IL-13 (170), and although all three cytokines have been linked with the development of fibrosis in various model systems (171–173), IL-13 has emerged as the dominant mediator of fibrotic tissue remodeling in several experimental and natural models of fibrosis (173, 174). IL-13 is implicated in the development of fibrosis in chronic asthma (174, 175), IPF (176), models of experimental lung fibrosis (177), systemic sclerosis (178), atopic dermatitis–induced skin fibrosis (179, 180), radiation-induced fibrosis (181), intestinal fibrosis associated with Crohn's disease and ulcerative colitis (182), and liver fibrosis resulting from persistent infections (173, 183) and from NASH (184). Mechanistically, IL-13 induces fibrosis by stimulating the production and activation of TGF-β (185, 186). However, studies have suggested that IL-13 can also promote fibrosis independently of TGF-β (93, 187). Indeed, IL-13 directly stimulates the synthetic and proliferative properties of fibroblasts, epithelial cells, and SMCs (173, 176, 186, 188–193). Additional mechanistic studies have established that the profibrotic activity of IL-13 is controlled by the relative expression of IL-13Rα1 (signaling receptor) versus IL-13Rα2 (decoy receptor) on important target cells such as myofibroblasts, epithelial cells, and SMCs (194, 195). When decoy receptor expression (IL-13Rα2) is low on these cells, IL-13-dependent fibrosis is exacerbated due to enhanced IL-13/IL-13Rα1-mediated signaling (196). These findings suggest that therapeutics that target the IL-13 pathway could be a viable strategy to ameliorate fibrosis.

THE ROLE OF MONOCYTE AND MACROPHAGE POPULATIONS IN FIBROSIS PROGRESSION AND RESOLUTION

At a basic level, all multicellular organisms recognize injuries through the neoepitope exposure of normally sequestered intracellular biochemical molecules. Examples include specific phospholipids that are normally expressed on the inner cell membrane that flip to the outer cell membrane during stress and apoptosis (e.g., phosphotidyl serine and phosphotidyl ethanolamine), as well as nuclear components such as DNA, histones, and other nuclear proteins that are exposed only during necrotic or apoptotic cell death. These so-called damage-associated molecular patterns (DAMPs) are up-regulated at sites of injury and/or infection and stimulate a highly conserved innate cellular response controlled by monocyte-derived cells. Whether this innate cellular response causes mild acute inflammation and healing, robust chronic inflammation and autoimmunity, or subchronic inflammation and fibrosis is determined by the type of cellular debris, the cellular activation pathways that are used to recognize these basic DAMP signals, and the surrounding cytokine and growth factor milieu in which they are found.

Monocyte-derived cell populations (macrophages, dendritic cells, and fibrocytes) can dynamically control the fibrotic process through both direct effects on matrix remodeling and indirect effects on the regulation of activated myofibroblasts, their precursor populations, and endothelial cells (4, 197–203). Monocyte-derived cells play an important role in inflammation, and the subsequent development of fibrosis, in a range of organ pathologies (4, 199–201, 204–208). For example, macrophages and fibrocytes in vivo are often found in close association with collagen-producing myofibroblasts, and alternatively they can produce cytokines and growth factors that either stimulate or suppress myofibroblast activity. Importantly, the identification of distinct functional subsets of macrophages (M1, inflammatory; M2a-like, profibrotic; M_{reg}/M2c-like, regulatory) (4, 198, 200, 201, 203, 205–207) and their relative impact on fibrosis progression and resolution indicate that the equilibrium between these different macrophage populations and other monocyte-derived cells (such as fibrocytes) probably determines whether the outcome of an injury response is productive reepithelialization and healing or pathogenic scarring.

Monocytes promote progression of fibrotic disease through differentiation into M2a-like macrophages and fibrocytes that produce various fibroblast stimulatory growth factors and cytokines, such as TGF-β1, PDGF, FGF2 (fibroblast growth factor 2), insulin-like growth factor–binding protein 5, CCL18, and Galectin-3 (Figure 6) (4, 208–216); increased levels of these macrophage-secreted factors can constitute peripheral biomarkers of fibrotic disease progression (217). Through the stimulation of additional local tissue injury, M1-type macrophages may also provoke a fibrotic wound-healing response in the neighboring tissue cells that is independent of the production of cytokines that drive myofibroblast activation. Indeed, conditional ablation of macrophages at early stages of fibrosis blocks fibrosis progression in several fibrosis model systems (4, 206, 218). Many individual and redundant stimuli, including cytokines such as IL-4 and IL-13, growth factors such as macrophage colony-stimulating factor, and chemokines such as CCL17 and CCL2, contribute to the milieu in fibrosis that activates the differentiation of monocytes into the downstream fibrocyte and profibrotic macrophage cell populations (4, 219–223). In addition, once activated, the fibrocytes and profibrotic macrophages amplify the level and number of profibrotic cytokines and growth factors produced, thereby driving and accelerating myofibroblast activation (Figure 6).

Fibrocytes constitute a distinct subset of collagen-producing, fibroblast-like cells derived from peripheral-blood monocytes that enter sites of tissue injury to promote angiogenesis, scar production, and collagen contraction (202, 224). They differentiate from a $CD14^+$

peripheral-blood monocyte precursor population and express markers of both hematopoietic cells (CD45, major histocompatibility class II, CD34 cells) and stromal cells (collagen I and III and fibronectin). In humans, fibrocytes have been detected in fibrotic tissue from many different sources, including cutaneous wounds, hypertrophic scars, scleroderma skin lesions, asthma, IPF, nephrogenic fibrosing dermopathy, and solid tumors (202, 224). Increased levels of fibrocyte precursors have also been detected in the peripheral blood of IPF patients and scleroderma patients with lung fibrosis (225–227). Interestingly, subsequent studies in IPF patients indicated a significant correlation between higher fibrocyte levels in blood and both exacerbation of disease and mortality (228). In animal models, fibrocytes are associated with experimental fibrosis induced by irradiation damage, bleomycin injections into skin or lung, intimal hyperplasia of the carotid artery, systemic acetaminophen administration, chronic granuloma formation following *Schistosoma japonicum* infection, and cutaneous wounds (202, 224). They have also been detected in other organs, including diseased kidney and liver, but their role in the direct deposition of fibrogenic matrix in these organ settings is controversial (21, 204). Fibrocytes may represent a subset of macrophages and dendritic cells, similar to M2a macrophages that exert profibrotic effects by mechanisms other than direct deposition of pathological matrix (21).

