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# Mechanisms of Hepatic Fibrogenesis

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## Abstract

Substantial improvements in the treatment of chronic liver disease have accelerated interest in uncovering the mechanisms underlying hepatic fibrosis and its resolution. Activation of resident hepatic stellate cells into proliferative, contractile, and fibrogenic cells in liver injury remains a dominant theme driving the field. However, several new areas of rapid progress in the past 5–10 years also have taken root, including: (1) identification of different fibrogenic populations apart from resident stellate cells, for example, portal fibroblasts, fibrocytes, and bone-marrow– derived cells, as well as cells derived from epithelial mesenchymal transition; (2) emergence of stellate cells as finely regulated determinants of hepatic inflammation and immunity; (3) elucidation of multiple pathways controlling gene expression during stellate cell activation including transcriptional, post-transcriptional, and epigenetic mechanisms; (4) recognition of disease-specific pathways of fibrogenesis; (5) re-emergence of hepatic macrophages as determinants of matrix degradation in fibrosis resolution and the importance of matrix cross-linking and scar maturation in determining reversibility; and (6) hints that hepatic stellate cells may contribute to hepatic stem cell behavior, cancer, and regeneration. Clinical and translational implications of these advances have become clear, and have begun to impact significantly on the management and outlook of patients with chronic liver disease.

The field of hepatic fibrosis is flourishing thanks to continued experimental advances complemented by exciting progress in the treatment of chronic liver disease.[1] Control of chronic hepatitis B and C by antiviral therapies has established that advanced fibrosis can regress in association with improved clinical outcomes,[2-4] thereby intensifying enthusiasm to uncover the mechanistic basis for hepatic fibrogenesis and its attenuation. At the same time, simple paradigms defining the cellular sources of extracellular matrix (ECM), and the roles of cytokines and paracrine interactions among resident liver cells and inflammatory cells have yielded a more nuanced understanding of how the liver responds to injury. These advances have had a collateral benefit towards understanding fibrosis in other organs, particularly the pancreas.[5] Thus, a review of the mechanisms underlying hepatic fibrosis is not only timely, but is more clinically relevant than ever. This article focuses on recent advances in the field, building on established principles from earlier reviews[6,7] while weaving in their clinical relevance, but also emphasizing the molecular subtleties that have emerged through continued progress in the field.

## General Principles

Fibrosis, or scarring of the liver, is a wound-healing response that engages a range of cell types and mediators to encapsulate injury. Although even acute injury will activate mechanisms of fibrogenesis, the sustained signals associated with chronic liver disease caused by infection,



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drugs, metabolic disorders, or immune attack are required for significant fibrosis to accumulate. Occasionally, fibrosis may be rapidly progressive over weeks to months, for example, as a result of drug injury, hepatitis C virus (HCV) after liver transplantation,[8] or human immunodeficiency virus (HIV)/HCV co-infection,[9] but for the most part this is a response that evolves over decades. The protracted nature of this response, in contrast to the more rapid progression of fibrosis in kidney or lung, has been classically ascribed to the liver's unique regenerative capacity, but the molecular underpinnings of this capacity remain mysterious.

In many ways, the liver's response to injury is an angiogenic one, with evidence of new blood vessel formation, sinusoidal remodeling, and pericyte (ie, stellate cell) expansion.[10] Thus, mediators familiar to the angiogenesis field are equally relevant in understanding hepatic fibrosis, including platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and their cognate receptors, as well as vasoactive mediators that include nitric oxide and carbon monoxide. For example, increased VEGF concentrations may contribute to accelerated progression of fibrosis in smokers who have hepatitis C.[11]

Cirrhosis, the most advanced stage of fibrosis, connotes not only more scar than fibrosis alone, but also distortion of the liver parenchyma associated with septae and nodule formation, altered blood flow, and risk of liver failure. However, cirrhosis still remains a dynamic and evolving state, as discussed later (see Clinical and Translational Implications section), such that interventions even at these advanced stages could regress scar and improve clinical outcomes.

Continued progress in the field also has exploited steady refinements in both cell culture and animal models of fibrosis.[12] Although there are no rodent models that closely mimic hepatitis B virus (HBV), HCV, or nonalcoholic steatohepatitis (NASH), the development of genetic mouse models has continued to accelerate progress by enabling reductionist approaches that focus on the role of individual gene products in fibrogenesis, and by permitting genetic lineage tracing to define cellular phenotypes and their evolution.[13] Animal and culture models also have benefited from improving technology, in particular the use of gene array and proteomics. For example, gene expression patterns from stellate cells (the key resident fibrogenic cell type) isolated from rats with fibrosis from either $CCl_4$ or bile duct ligation are remarkably similar, but differ substantially from ultrapurified (ie, using flow cytometry) culture-activated stellate cells.[14] However, stellate cells isolated using standard gradient methods more closely resembled activated cells from fibrotic liver, indicating that standard cell isolation methods yield gene expression data that are relevant to the biology of stellate cells during fibrosis in vivo.[14]

## Liver Injury and Inflammation: Established Mediators and New Players

Fibrosis requires some element of liver injury, albeit not necessarily defined by the presence of inflammatory cells. For example, although the most prevalent diseases in clinical practice (viral hepatitis, alcoholic steatohepatitis [ASH], and NASH) are characterized by leukocyte infiltration, metabolic diseases such as hemachromatosis are notable for their lack of inflammatory cells, yet they too lead to cirrhosis and risk of hepatocellular carcinoma. Thus, any chronic perturbation of hepatic homeostasis, whether visible by light microscopy or not, may elicit the signals necessary to stimulate fibrogenesis.

One family of such established mediators are reactive oxygen species. These unstable compounds include superoxide and hydroxyl radicals, hydrogen peroxide, and aldehydic end products including 4-hydroxy-2,3-nonenal and 4-hydroxy-2,3-alkenals. These mediators are generated through lipid peroxidation, and can derive from hepatocytes, macrophages, stellate cells, and inflammatory cells.[15,16] Several substrates may enhance reactive oxygen species production including ethanol, polyunsaturated fatty acids, and iron. The classic pathway of reactive oxygen species generation in hepatocytes results from induction of cytochrome P450

2E1, especially in either ASH or NASH,[17,18] leading to pericentral (zone 3) injury. More recently, however, reduced nicotinamide adenine dinucleotide phosphate oxidase has emerged as another source of oxidant stress that mediates pathways of fibrogenic activation in hepatic stellate cells, as well as in Kupffer cells, the resident liver macrophages.[19] Reduced nicotinamide adenine dinucleotide phosphate oxidase also may mediate liver injury and fibrosis generated by angiotensin signaling because animals genetically lacking the p47 subunit of reduced nicotinamide adenine dinucleotide phosphate oxidase have reduced superoxide production and attenuated hepatic fibrosis after injury.[20]

More recently, increasing attention also has been directed to nitrosative stress generated by hepatocyte mitochondrial injury and induction of nitric oxide synthase 2,[21,22] although links from this pathway to fibrogenesis are not as well defined.