The identification of M_{reg}/M2c-like macrophages that can promote resolution of fibrotic disease is a relatively recent advance in the field of fibrotic research. Macrophage depletion at the start of the recovery phase of liver injury severely suppresses ECM degradation and the reduction in myofibroblasts necessary for resolution (205), whereas transfer of nonfibrotic macrophages into mice reduces fibrosis in both the kidney and lung fibrosis models (222, 229). Macrophage-mediated resolution of liver fibrosis has also been directly linked to the production of MMP-13 (207), and MMP-9 overexpression in macrophages substantially reduces lung fibrosis (230). In models of liver fibrosis, macrophage phagocytosis of apoptotic hepatocytes also reduces inflammation and prevents the development of fibrosis (231), and phagocytosis of apoptotic cholangiocytes reverses existing fibrosis (232). A defining marker of regulatory macrophage function is the secretion of IL-10; direct IL-10 treatment, genetically modified or transfused IL-10-stimulated macrophages, or in vivo induction of macrophage IL-10 expression can ameliorate fibrosis and inflammation in kidney, gut, and brain (201, 204, 233–235). Arginase-1-expressing M2 macrophages have also been implicated in the amelioration of liver fibrosis induced by chronic *S. mansoni* infection (236). Therefore, monocytes can promote resolution of fibrotic disease by differentiating into regulatory macrophages that locally produce suppressor cytokines, including IL-10, by producing MMPs that can directly degrade interstitial collagen (e.g., MMP-1, MMP-2, MMP-8, MMP-9, and MMP-13), by locally depleting essential amino acids required for T cell and fibroblast proliferation, by actively promoting apoptosis of myofibroblasts, and by phagocytosing ECM and cellular debris that would otherwise stimulate inflammatory and fibrotic cell activation. This critical discovery has led investigators to search for mechanisms through which regulatory macrophage activity can be stimulated or amplified to naturally treat fibrotic disease.

THE ROLE OF PENTRAXIN-2 IN FIBROSIS REGULATION

Pentraxin-2 (PTX-2), commonly referred to as serum amyloid P, is a naturally circulating plasma protein and a soluble PRR of the innate immune system that controls monocyte differentiation and activation in response to damaged tissue. PTX-2 potently inhibits the differentiation of M2a-like macrophages and fibrocytes (34, 222), as well as M1 macrophages (237), while promoting the differentiation of IL-10-secreting M_{reg}/M2c-like macrophages (204, 222, 238). The unique pentameric structure of PTX-2 allows recognition of DAMP ligands through one face of the molecule to localize PTX-2 specifically to damaged tissue at sites of injury (204), and subsequent Fcγ receptor (FcγR) binding to the

opposite face of the molecule promotes phagocytosis (239–244) and regulatory monocyte function at those sites (200, 204).

Initial studies in preclinical models of pulmonary fibrosis demonstrated that purified PTX-2 exerts potent therapeutic effects in blocking fibrotic collagen deposition; these effects strongly correlated with both improved lung function and decreased numbers of fibrocytes, macrophages, and myofibroblasts in the lung (245). Subsequent studies confirmed that PTX-2 has preventative and therapeutic antifibrotic effects in multiple models of fibrosis in lung (218, 222, 227, 245), heart (246, 247), skin (33), and kidney (204, 248), as well as strong anti-inflammatory effects in an EAE (experimental autoimmune encephalomyelitis) model of multiple sclerosis (237). Importantly, the therapeutic effect of PTX-2 was consistent across both acute and chronic model systems; the widely varying modes of induction included obstructive injury, toxin exposure, reperfusion, allergic autoimmunity, and chronic transgenic TGF-β1 expression. In animals that have been treated with PTX-2, macrophages isolated from injured kidney (204), monocytes stimulated in vitro with cytokine cocktails (222), and macrophages analyzed in situ from injured lung (218, 222, 227) demonstrate a substantial suppression of profibrotic and proinflammatory activation markers. PTX-2 promoted a regulatory macrophage phenotype associated with increased expression of the antifibrotic cytokine IL-10 (204, 222, 227, 248) and the antifibrotic chemokine IP-10 (IFN-γ-induced protein 10) (218). Furthermore, the therapeutic effect of PTX-2 on kidney fibrosis was attenuated in IL-$10^{-/-}$, whereas IL-10 transgenic expression was itself therapeutic in vivo and directly inhibited myofibroblast expression of collagen in vitro (204). PTX-2 therapeutic activity was also substantially reduced in both cardiac (247) and kidney (204) fibrosis models when FcγR signaling was compromised (FcγR$\gamma^{-/-}$ mice), and adoptive transfer of monocytes treated in vitro with PTX-2 was therapeutic in reducing fibrosis in a fungus-induced asthma model (222). Together, these data indicate that PTX-2 couples recognition of DAMP ligands bound to one face of the molecule with binding to monocyte FcγRs at the opposite face of the pentraxin molecule, thereby affecting monocyte signaling events specifically at the sites of tissue injury (204, 249) and promoting a proresolution macrophage phenotype.

Initial human studies have also demonstrated a potential association between decreased PTX-2 levels in blood and fibrotic pathology. Patient and control samples were collected in two independent studies, and the serum levels of PTX-2 were determined (204, 218). In the circulation of patients with kidney disease, the level of PTX-2 was lower than in patients with minimal kidney disease (204), and it correlated with the glomerular filtration rate (a measure of kidney function). This finding suggests that, like complement proteins, binding and turnover at sites of inflammation may lead to consumption from the circulation (204). Human kidney biopsy specimens were also tested and showed deposition of PTX-2 in areas of damage in a range of kidney diseases (200, 204). PTX-2 concentrations were also reduced in the circulation of patients with histologically confirmed IPF, compared with concentrations in age-matched controls, and in the sera of IPF patients inversely correlated with disease severity (218). Given the associations between dysregulated M2 monocyte and fibrocyte levels that were also observed in IPF patients (202, 213, 217, 225–228), these data suggest that an inverse relationship may exist between PTX-2 levels in patients and insufficiently controlled monocyte biology in human fibrotic disease and that potential therapeutic intervention with PTX-2 may be warranted. A fully recombinant form of the human PTX-2 protein, PRM-151, is currently in multiple human clinical trials of fibrotic disease (http://www.promedior.com).

Several other signaling pathways in dendritic cells and macrophages regulate the monocyte response to activation by pathogen-associated molecular patterns (PAMPs) or DAMPs. Studies in several organs show that the canonical WNT signaling pathway transcriptional

regulator β-catenin can play a pivotal role in myeloid cells in the switch to an M_{reg}-type leukocyte in response to DAMP or PAMP activation. This transcriptional regulator may be activated by numerous cell signaling pathways, including the WNT pathway, as well as by other signaling pathways that act through the inhibitory cytosolic protein glycogen synthase kinase 3β (250). The effect of manipulating this pathway in myeloid cells in chronic inflammation and the consequences for fibrosis have not been established. Recent studies have established that signaling through the autophagy pathway in macrophages can promote an M_{reg} phenotype by mechanisms that are not yet understood (251, 252). Promoting signaling through these pathways during tissue injury may promote resolution of inflammation and inhibit fibrogenesis.

THERAPEUTIC APPROACHES TO THE TREATMENT OF FIBROSIS

On the basis of the large number of biochemical pathways reviewed in the sections above, an equally large number of therapeutic approaches to treat fibrotic pathology are currently in various stages of development (Table 5). To date, despite the enormous unmet medical need for an effective antifibrotic agent, the only approved drug treatment for fibrotic pathology in the United States is direct injection of collagenase (http://www.Xiaflex.com) into the joints of patients with Dupuytren's contracture, a proliferative fibrotic disorder that involves the palmar fascia of the hand, causing the fingers to curl. All other potential drug products are still undergoing clinical trials to prove their efficacy and safety.

One busy clinical area has been the study of treatments for IPF. Notably, outside the United States another drug, pirfenidone (http://www.intermune.com/pirfenidone), a relatively primitive p38 kinase inhibitor that reduces TGF-β synthesis, has been approved for the treatment of IPF in Japan (trade name, Pirespa™) and more recently in Europe (trade name, Esbriet™). Interestingly, the use of pirfenidone in the United States was rejected by the Food and Drug Administration in 2011 due to failure in one of two Phase III clinical studies; this drug is currently being retested in a new Phase III clinical trial. Some other notable failures in clinical development for IPF include recombinant human IFN-γ (trade name, ActImmune™; http://www.intermune.com), bosentan (trade name, Tracleer™, an endothelin antagonist; http://www.actelion.us), and imatinib mesylate (trade name, Gleevec, a mixed kinase inhibitor with selectivity for PDGFRs, Abl, and c-kit; http://www.novartis.com).

However, there is evidence that other molecules are making progress in the clinic. Vargatef (BIBF-1120, a mixed kinase inhibitor with selectivity for PDGFR, VEGFR, and FGF receptor; http://www.boehringer-ingelheim.com) recently showed a statistically significant delay in the rate of decline in lung function in IPF patients in a Phase II study and has advanced into Phase III testing. Many additional drugs based on more recently identified targets are in the initial stages of development; these include drugs that target inhibition of the TGF-β1, IL-13, LPA (lysophosphatidic acid), CTGF, $\alpha_v\beta_6$ integrin, Galectin-3, LOXL2, transglutaminase 2, NOX4, and JNK pathways, as well as those that stimulate the PGE2 (prostaglandin 2), HGF (hepatocyte growth factor), Nrf2, and PTX-2 pathways (Table 5). Only time will tell which pathways and molecules show the best promise, but given our rapid progress in the field of fibrotic research, the future looks bright indeed.

CONCLUSIONS

Although myofibroblasts from distinct organ sources are the primary drivers of fibrosis, it is becoming increasingly clear that various monocyte and macrophage populations also play key roles in the pathogenesis of fibrosis. Inflammatory macrophages promote fibrosis by producing specific MMPs, such as MMP-2 and MMP-9, that degrade the basement

membrane, allowing inflammatory cells and fibroblasts to migrate into areas of tissue injury. They also produce ROS and nitrogen species that are toxic to invading organisms. However, these cells also induce significant bystander damage to neighboring healthy tissues if they are not quickly controlled. Macrophages subsequently take on features of wound-healing macrophages. These cells remove cellular debris that would otherwise perpetuate the inflammatory response, antagonize the activity of M1 macrophages, engulf and digest ECM components, and induce the transformation of fibroblasts into ECM-secreting myofibroblasts. They also secrete various profibrotic mediators, such as TGF-β1, PDGF, and chemokines that recruit and activate cells involved in wound repair. In the final phase, macrophages take on a regulatory phenotype and express various mediators, such as IL-10, MMP-13, Relma-α, PD-L2, and Arginase-1, which are critical to the resolution of the fibrotic response and can direct the reversal of established fibrosis. Thus, future efforts should focus on better characterizing the macrophage subpopulations that induce, maintain, suppress, and ultimately reverse the profibrotic activity of myofibroblasts. This information could help guide the development of novel antifibrotic treatments, which would be based on modifying the wound-healing response so that tissue regeneration is favored over pathological fibrosis.