Apoptosis of parenchymal cells is no longer viewed as a silent consequence of liver injury, but rather as an important inflammatory stimulus[23] that activates stellate cells, which display a surprising capacity to phagocytose apoptotic bodies,[24] leading to induction of reduced nicotinamide adenine dinucleotide phosphate oxidase.[25] This response to apoptotic hepatocytes in part reflects the interaction of hepatocyte DNA with Toll-like receptor 9 (TLR9) expressed on stellate cells.[26] A profibrogenic response also can be elicited by hepatocyte apoptosis after disruption of the anti-apoptotic mediator Bcl-xL,[27] and by Fas.[28] Thus, efforts to block hepatocyte apoptosis therapeutically are being developed as a potential antifibrotic strategy.[29] On the other hand, selective stimulation of apoptosis in stellate cells rather than hepatocytes would be antifibrotic, mediated by either tumor necrosis factor-related apoptosis-inducing ligand,[30] gliotoxin,[31] or proteasome inhibitors.[32]

Although necrosis of cells is a classic morphologic feature of liver injury, its pathogenic contribution to hepatic fibrosis has been overlooked, largely because there are no classic biochemical or molecular hallmarks of necrosis similar to those that have been uncovered for apoptosis.[33] Yet, in human disease both necrosis and apoptosis are evident in liver sections, although specific inflammatory pathways of necrosis have not been identified. Necrosis may simply represent a more severe cellular response than apoptosis when concentrations of injurious stimuli are higher,[15,16] but the relative potencies of necrosis compared with apoptosis in stimulating fibrogenesis are unknown.

Among the most compelling pathways of injury are those recently uncovered for innate immune signaling in liver. Specifically, the discovery of TLRs has led to major advances in understanding how the human organism responds to pathogens.[34] The identification of TLR4, the receptor for bacterial lipopolysaccharide, on Kupffer cells, was therefore not a surprise, but its expression on stellate cells was unexpected.[35] Moreover, although TLR4 signaling in macrophages may be essential for inflammatory responses,[36] recent studies have indicated that signaling by stellate cells in response to lipopolysaccharide and possibly endogenous ligands of TLR4 (eg, high-mobility group box 1, biglycan, and heparan sulfate) may be more important than in Kupffer cells in eliciting a fibrogenic response by down-regulating bone morphogenic protein (BMP) and activin membrane-bound inhibitor, a transmembrane suppressor of transforming growth factor $\beta$1 (TGF$\beta$1), which is the major fibrogenic cytokine in the liver.[37] This finding has converged with evidence that specific single-nucleotide polymorphisms of TLR4 contribute to the rate of fibrosis progression in HCV infection,[38] thereby linking a genetic risk marker to disease pathogenesis.

Evidence that stellate cells play a pivotal role in orchestrating hepatic immune responses extends beyond the TLR pathway and is among the most surprising findings of the past 5 years. Stellate cells produce a host of chemotactic peptides (especially chemokines) that amplify infiltration by inflammatory cells. They also interact directly with lymphocyte subsets,

including natural killer cells (see Mehal and Friedman[39] for detailed review). Indeed, CD8 T cells appear to be more fibrogenic towards stellate cells than CD4 cells,[40] which could account for the increased rate of fibrosis in patients with HIV and HCV, in whom the CD4/CD8 ratio typically is reduced.[41] In contrast, natural killer cells play an important role in clearing activated stellate cells,[42,43] an activity enhanced by gamma interferon and retinoic acid[44] and abrogated by ethanol.[45] Intriguingly, stellate cells also are antigen-presenting cells,[46] and may contribute to the liver's immunotolerant properties through T-cell suppression.[47] Finally, B lymphocytes, which comprise up to 50% of the total lymphocyte pool in liver, contribute to the fibrogenic milieu because mice lacking B cells (JH−/− animals) have attenuated fibrosis, apparently by more rapid ECM degradation after $CCl_4$ injury, implicating an unknown interaction with pathways of matrix degradation.[48]

## Cellular Sources of ECM in Hepatic Fibrosis: An Evolving Paradigm

The discovery of stellate cell activation—a transdifferentiation from a quiescent vitamin A–storing cell to a proliferative myofibroblast—remains among the most informative discoveries to date in unlocking the basis for hepatic fibrogenesis. However, the simple paradigm conceived 15 years ago[6] that all fibrosis derives from activated stellate cells has grown far more multifaceted, both in terms of the pathways of activation and the overall contribution of stellate cells to the total fibrogenic population during liver injury. It is increasingly clear that these fibrogenic cells derive not only from resident stellate cells, but also from portal fibroblasts,[49-51] circulating fibrocytes,[52] bone marrow,[53] and epithelial–mesenchymal cell transition.[13] Although the relative contribution of each source varies, these differences are likely to reflect differing contributions with disease progression and among different etiologies (Figure 1). For example, portal fibroblasts appear to be especially important in cholestatic liver diseases and ischemia,[51] where paracrine interactions between cholangiocytes and fibroblasts involve both chemokines[54] and extracellular nucleotides.[55] In contrast, progressive recruitment of bone-marrow– derived cells may occur over time, such that these cells can represent a substantial fraction of the total fibrogenic population in more chronic injury. Bone marrow recruitment of mesenchymal cells remains a somewhat confusing event because of contradictory findings that on the one hand, bone marrow may provide fibrogenic cells, yet on the other hand autologous bone marrow[56,57] and marrow-derived endothelial progenitor cells[58] can be antifibrotic. It remains unclear which cells within marrow contribute to fibrogenesis and which might be antifibrotic, and/or what mediators regulate these apparently divergent activities of bone-marrow– derived cells.

Because cellular sources of fibrogenic cells may differ among different etiologies, the relative value of particular antifibrotic therapies also may depend on the underlying disease. For example, the integrin $\alpha v\beta 6$ is up-regulated only on biliary epithelium during experimental cholestatic liver fibrosis,[59] implying that antagonists to this integrin might be more rational in biliary than in parenchymal liver diseases.

## Pathways of Stellate Cell Activation: A Moving Target

### Initiating Pathways

Stellate cell activation unfolds progressively in sequential stages; this paradigm provides a useful framework for defining fibrogenic events after liver injury (Figure 2). In particular, the initiation phase, which refers to early events that render the quiescent stellate cell responsive to a range of growth factors, remains an important focus. Rapid induction of $\beta$-PDGF receptor, development of a contractile and fibrogenic phenotype, as well as modulation of growth factor signaling are the cardinal features of this early response. Initiating stimuli include paracrine signals such as reactive oxygen species from apoptotic hepatocytes and injured cholangiocytes, as detailed earlier (see Liver Injury and Inflammation section).

**Changes in ECM composition—**Initiating events in stellate cell activation are occurring on a background of progressive changes in the surrounding ECM within the subendothelial space of Disse. Over time, the subendothelial matrix composition changes from one comprised of type IV collagen, heparan sulfate proteoglycan, and laminin (the classic constituents of a basal lamina) to one rich in fibril-forming collagens, particularly types I and III. One important yet subtle change is the deposition of a specific isoform of cellular fibronectin from sinusoidal endothelial cells, which has an activating effect on stellate cells.[60] Because this response is TGF-$\beta$ dependent,[61] however, induction of this cytokine must occur first, either from autocrine or paracrine sources.