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Glossary

ECM	extracellular matrix
Matrix metalloproteinases (MMPs)	proteases that degrade ECM proteins, process numerous bioactive molecules, and cleave cell-surface receptors
TGF	transforming growth factor
α-SMA	α–smooth muscle actin
Pericytes	cells that surround the endothelial cell layers of the capillary network and play a critical role in scar formation
ROS	reactive oxygen species
Epithelial-to-mesenchymal transition (EMT)	a process in which epithelial cells are stimulated to differentiate into myofibroblast-like cells
Fibrocyte	a monocyte-derived cell that expresses both hematopoietic cell markers (CD34 and CD45) and collagen I
GFP	green fluorescent protein
NADPH oxidase 4 (NOX4)	an enzyme that produces ROS that exhibit antimicrobial activity but promote myofibroblast differentiation
IPF	idiopathic pulmonary fibrosis
Pentraxin-2 (PTX-2)	a naturally circulating plasma protein that can inhibit the differentiation of profibrotic macrophages and fibrocytes

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SUMMARY POINTS

1. Myofibroblasts are the primary effector cells in tissue remodeling and fibrosis.
2. Genetic fate mapping experiments suggest that mesenchyme-derived cells known as resident fibroblasts and pericytes are the primary precursors of scar-forming myofibroblasts.
3. Epithelial cells, endothelial cells, and myeloid leukocytes (fibrocytes) contribute to fibrogenesis by producing key fibrogenic cytokines and by promoting cell-to-cell communication.
4. Hematopoietic cells participate in the activation of myofibroblasts by producing various cytokines, including TGF-β1, TNF-α, IL-1, IL-6, PDGF, IL-17A, and IL-13.
5. Increased matrix stiffness can also perpetuate the activation of myofibroblasts by promoting the activation of latent TGF-β1.
6. Resolution of the reparative phase of wound healing requires apoptotic clearance of myofibroblasts. Therapeutic approaches that induce apoptosis of myofibroblasts and/or interfere with myofibroblast contractility and tissue stiffness may prove effective in progressive fibrotic disorders.
7. Circulating bone marrow–derived monocytes differentiate into macrophages and dendritic cells and can regulate all stages of fibrogenesis and repair. They can amplify epithelial cell damage by functioning as classical inflammatory cells; they can promote myofibroblast activation and proliferation through release of profibrotic cytokines and growth factors; and they can also reverse the disease by dismantling and degrading established fibrosis and by stimulating inactivation of myofibroblasts through production of IL-10.
8. Many antifibrotic drugs are at various stages of development, including those targeting the TGF-β1, IL-13, LPA, CTGF, αvβ6 integrin, Galectin-3, LOXL2, transglutaminase-2, NOX4, and JNK pathways, as well as those stimulating the PGE2, HGF, Nrf2, and PTX-2 pathways.

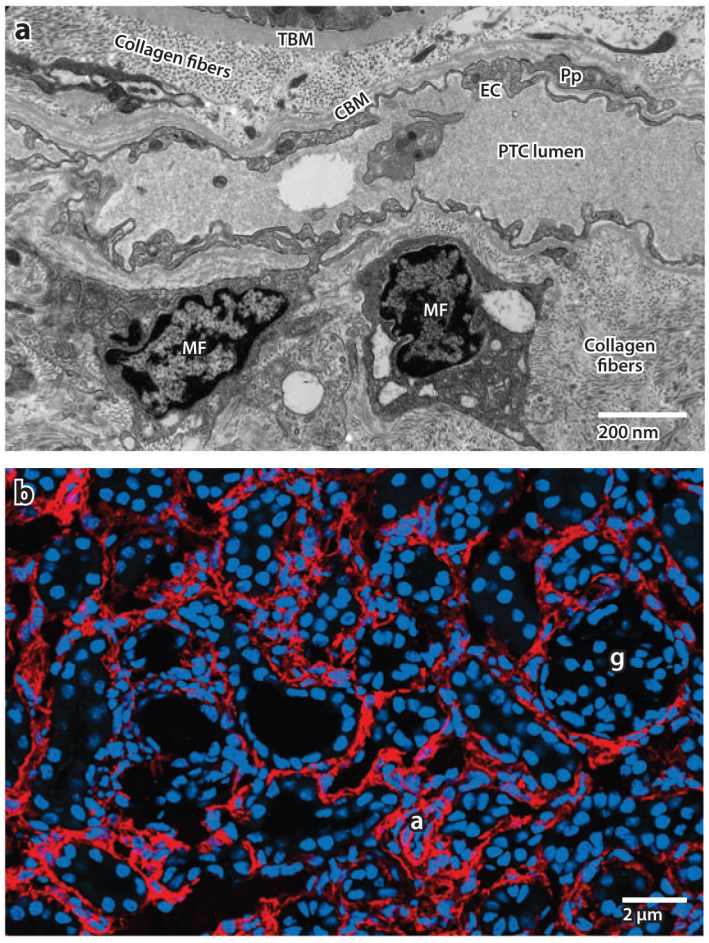

Figure 1.

(*a*) Electron microscope image of myofibroblasts (MFs) in the interstitial space of a kidney from a patient with chronic kidney disease. Note the abundance of rough endoplasmic reticulum in these cells due to high ribosomal activity, and note the markedly expanded interstitial space with collagen fibers.

Abbreviations: CBM, capillary basement membrane; EC, endothelial cell; PTC, peritubular capillary; TBM, tubule basement membrane. (*b*) Confocal image of α–smooth muscle actin–expressing MFs (*red*) in adult diseased mouse kidney. Abbreviations: a, arteriole; g, glomerulus.