These progressive changes in ECM composition as fibrosis accumulates instigate several positive feedback pathways that further amplify fibrosis. First, dynamic changes in membrane receptors, in particular integrins, sense altered matrix signals that provoke stellate cell activation and migration through focal adhesion disassembly[62-64] while also linking to other growth factor receptors through integrin-linked kinase.[65,66] Matrix-provoked signals also engage membrane-bound guanosine triphosphate binding proteins, in particular Rho[67] and Rac,[68] which transduce signals to the actin cytoskeleton that promote migration and contraction. Second, activation of cellular matrix metalloproteases leads to release of growth factors from matrix-bound reservoirs in the extracellular space that may stimulate cellular growth and fibrogenesis.[69,70] Third, the enhanced density of ECM leads to increasing matrix stiffness, which is a significant stimulus to stellate cell activation, at least in part through integrin signaling.[71] Interestingly, however, increased stiffness caused by edema and inflammation in animal models and human beings may precede the increase in matrix content.[72,73] These experimental findings nicely complement the increasing use of Fibroscan[74,75] (Echosens, Paris, France) and magnetic resonance elastography,[76,77] two clinical techniques that noninvasively assess hepatic stiffness as a reflection of ECM content.

**Molecular mechanisms underlying stellate cell activation—**Because activation occurs so rapidly, attention has focused on regulatory pathways that can respond quickly to injurious stimuli, either by activating or repressing gene transcription, by epigenetic regulation, or by posttranscriptional control. In addition, although microRNAs have emerged as important layers of regulatory control in many systems,[78] their roles in liver injury and stellate cell activation have not yet been explored.

Among the many target genes of transcription factors described in stellate cells, those most comprehensively characterized include type I collagen (alpha 1 and alpha 2 chains), $\alpha$-smooth muscle actin, TGF$\beta$1, and TGF$\beta$ receptors, matrix metalloproteinase (MMP)-2, and tissue inhibitors of metalloproteinases (TIMPs) 1 and 2 (see Rippe and Brenner,[79] Mann et al,[80] and Tsukamoto et al[81] for reviews).

Foxf1, JunD, and C/EBP$\beta$ are among the clearest examples of activating transcription factors. Deletion of one Foxf1 allele reduces stellate cell activation and fibrosis.[82] Similarly, knockout of JunD, a member of the AP-1 transcription factor complex,[83] protects mice from $CCl_4$induced hepatic fibrosis, which is associated with reduced numbers of activated stellate cells and diminished expression of hepatic TIMP-1.[84] Finally, phosphorylation of the transcription factor C/EBP$\beta$ by the RSK kinase promotes stellate cell survival.[85]

The LIM homeodomain protein, Lhx2, on the other hand, is a protein that preserves stellate cell quiescence, and whose loss leads to activation of stellate cells. In fact, Lhx2−/− mice develop spontaneous congenital fibrosis.[86] A similar role has been uncovered for the transcription factor FoxO1; viz. Stellate cell activation and fibrogenesis are amplified in cells from mice with reduced FoxO1 activity.[87]

Similar to Lhx2, the peroxisome proliferator activated receptor $\gamma$ nuclear receptor down-regulates stellate cell activation and reduces collagen gene expression.[88] This and related observations[89] have prompted the use of peroxisome proliferator activated receptor $\gamma$ ligands in clinical trials not only in fibrosis associated with NASH, but also as a candidate antifibrotic in patients with HCV fibrosis. Other nuclear receptors regulating stellate cell behavior include pregnane X receptor,[90,91] and retinoid receptors.[92,93]

Epigenetic regulation is a tightly controlled pathway that modulates stellate cell activation in part through induction of the molecules CBF1 and MeCP2.[94,95] These proteins repress gene expression of the inhibitory protein inhibitor kappa beta (I$\kappa$B) by CpG island methylation, thereby unleashing nuclear factor $\kappa$ B activity, which promotes stellate cell survival and thus increases fibrosis. Interestingly, sulfasalazine inhibits the kinase (inhibitor kappa kinase, I$\kappa$K) that activates I$\kappa$B, and thus may accelerate recovery from experimental fibrosis by clearance of activated stellate cells through apoptosis.[94,96] Because these activated cells typically express high levels of TIMP-1, a metalloproteinase inhibitor, their clearance leads to increased net activity of matrix degrading proteases.

Finally, messenger RNA (mRNA) stabilization also contributes to increased gene expression during stellate cell activation. Specifically, there is a 16-fold increase in collagen alpha 1(I) mRNA stabilization during stellate cell activation as a result of interaction of a specific protein, $\alpha$CP, to a specific sequence in the 3′ untranslated region of the mRNA,[97] and also involving the interaction of a 120-kilodalton protein with the 5′ stem-loop structure.[98] Similarly, there is enhanced interaction of the RNA binding protein, RBMS3, with the 3′ untranslated region of the homeobox protein Prx1, thereby increasing its mRNA stability.[99]

## Perpetuating Pathways

The stellate cell that is activated by initiating stimuli then is primed to respond to a host of cytokines and growth factors. These signals conspire to generate scar through enhanced proliferation, contractility, fibrogenesis, matrix degradation, and proinflammatory signaling. Although earlier models suggested that the pathways of activation were identical regardless of the disease, it is now clear that there are disease-specific pathways of fibrosis (see Disease-Specific Pathways of Hepatic Fibrosis section), and, moreover, that not all cytokine pathways are necessarily activated in parallel. For example, although PDGF stimulation may drive cellular proliferation in parallel with fibrogenic stimulation in some settings, TLR9 activation blocks PDGF-mediated migration while provoking fibrogenesis,[26] thereby providing a stop signal that allows activated cells to accumulate at sites of injury where they can deposit more scar.

**Proliferation—**Autocrine signaling by PDGF was the first cytokine loop uncovered during stellate cell activation and remains among the most potent.[100] Both the PDGF ligand and the beta isoform of its receptor are rapidly induced in vivo and in culture.[101,102] In addition to the well-characterized A and B chains of plateletderived growth factor (PDGF), C and D isoforms also have been discovered more recently; in fact, PDGF-D may be the most potent and physiologically relevant PDGF subunit in stellate cell activation.[100] Interestingly, although both mice with transgenic expression of either PDGF-B[103] or PDGF-C have hepatic fibrosis,[104] the PDGF-C transgenic animals also develop hepatocellular carcinoma,[104] mimicking the progression from fibrosis to cancer that occurs in human beings. Downstream consequences of PDGF signaling in stellate cells include signaling by PI3 kinase, ERK, and other pathways,[105-107] as well as stimulation of $Na^+/H^+$ exchange, providing a potential site for therapeutic intervention by blocking ion transport.[108] Other stellate cell mitogens include VEGF,[109] thrombin and its receptor,[110,111] epidermal growth factor, TGF$\alpha$, keratinocyte growth factor,[112] and basic fibroblast growth factor.[113]

**Chemotaxis—**Stellate cells can migrate towards sites of injury driven by chemoattractants that include PDGF,[114,115] monocyte chemoattractant protein-1,[116] and CXCR3 ligands.[117] Functionally, PDGF-stimulated chemotaxis provokes cell spreading at the tip, movement of the cell body towards the stimulant, and retraction of trailing protrusions associated with transient myosin phosphorylation.[118]

In contrast to PDGF, adenosine blunts chemotaxis, thereby providing a counter-regulatory pathway that fixes cells at sites of injury.[119] Paradoxically, enhanced adenosine signaling also may contribute to alcoholic fibrosis by stimulating stellate cell fibrogenesis,[120] which not only represents a potential fibrogenic mechanism, but also may explain the protective effect of caffeine (which inhibits adenosine generation) reported in epidemiologic surveys.[121]

**Fibrogenesis—**Collagen type I is the prototype constituent of the fibril-forming matrix in fibrotic liver, and its expression is regulated both transcriptionally and posttranscriptionally as described earlier and in several reviews.[122-124]

TGF$\beta$1, derived from both paracrine and autocrine sources, remains the classic fibrogenic cytokine (see Inagaki and Okazaki[124] and Breitkopf et al[125] for reviews). Signals downstream of TGF$\beta$ converge on Smad proteins, which fine-tune and enhance the effects of TGF$\beta$ during stellate cell activation; Smads 2 and 3 are stimulatory whereas Smad 7 is inhibitory[124,126,127] and is antagonized by Id1.[128] TGF$\beta$1 also stimulates collagen transcription in stellate cells through a hydrogen peroxide– and C/EBP$\beta$-dependent mechanism.[129] The response of Smads in stellate cells evolves as injury becomes chronic, further enhancing fibrogenesis.[126,130]

Connective tissue growth factor (CTGF/CCN2) is also a potent fibrogenic signal towards stellate cells[131-133] that is up-regulated by hyperglycemia and hyperinsulinemia.[134] Although stimulation of CTGF production traditionally has been considered TGF$\beta$-dependent,[135] TGF$\beta$-independent regulation is increasingly likely as well.[136] Moreover, TGF$\beta$ stimulates CTGF primarily in hepatocytes, not stellate cells,[137,138] a notable exception to the general rule that cytokine signaling in stellate cell activation typically is autocrine.

Neurohumoral signaling contributes to stellate cell responses.[139] In particular, cannabinoids have emerged as potent mediators of hepatic steatosis, stellate cell activation, and fibrosis (reviewed in Mallat et al[140]), as well as provoking the hemodynamic alterations associated with advanced liver disease.[141] Two receptors, CB1 and CB2, exert opposing effects, with CB1 a fibrogenic pathway and CB2 antifibrotic. Thus, antagonism of CB1 signaling in stellate cells has emerged as a promising antifibrotic strategy, as exemplified by the clinical agent Rimonabant (Sanofi-Aventis, Paris, France).[142] Conversely, agonism of CB2 receptors, which also are expressed by stellate cells, reverses fibrosis in experimental animals.[143] The fundamental challenge of developing cannabinoid therapeutics for liver disease is to minimize central nervous system effects because CB1 and CB2 receptors are expressed abundantly in brain. Similarly, opioids signal in stellate cells and promote fibrogenesis,[144,145] which is antagonized by naltrexone. Finally, sympathetic neurotransmitters also contribute to activation pathways.[146]

**Contractility—**Contraction of stellate cells contributes to increased portal resistance during liver fibrosis that presumably is reversible before the thickened septae, intrahepatic shunts, and lobular distortion of cirrhosis develop, leading to fixed increases in portal pressure. Even in earlier stages of fibrosis, activated stellate cells already show features of smooth muscle–like cells, characterized by expression of a number of contractile filaments including $\alpha$ smooth muscle actin[147] and myosin,[148] which generate calcium-dependent and calcium-independent contractile forces that contribute to cellular contractility.[149-151] Culture models increasingly recapitulate many of these smooth muscle features, in part by restoring a more physiologic

substratum, as well as by including other resident cell types in a co-culture system, especially Kupffer cells.[152]

As reviewed recently, stellate cells are recognized as liver-specific pericytes that contribute to angiogenesis in liver development, regeneration, and the response to injury.[10] After partial hepatectomy, stellate cells migrate along with endothelial cells to establish vascular connections with hepatocytes, thereby creating new sinusoidal branches.[153] Although it is unclear to what extent these responses occurring during pure regeneration are mounted during liver injury, the 2 responses—repair and regeneration— occur concurrently in chronic liver disease, and thus similar angiogenic behavior is likely to underlie repair. Moreover, progressive fibrosis with angiogenesis also contributes to tumor vascularization in which activated stellate cells play a vital role.[10,154]

As fibrosis advances, the collagenous bands typical of end-stage cirrhosis contain large numbers of activated stellate cells.[155] These cells progressively impede portal blood flow by both constricting individual sinusoids and by contracting the cirrhotic liver, mediated by pathways that allow interaction with the ECM.[156,157] At the same time, stellate cell density and coverage of the sinusoidal lumen increases.[10,153] Endothelin-1 and nitric oxide are the key opposing counter-regulators that control stellate cell contractility, in addition to angiotensinogen II, eicosanoids, atrial natriuretic peptide, somatostatin, and carbon monoxide, among others (see Rockey[155] and Reynaert et al[158] for reviews). Progressive development of intrahepatic shunts also is likely to require angiogenic responses driven by stellate cells.[159,160]

The therapeutic implications of elucidating angiogenic signaling in liver fibrosis are not so clear. Although a simple paradigm would suggest that anti-angiogenic agents, for example, VEGF inhibitors, are an effective anti-inflammatory and antifibrotic strategy,[161] their long-term impact on regeneration is uncertain. For example, sustained and complete inhibition of angiogenesis might compromise hepatic blood flow and oxygen delivery, especially because the liver has high metabolic demands and receives primarily venous blood. Thus, there is a delicate balance between the requirement for angiogenesis to preserve blood flow, and its negative impact on liver structure and function. Moreover, strategies to antagonize contractile proteins also may have unintended consequences. For example, mice lacking $\alpha$ smooth muscle actin protein in myofibroblasts have increased renal fibrosis in experimental glomerulonephritis,[162] suggesting that $\alpha$-actin induction may be a counter-regulatory response to enhanced fibrogenesis. Moreover, missense mutations of this protein have been linked to aortic aneurysms in human beings,[163] indicating that the protein also may contribute to vascular integrity.