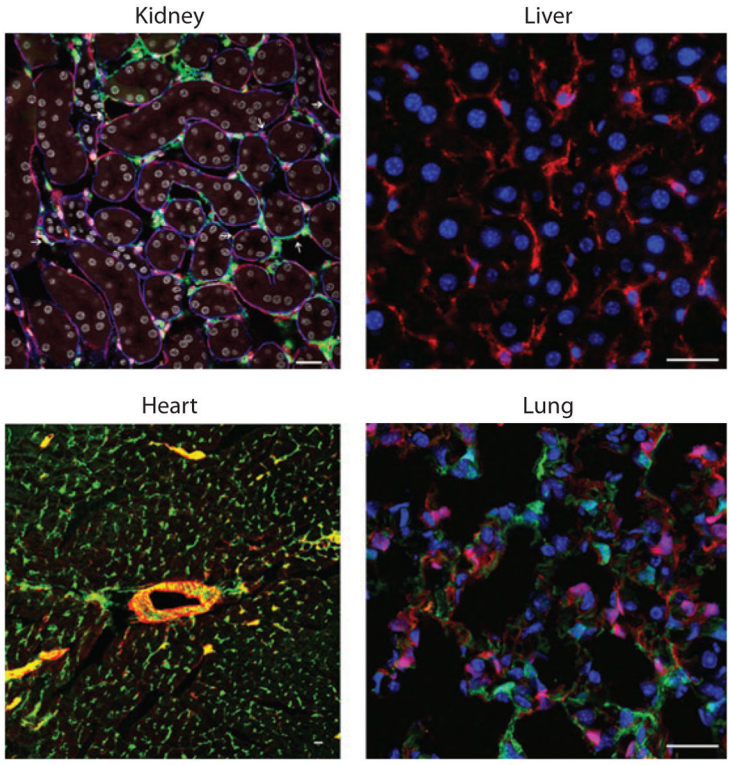

Figure 2.
Mesenchyme cells in normal organs of the mouse. Confocal fluorescence images showing normal adult liver, kidney, lung, and heart with fluorescent labels of the resident mesenchyme cells. All the cells lack markers of leukocytes, endothelial cells, and epithelial cells. The kidney image shows pericytes (*green*), endothelium (*red*), basement membrane (*blue*), and nuclei (*white*). The liver image shows hepatic stellate cells labeled with anti-PDGFR-β (platelet-derived growth factor receptor β) antibodies (*red*) and nuclei (*blue*). The heart image shows normal lung fibroblasts labeled with anti-PDGFR-β antibodies (*green*) and SM22 (*red*). The lung image shows alveolar spaces with two populations of stromal cells, one expressing collagen Iα1 (*green*) and the other PDGFR-β (*red*). Scale bars, 25 μm. Images reproduced courtesy of Dr. Michelle Tallquist, University of Texas, San Antonio.

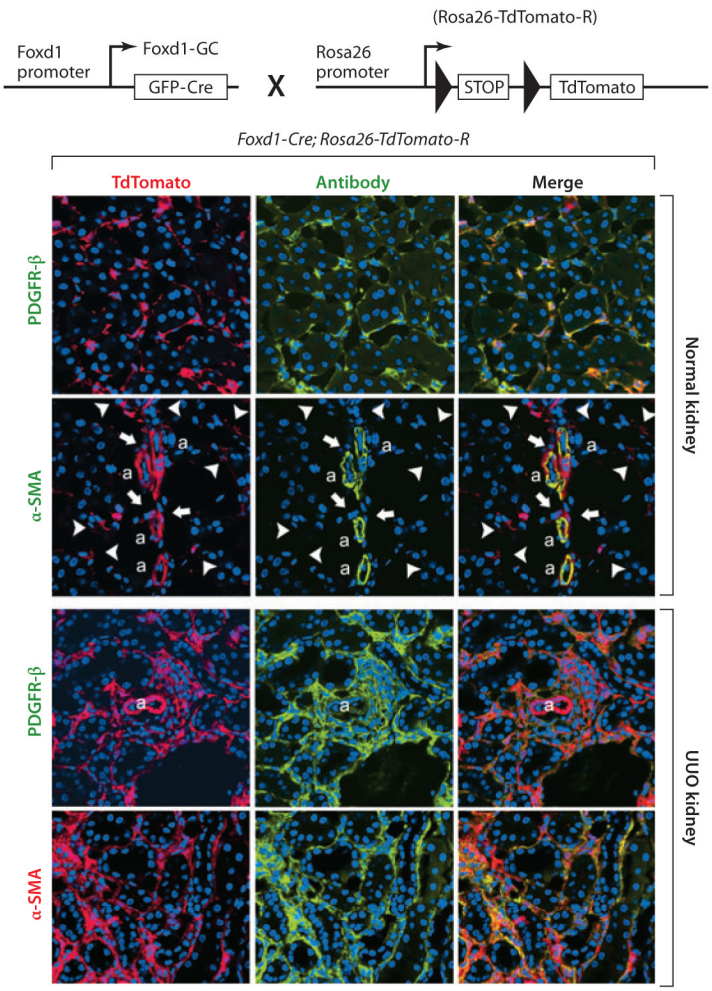

Figure 3.

Genetic fate mapping of mesenchymal progenitors in normal adult and injured kidney by use of the *Foxd1-Cre;Rosa26-TdTomato-R* mouse. (*Top*) The cross of the *Foxd1–Cre* recombinase allele with the *TdTomato* reporter allele, driven by the universal promoters at the Rosa26 locus. Bigenic mice recombine genomic DNA at the *Rosa* locus only in cells that have activated *Foxd1* in nephrogenesis. (*Bottom*) Confocal images of kidney cortex in normal adult kidney show large numbers of perivascular cells, all of which coexpress platelet-derived growth factor receptor β (PDGFR-β). Vascular smooth muscle cells of the kidney arterioles are also derived from Foxd1 progenitors and coexpress α–smooth muscle actin (α-SMA) intermediate filament in normal kidney, but none of the Foxd1-derived pericytes (*arrowheads*) or perivascular fibroblasts (*arrows*) express α-SMA. In kidney injury [shown here is unilateral ureteral obstruction (UUO) day 7], the pericyte and perivascular fibroblast populations expand and continue to express PDGFR-β. However, the entire expanded population of interstitial Foxd1 progenitor–derived cells coexpress α-SMA, the marker that defines these cells as myofibroblasts. Abbreviations: a, arteriole; GFP, green fluorescent protein.