**Inflammatory signaling—**As reviewed earlier (see Liver Injury and Inflammation: Established Mediators and New Players section), stellate cells have emerged as central modulators of hepatic inflammation and immunity, and not just passive targets of inflammatory cytokines. In particular, a growing list of chemokines and their cognate receptors serve the dual function of provoking further fibrogenesis, as well as interacting with inflammatory cells to modify the immune response during injury.[164-166]

## Matrix Degradation and Resolution of Fibrosis

Evidence that fibrosis and even cirrhosis are reversible has intensified interest in understanding the regulation of matrix degradation and fibrosis resolution, in hopes that therapies might exploit those endogenous pathways that reverse disease (Figure 3). Simplistically put, this response would be considered therapeutic matrix degradation, whereas early liver injury is marked by pathologic matrix degradation that disrupts hepatic homeostasis. Thus, in early liver injury matrix-degrading proteases with activity towards type IV collagen (eg, MMP-2),

degrade the low-density basement membrane present in the subendothelial space. Its replacement with fibril-forming matrix has deleterious effects on differentiated cell function, in particular on hepatocytes.

Enzymes controlling matrix degradation comprise a family of matrix-metalloproteinases (also known as *matrixins*), which are calcium-dependent enzymes that specifically degrade collagens and noncollagenous ECM substrates.[12,167]

Stellate cells are a key source of the basement membrane proteases MMP-2,[168] MMP-9,[169] and stromelysin (MMP-3),[170] as well as the interstitial collagenase MMP-13 (the rodent equivalent of MMP-1).[171]

A major determinant of progressive fibrosis is failure to degrade the increased fibril-forming, or interstitial, scar matrix. MMP-1 is the main protease that can degrade type I collagen, the principal collagen in fibrotic liver. However, sources of this enzyme are not as clearly established as for the type IV collagenases MMP-2 and MMP-9. Stellate cells express modest levels of MMP-1 mRNA and thus it is uncertain whether this represents the primary interstitial collagenase responsible for matrix resorption as liver fibrosis regresses. Alternative interstitial proteases might include either matrix type 1 MMP or even MMP-2, which also displays some interstitial collagenase activity.

The cross-linking of collagen by lysyl oxidase and tissue transglutaminase, and the maturation of hepatic scar through the action of a disintegrin and metalloproteinase with thrombospondin–type repeats metalloproteinase with thrombospondin type I motif (ADAMTS2) are important determinants of hepatic fibrosis reversibility. The long-standing clinical dogma that the slower the pace of injury, the less reversible the scar, is borne out by animal studies in which even advanced fibrosis of short duration is reversible, which is limited primarily by the extent of collagen cross-linking caused by tissue transglutaminase.[172] Moreover, as advanced fibrosis resolves, the micronodules typical of active cirrhosis dissolve, coalescing into macronodules.[172] This finding correlates remarkably well with recent clinical data showing that increased septal thickness and smaller nodule size are significant predictors of poorer clinical outcomes.[173] Similar studies have been performed in knockout mice lacking ADAMTS2, which catalyzes the N-propeptide excision of procollagens I, III, and V, which allows the polymerization of collagen fibrils.[174] In the ADAMTS2 knockout animals, the extent of liver injury after $CCl_4$ was similar to that of wild-type mice, yet fibrosis reversal was slightly faster, associated with much less dense collagen fibrils, and smaller fibril diameters.[175] Recently, increased proteolytic activity ascribed to ADAMS-13 also has been reported in activated stellate cells.[176] However, the native substrate(s) and biological role of this protease are not known.

Hepatic macrophages are re-emerging as critical regulators of matrix remodeling. An elegant study using a genetic model that allows for the selective, timed depletion of macrophages during different stages of liver injury and resolution has shown divergent roles for these cells.[177] During progression of liver fibrosis, macrophages augment fibrogenesis, whereas during resolution they hasten matrix degradation, primarily through increased production of MMP-13.[178]

Inactivation of proteases by binding to TIMPs also is emerging as an important locus of control[12] because sustained production of these proteins during liver injury could inhibit the activity of interstitial collagenases, leading to reduced degradation of the accumulating matrix. In addition, TIMP-1 is anti-apoptotic towards stellate cells,[179] and thus its sustained expression in liver injury will enlarge the population of activated stellate cells by preventing their clearance. In support of TIMP's role in vivo, either transgenic over expression of TIMP-1 in liver, or administration of TIMP-neutralizing antibodies, delay regression of liver fibrosis in

experimental animals.[180] Conversely, the use of MMP-9 mutant proteins as TIMP-1 scavengers reduces fibrosis accumulation by enhancing matrix resorption.[181]

Stellate cells express uroplasminogen activator receptor and its inhibitor, as well as other components of the plasmin system.[182-184] Collectively, these findings suggest that stellate cells contain most, if not all, of the molecules necessary to either activate or inhibit metalloproteinases.

Clearance of activated stellate cells by apoptosis remains an appealing target for antifibrotic therapy because this would use an endogenous pathway of fibrosis regression. In addition, data from cultured stellate cells[185,186] and liver slices[187] suggest that activated cells also can revert to a quiescent phenotype, such that this response may be an antifibrotic pathway worth exploiting.

## Disease-Specific Pathways of Hepatic Fibrosis

Although key pathways of stellate cell activation are common to all forms of liver injury and fibrosis, disease-specific pathways are being unearthed as well, particularly in ASH and NASH, and in HCV disease.

### NASH and ASH

The accelerating obesity epidemic is tied to an increasing prevalence of NASH and subsequent cirrhosis.[188] Adipokines mediate fibrogenesis and many hepatic manifestations of obesity.[189,190] Specifically, leptin, a circulating adipogenic hormone, promotes stellate cell fibrogenesis and enhances TIMP-1 expression,[191-193] which is associated with increased leptin signaling.[191] Concurrently, adiponectin, a counter-regulatory hormone that antagonizes the fibrogenic activity of leptin is reduced in hepatic fibrosis,[194] and mice lacking adiponectin have enhanced fibrosis after toxic liver injury.[195] Equally important to fibrosis is insulin resistance per se, whether associated with steatosis[196] or HCV.[197,198] As noted earlier, cannabinoids also mediate steatosis, and daily cannabis use is a risk factor for steatosis and fibrosis in HCV.[199,200] Interestingly, a recent study directly links CB1 receptor signaling to alcoholic steatosis in rodents fed a liquid ethanol diet.[201] Moreover, in these animals, activated stellate cells are a key source of the endogenous cannabinoid, 2-AG, which drives increased CB1 signaling.[201] As noted earlier, oxidant stress associated with ethanol metabolism is an important stimulus to fibrogenesis. In contrast, aldehydes, although fibrogenic, are unlikely to account entirely for ethanol-induced fibrosis.[15]

### HCV and HIV

Stellate cells express the putative HCV receptors CD80, LDL receptor, and C1q, raising the possibility of direct HCV infection in vivo, which has not yet been established. Moreover, expression of HCV nonstructural and core proteins induces stellate cell proliferation, release of inflammatory signals,[202] and CTGF,[203] although interaction of HCV E2 protein stimulates MMP-2 expression.[204] Furthermore, hepatocytes harboring replicating HCV in culture produce fibrogenic stimuli towards stellate cells.[205] In HCV-infected liver, chemokines promote lymphocyte recruitment.[165] HCV proteins also may interact directly with sinusoidal endothelium.[206] Remarkably, no studies have reported potential interactions between HBV and stellate cells, or HBV-specific pathways of fibrogenesis.

The increased rate of fibrosis in patients co-infected with HCV and HIV compared with those with HCV alone has been well documented.[41] The reduced CD4/CD8 ratio typical of HIV infection has been invoked as a cause, because CD8 cells may be relatively fibrogenic compared with CD4 cells; however, recent preliminary data additionally suggest that hepatic stellate cells may be infected directly by HIV,[207] which also might account for why fibrosis

progression is slowed when HIV is suppressed by antiretroviral therapy in co-infected patients.[208]

## Links Between Stellate and Progenitor Cells, Fibrosis, and Cancer

The remarkable phenotypic plasticity of stellate cells, combined with the recent demonstration that they express the stem cell marker CD133,[209] have raised the fascinating prospect that they are true progenitor cells (Figure 4). Further studies are required to establish bona fide and robust pluripotency, but intriguing possibilities are raised. First, activated stellate cells appear to contribute to the stem cell niche based on histologic studies,[210] raising the possibility that they are actually differentiating into stem cells directly. Second, could this cellular behavior provide a missing link between fibrosis and hepatocellular cancer that is derived from stem cells? Although epithelial to mesenchymal transition is well established, the possibility of mesenchymal to epithelial transition, while still quite speculative at this point, should not be ignored. Support for this suggestion includes the presence in stellate cells of both hedgehog[211,212] and Wnt signaling,[213] two pathways implicated in stem cell differentiation and cancer.[214] Moreover, the near-absolute requirement for fibrosis to occur before hepatocellular carcinoma develops in patients with chronic HCV remains completely unexplained. Potential explanations have included the presence of secreted survival factors that prevent apoptosis of DNA-damaged hepatocytes and activated stellate cells (eg, Gas6[215]), reduced tumor surveillance owing to decreasing natural killer cell number and function, and/or the accelerated shortening of telomeres that accompanies progressive fibrosis. The question of how fibrosis promotes hepatocellular carcinoma is a vital one that demands clearer answers.

## Clinical and Translational Implications

The tightening links between the biology of hepatic fibrosis and clinical expression of disease attest to the importance of continued basic and translational research into mechanisms of hepatic fibrogenesis. In particular, newly uncovered correlations between matrix stiffness and fibrogenesis, ECM cross-linking and reversibility, and both cirrhotic nodule size and septal thickness with clinical outcomes, have emerged from cell culture and animal studies, yet they lead directly to new modes of diagnosis and therapy. Moreover, cirrhosis can no longer be viewed as a single, irreversible end stage of disease but rather as a much broader category subdivided by progressive stages of ECM accumulation, nodule size, portal pressure, reversibility, and clinical risk (Figure 5). More refined and rigorous characterization of cirrhosis will be essential for accurate randomization of patients in clinical trials of antifibrotic therapies and stratification of disease risk, possibly including the risk of hepatocellular carcinoma. Indeed, a key lesson from antifibrotic trials to date is that fibrosis may continue to accrue rapidly even in patients with cirrhosis when therapy is not effective.[216] Based on this lesson, it is clear that trials in such cirrhotic patients will need to be lengthy, with the use of more sensitive and specific biomarkers that do not rely on biopsy alone.[217] Further progress in understanding, diagnosing, and treating hepatic fibrosis will continue to rely on the exploration of fundamental mechanisms of fibrogenesis, which is certain to lead to a meaningful impact on the prognosis of patients with chronic liver disease.

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## Biography

## Abbreviations used in this paper

| | |
|---|---|
| ADAMTS2 | a disintegrin and metalloproteinase with thrombospondin–type repeats metalloproteinase with thrombospondin type I motif |
| ASH | alcoholic steatohepatitis |
| CTGF/CCN2 | connective tissue growth factor |

| | |
|---|---|
| ECM | extracellular matrix |
| HIV | human immunodeficiency virus |
| MMP | matrix metalloproteinase |
| NASH | nonalcoholic steatohepatitis |
| PDGF | platelet-derived growth factor |
| TGF | transforming growth factor |
| TIMP | tissue inhibitors of metalloproteinase |
| TLR | Toll-like receptor |
| VEGF | vascular endothelial growth factor |

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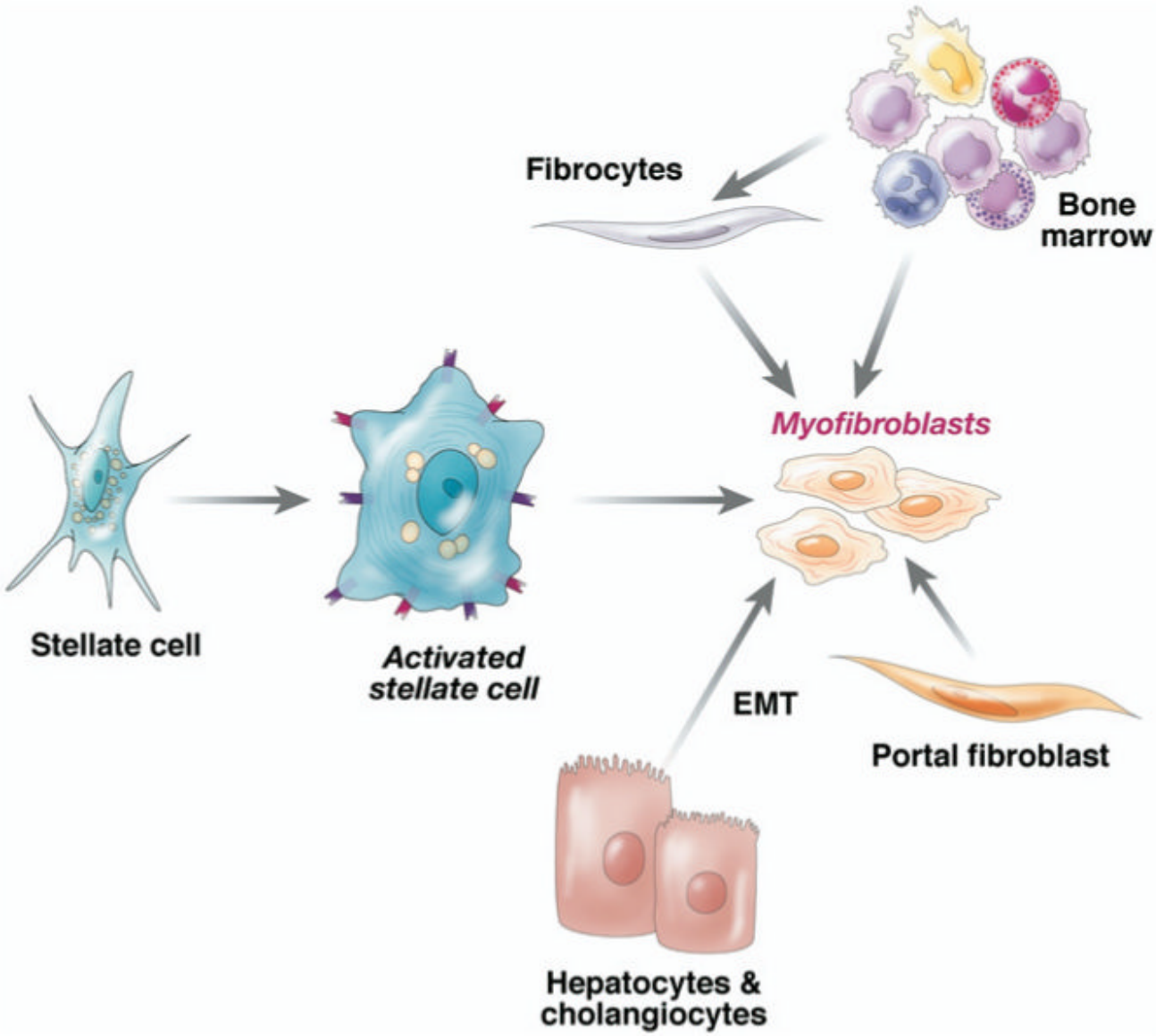