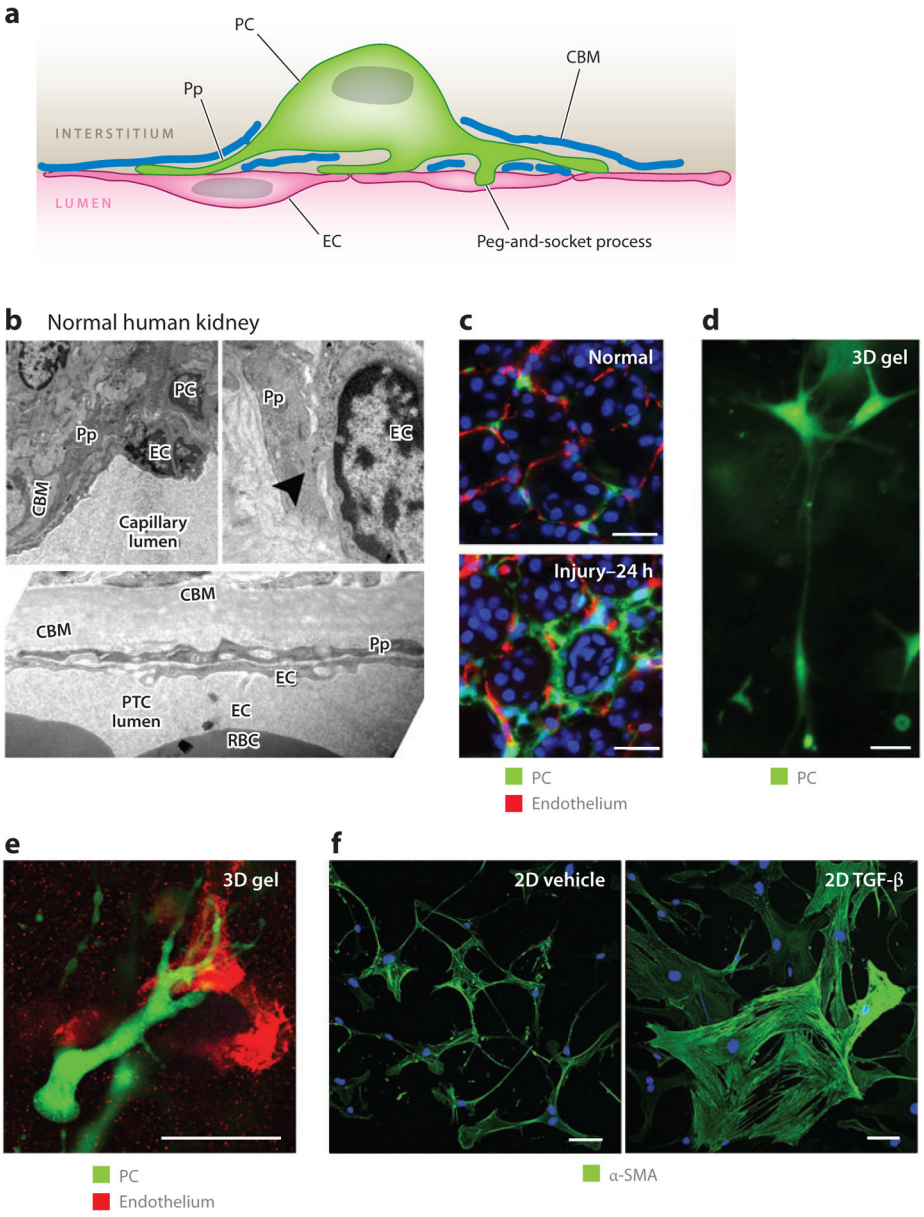

Figure 4.

Pericytes (PCs) in the kidney: definitions, functions, and response to cytokines. (*a*) A PC attached to an endothelial cell (EC) and partially embedded in a duplication of the capillary basement membrane. Note the specific attachment of PC processes (Pp) and the cell body to the EC at several different sites. (*b*) Electron microscope images of normal human kidney cortex. Shown are peritubular capillaries (PTCs), in which ECs and PCs or Pp are visible. The tubule basement membrane is clearly visible. Note the peg-and-socket process (*arrowhead*) at the upper right. (*c*) PCs attach to ECs in normal mouse kidney, but only 24 h (prior to mitosis) after the onset of obstructive injury, the PCs detach, spread, and migrate from the ECs. (*d*) PCs in three-dimensional (3D) collagen gel that exhibit long cytoplasmic processes that extend the length of more than 10 cell bodies. (*e*) PCs in 3D collagen gel home to and bind by attachment specifically to capillary tubes composed of endothelial cells. (*f*) Kidney PCs cultured on gelatin matrix, stained for α–smooth muscle actin (α-

SMA). Note that, in control conditions, PCs show weak α-SMA expression and many long fine processes and elongations, but 24 h after exposure to transforming growth factor (TGF)-β, the PCs change shape, spread, lose their long cytoplasmic processes, upregulate α-SMA, and show distinct cytoplasmic filaments. Scale bars, 25 μm. Abbreviations: 2D, two-dimensional; CBM, capillary basement membrane; RBC, red blood cell.

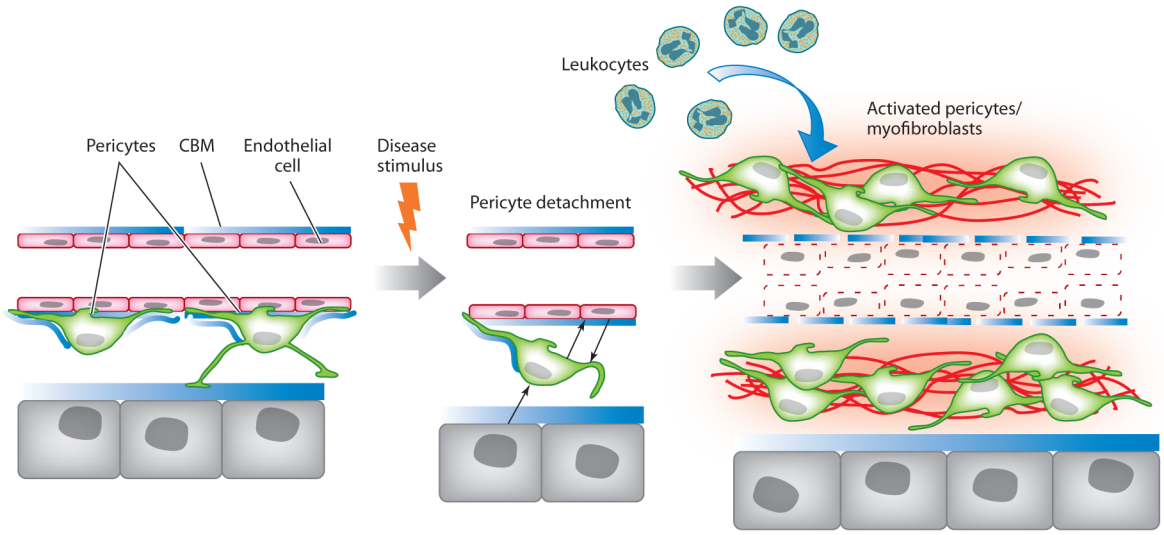

Figure 5.