**Figure 1.**
Contributions of activated stellate cells and other fibrogenic cell types to hepatic fibrosis. Quiescent stellate cell activation is initiated by a range of soluble mediators (Figure 2). The activated cell is stimulated further by key cytokines (detailed further in Figure 2) into myofibroblasts (which contain contractile filaments). Over time, however, other sources also contribute to fibrogenic populations in liver, including bone marrow (which likely gives rise to circulating fibrocytes), portal fibroblasts, and epithelial mesenchymal transition ($_{EMT}$) from hepatocytes and cholangiocytes. Relative contributions and the stages at which these cell types add to the myofibroblast population is likely to differ among various etiologies of liver injury (see text).

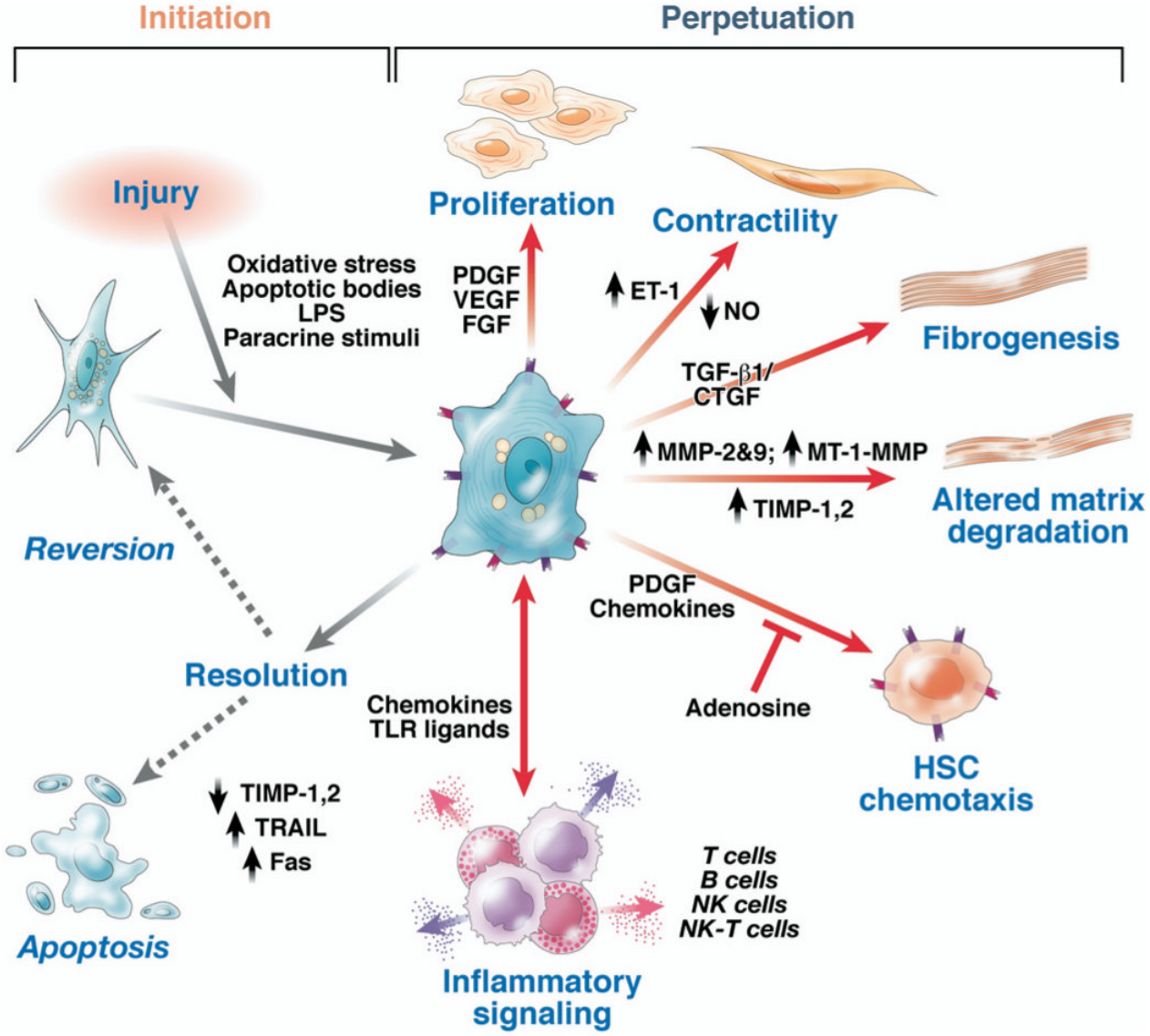


**Figure 2.**

Pathways of hepatic stellate cell activation. Features of stellate cell activation can be distinguished between those that stimulate initiation and those that contribute to perpetuation. Initiation is provoked by soluble stimuli that include oxidant stress signals (reactive oxygen intermediates), apoptotic bodies, lipopolysaccharide (LPS), and paracrine stimuli from neighboring cell types including hepatic macrophages (Kupffer cells), sinusoidal endothelium, and hepatocytes. Perpetuation follows, characterized by a number of specific phenotypic changes including proliferation, contractility, fibrogenesis, altered matrix degradation, chemotaxis, and inflammatory signaling. FGF, fibroblast growth factor; ET-1, endothelin-1; NK, natural killer; NO, nitric oxide; MT, membrane type. Modified with permission from Friedman.[7]

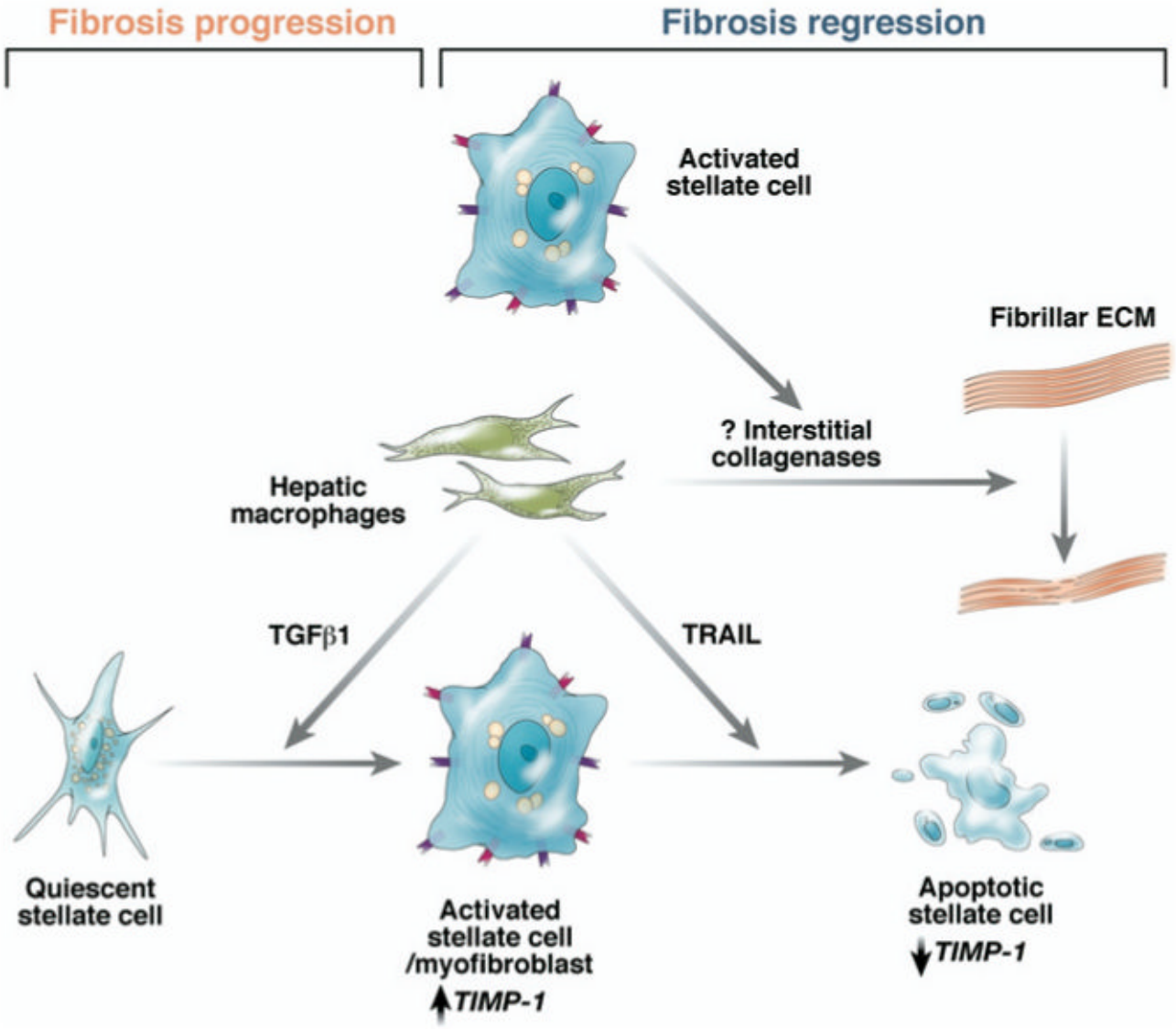


**Figure 3.**

Pathways of matrix degradation in fibrosis progression and regression. Macrophages have assumed an important role in matrix degradation, which is profibrogenic during progression of fibrosis but antifibrotic during fibrosis resolution. Although key sources of matrix degrading activity are uncertain, it seems increasingly likely that both scar-associated macrophages and stellate cells are sources of interstitial collagenases. At the same time, decreased TIMP-1 fosters apoptosis of fibrogenic myofibroblasts. Figure adapted from studies of Duffield and Iredale[177] (and accompanying editorial, pp 29–32).

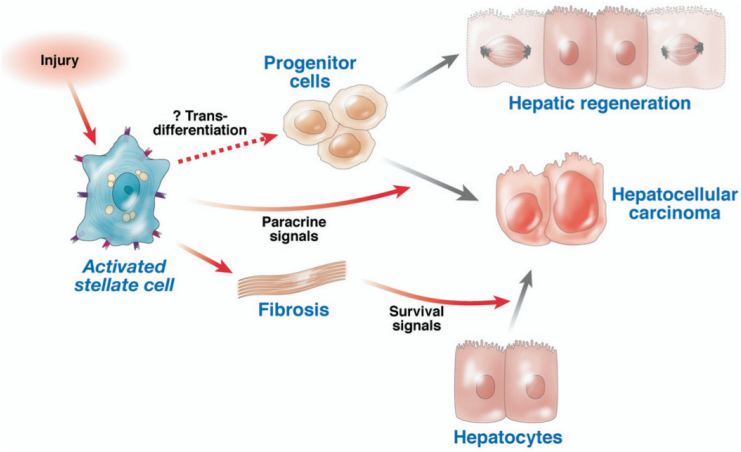


**Figure 4.**

Possible links between stellate cells, fibrosis, regeneration, and cancer. Upon liver injury, activated stellate cells release paracrine factors that may promote progenitor cell expansion, the outcome of which could be either hepatic regeneration and/or promotion of hepatocellular cancer. The possibility also exists that stellate cells may harbor the potential to transdifferentiate into progenitor cells directly, which remains speculative. Moreover, fibrosis promotes hepatocarcinogenesis through unknown mechanisms that may include release of survival signals.

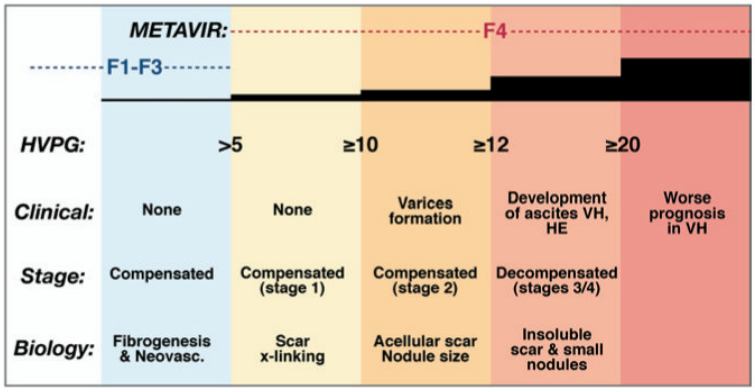


**Figure 5.**

Cirrhosis is a series of progressive stages, not a single stage. Within the spectrum of cirrhosis, the disease is characterized by progressive increases in hepatic venous pressure gradient (HVPG), decompensation, and matrix cross-linking, associated with shrinking nodule size, thickening septae, and enhanced risk of decompensation. For each 1-mm increase in HVPG the risk of decompensation increases by 11%. Concepts presented here are not rigorously supported by primary data for all features, but rather are intended to convey the progressive changes that underlie deterioration in patients with chronic hepatic injury and fibrosis. Stages are based on data from D'Amico et al.[218] HE, hepatic encephalopathy; VH, variceal hemorrhage.