Schema of pericyte activation by a disease stimulus (based around kidney injury). In response to injury, pericytes become activated and detach from capillaries. This process requires bidirectional signaling between endothelial cells and pericytes. Epithelial cells can also signal to pericytes, and it is unknown whether pericytes signal to epithelial cells. In the presence of persistent injury, activated pericytes proliferate, migrate, and activate genes that give them the myofibroblast phenotype, including upregulated expression of pathological matrix genes, contractile machinery, and immune response genes. This process results in pathological matrix deposition in the virtual interstitial space; recruitment of inflammatory cells; and the loss of pericyte coverage of the endothelial cells, which causes an unstable endothelium that in turn leads to dysangiogenesis and, potentially, rarefaction. Abbreviation: CBM, capillary basement membrane.

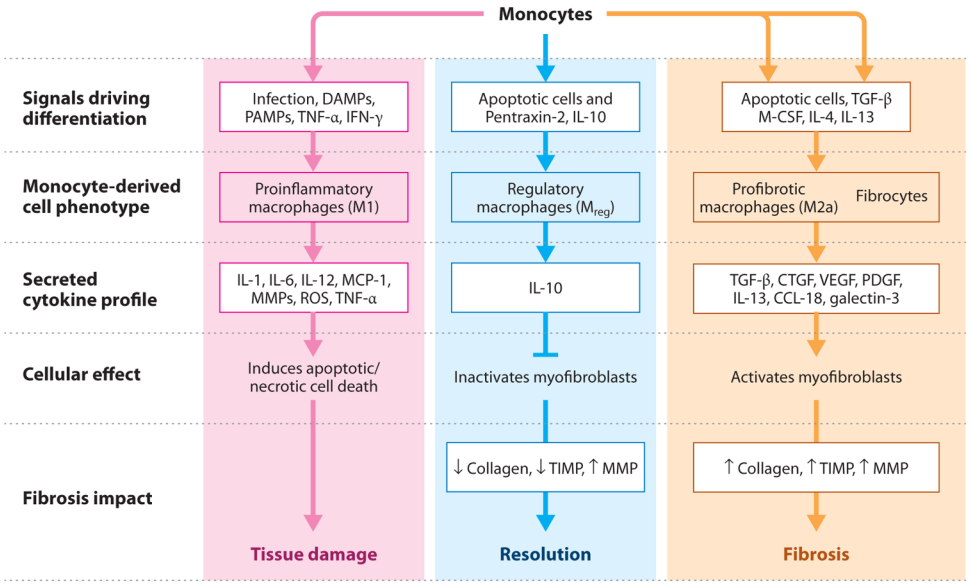

Figure 6.

Monocyte/macrophage activation pathways involved in fibrosis progression and resolution. Monocytes are recruited to sites of tissue injury and differentiate into distinct specialized effector macrophage populations, depending on the extracellular milieu present at the site of injury. These different effector cell populations can have dramatically different impacts on fibrosis initiation, propagation, and resolution. (*Left*) Monocytes promote the initiation of fibrosis through differentiation into M1-type macrophages that release cytokines and reactive oxygen species (ROS) that cause additional local tissue injury, and they promote myofibroblast resistance to apoptosis. (*Center*) Monocytes promote the resolution of fibrotic disease through differentiation into regulatory macrophages (M_{reg}) that inactivate myofibroblasts and inhibit M1- and M2-type macrophages through local production of interleukin (IL)-10 and/or Arginase-1. (*Right*) Monocytes promote the progression of fibrotic disease through differentiation into profibrotic (M2a-like) macrophages and fibrocytes that produce various fibroblast stimulatory growth factors and cytokines. Abbreviations: CTGF, connective tissue growth factor; DAMP, damage-associated molecular pattern; IFN, interferon; M-CSF, macrophage colony-stimulating factor; MCP, monocyte chemotactic protein; MMP, matrix metalloproteinase; PAMP, pathogen-associated molecular pattern; PDGF, platelet-derived growth factor; TIMP, tissue inhibitor of metalloproteinase; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

Table 1

Markers expressed by resident mesenchymal cells, including cells known as pericytes and resident fibroblasts (differ between organs and between subpopulations)

Restricted by cell type, expressed in homeostasis	Restricted by cell type, expressed only in activated state	Not restricted by cell type, expressed in homeostasis
PDGFR-β	NG2	VEGFA
PDGFR-α	P75 NGFR	ANGPT1
CD248	α-SMA	CTGF
CD73[a]	ADAMTS1[a]	TIMP-3
CD105		CD44
Glial fibrillary acidic protein		
Synaptophysin		
gp38 (synaptopodin)		
Sprouty1[a]		
ColIa1		
RGS5		
Desmin		

[a]Somewhat restricted. Abbreviations: CTGF, connective tissue growth factor; NGFR, nerve growth factor receptor; PDGFR, platelet-derived growth factor receptor; RGS, regulator of G protein signaling; SMA, smooth muscle actin; TIMP, tissue inhibitor of metalloproteinase; VEGF, vascular endothelial growth factor.

Table 2

Pericyte functions in development and homeostasis

Function	Reference(s)
Migrate to and bind to capillaries	87, 95
Regulate angiogenesis by cell cross talk involving PDGFR-β, Tie-2, TGF-β receptor, VEGFR, S1P	98
Regulate vascular permeability	89, 93
Regulate capillary flow by contractile functions	85, 86
Stabilize vessels:	
Inhibit and regulate angiogenesis	—
Synthesize the CBM and stimulate ECs to deposit the CBM	73, 87, 95–97
Prevent hemorrhage, aneurysm, dilatation, rarefaction, EC death	72, 90

Abbreviations: CBM, capillary basement membrane; EC, epithelial cell; PDGFR, platelet-derived growth factor receptor; S1P, sphingosine 1-phosphate; TGF, transforming growth factor; VEGFR, vascular endothelial growth factor receptor.

Table 3

Candidate epithelial signaling molecules

Developmental pathway factors	Cytokines	Chemokines, small-molecular mediators
Hedgehog	IL-1β	CCL3–6
VEGF	Oxygen radicals	CCL8
PDGF	IL-6	
TGF-β	S1P	
CTGF family	Nitric oxide	
WNT	TNF-α	
Notch		
FGF		

Abbreviations: CTGF, connective tissue growth factor; FGF, fibroblast growth factor; IL, interleukin; PDGF, platelet-derived growth factor; S1P, sphingosine 1-phosphate; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

Table 4

Immune response gene categories activated in pericytes in response to injury or platelet-derived growth factor receptor (PDGFR) signaling

Adult kidney pericytes in response to organ injury	Newborn brain pericytes in response to PDGFR-β overactivity
Immune response	Antigen presentation pathway
Response to wounding	Interferon signaling
Defense response	Dendritic cell maturation
Inflammatory response	Pattern-recognition receptors
Response to stress	Complement system
Regulation of cytokine production	
Innate immune response	
Adaptive immune response	
Cytokine activity	
Chemotaxis	

Table 5

Antifibrotics in clinical development

Company	Compound	Mechanism of action	Route	Indications	Status of project
Intermune, Shionogi	Pirfenidone	Unknown, reduces growth factors/cytokines	Oral	IPF, DN, FSGS	Approved in Japan and Europe for IPF, Phase III for IPF in United States, Phase II for DN and FSGS
Intermune	ActImmune™	Recombinant human IFN-	Subcut.	IPF	Failed in Phase III for IPF
Actelion Pharmaceuticals	Bosentan (Tracleer™)	Endothelin antagonist	Oral	IPF	Failed in Phase III for IPF
Gilead	Ambrisentan (Letiaris)	Endothelin antagonist	Oral	IPF	Phase III
Pfizer	Sildenafil (Revatio)	PDE5 inhibitor	Oral	PAH in IPF	Phase III
Boehringer Ingelheim	BIBF-1120 (Vargatef)	VEGFR, FGFR, PDGFR inhibitor	Oral	IPF	Phase III
Abbott/Reatta	Bardoxolone methyl	Nrf2 agonist, PDJ2 mimetic	Oral	CKD in DN	Phase III
Novartis	Imatinib mesylate (Gleevec)	PDGFR-α/β inhibitor	Oral	IPF, DN	Failed in Phase II for IPF, Phase II for DN
Celgene	Thalidomide (Thalomid)	Unknown, reduces collagen production	Oral	IPF, MF	Phase II in IPF, Phase II in MF
Celgene	CC-930	JNK inhibitor	Oral	IPF	Phase II
Gilead/Arresto	GS-6624	Anti-LOXL2 monoclonal antibody	IV	MF, LF, IPF	Phase II in MF, Phase I/II in LF, and Phase I in IPF
Amarillo Biosciences	IFN-α	Anti-inflammatory	Oral	IPF	Phase II
Wyeth	Etanercept (Enbrel)	Soluble TNF-α receptor	Subcut.	IPF	Phase II
Cornerstone Therapeutics/Skyepharma	Zileuton (Zyflo)	Leukotriene synthesis inhibitor, 5-LO inhibitor	Oral	IPF	Phase II
Adeona/Pipex Therapeutics	Tetrathiomolybdate (Coprexa)	Copper chelator	Oral	IPF, DN	Phase II
Centocor	CNTO-888	Anti-CCL2 monoclonal antibody	IV	IPF	Phase II
Novartis	QAX576	IL-13 inhibitor	IV	IPF, asthma	Phase II
Pharmaxis	PXS25	Leukocyte extravasation inhibitor	IV	IPF	Phase II
Mondobiotech	Vasoactive intestinal peptide	Anti-inflammatory	Inhaled	IPF	Phase II
Lilly	LY2382770	Anti-TGF-β monoclonal antibody	Subcut.	DN	Phase II
Genzyme	GC-1008 (fresolimumab)	Anti-TGF-β monoclonal antibody	IV	IPF, MF	Phase II in IPF, Phase I in MF
FibroGen	FG-3019	Anti-CTGF human monoclonal antibody	IV	IPF, DN, LF in HBV	Phase II in IPF, Phase II in DN, Phase II in LF
Insys Therapeutics	Cintredekin besudotox	IL-13 receptor^{+} cell destruction	Inhaled	IPF	Phase II, appears discontinued
Stromedix	STX-100	Anti–$\alpha_v\beta_6$ integrin monoclonal antibody	IV	IPF	Phase Ib/IIa

Company	Compound	Mechanism of action	Route	Indications	Status of project
Promedior	PRM-151	Recombinant human SAP, monocyte inhibitor	IV	IPF	Phase Ib
Bristol-Myers Squibb	—	Anti-IL-4/IL-13 receptor monoclonal antibody	IV	IPF	Phase I

Abbreviations: CTGF, connective tissue growth factor; DN, diabetic nephropathy; FGFR, fibroblast growth factor receptor; FSGS, focal segmental glomerulsclerosis; IFN, interferon; IL, interleukin; IPF, idiopathic pulmonary fibrosis; IV, intravenous; LF, liver fibrosis; MF, myelofibrosis; PAH, pulmonary arterial hypertension; PDE, phosphodiesterase; PDGFR, platelet-derived growth factor receptor; SAP, serum amyloid protein; subcut., subcutaneous; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGFR, vascular endothelial growth factor receptor; HBV, hepatitis B virus infection.