Novel perspectives on the origins of the hepatic myofibroblasts

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Abstract: Liver fibrosis results from chronic liver injury that causes hepatocellular damage. Damaged hepatocytes apoptose, and release factors that facilitate recruitment of leukocytes to the site of injury, which in turn mediate recruitment and activation of liver-resident (Kupffer cells) and bone marrow (BM)-derived macrophages. Activated macrophages secrete TGF-β1, the major profibrogenic cytokine, which activates hepatic myofibroblasts, which are not present in the liver under physiological conditions. Several sources of myofibroblasts have been identified, but it is believed that liver-resident hepatic stellate cells (HSCs) and portal fibroblasts (PFs) are the major source of hepatic myofibroblasts in fibrotic liver. Fibrocytes, designated as BM-derived collagen Type I producing cells, were also implicated in liver fibrosis; hence, their contribution to liver fibrosis remains controversial. Upon removal of the etiological agent, myofibroblasts either undergo apoptosis or inactivate into a quiescent-like state, followed by resorption of the fibrous scar. However, prolonged/repeated liver injury triggers irreversible cross-linking of collagen fibers that prevents fibrous scar from collagenase-mediated degradation. This review will discuss several types of fibrogenic cells contributing to the myofibroblast population, and the signaling pathways regulating their activation and collagen deposition.

Keywords: Liver fibrosis, TGF-β1 signaling, hepatic stellate cells, portal fibroblasts, fibrocytes, collagen Type I deposition

Introduction
Liver fibrosis results from chronic liver injury, and is characterized by tissue remodeling, formation of fibrous scar, and activation of myofibroblasts. Activated myofibroblasts produce extracellular matrix (ECM), mainly collagen Type I. In addition, myofibroblasts express stromal markers, alpha smooth muscle actin (α-SMA), nonmuscle myosin, and fibronectin and exhibit spindle-like morphology.1–3 They contain rough endoplasmic reticulum (rER) and Golgi apparatus that are required for ECM production. Data obtained from patient biopsies and experimental models implicate myofibroblasts in the pathogenesis of liver fibrosis, and demonstrate that increased numbers of myofibroblasts correlate with severity of liver fibrosis.3 Furthermore, clinical and experimental studies have suggested that liver fibrosis can regress upon cessation of liver injury. Reversibility of liver fibrosis is associated with the disappearance of hepatic myofibroblasts.4,5 Therefore, myofibroblasts become an attractive target for antifibrotic therapy. Although all myofibroblasts share similar characteristics, such as expression of α-SMA, myofibroblasts may originate from different sources. Heterogeneity of the myofibroblast population may provide challenges for drug design. Five sources of myofibroblasts have been suggested to contribute to liver fibrosis: 1) Hepatic stellate...
cells (HSCs), which were reported to be a major source of myofibroblasts during hepatoxic liver injury.6,7 2) Portal fibroblasts (PFs), which arise in fibrotic liver in response to cholestatic liver injury, 3) Fibrocytes, a population of bone marrow (BM)-derived collagen Type I producing cells that are capable of differentiating into myofibroblasts, 4) Epithelial-to-mesenchymal transition, a mechanism described during embryonic development when epithelial cells undergo transdifferentiation into myofibroblasts, and 5) Endothelial cells that may undergo similar changes via endothelial-to-mesenchymal transition (EndMT). Hence, most recent cell fate mapping-based studies did not support a concept of EMT contribution to myofibroblasts. Although the composition of myofibroblasts varies depending on the etiology of liver fibrosis, their cellular origin can be identified by the expression of unique lineage markers. Identification of the origin and composition of myofibroblasts is critical for their targeting by antifibrotic therapies. In this review, we will discuss the characteristics of myofibroblasts of different origins, and the methodologies to isolate and target each population.

**Hepatic stellate cells**

HSCs, liver-resident mesenchymal cells, are considered to be a major source of myofibroblasts in fibrotic liver in response to toxic liver injury.7–9 In the healthy liver, HSCs exhibit a quiescent phenotype: they reside in the space of Disse (located between hepatocytes and sinusoidal endothelial cells), contain lipid droplets, and serve as a major storage of vitamin A in the mammalian body.10 Under physiological conditions, quiescent HSCs do not express collagen, but can be identified by the expression of neuronal markers (glial fibrillar acidic protein, GFAP, synemin, and neural growth factor p75) and type III intermediate filament, desmin. In response to liver injury, they downregulate vitamin A droplets, migrate to the injury foci, and elevate the expression of collagen and α-SMA for ECM deposition. The activated HSCs obtain a myofibroblast phenotype,1 and upregulate α-SMA, Col1a1, TIMP1, Ctrfl, Spp1, IL1r1, IL-17ra, and Itga5.4,5 In agreement, partial deletion of HSCs in mice (achieved by overexpression of the herpes simplex virus thymidine kinase gene under the control of GFAP promoter) results in inhibition of CCl4-induced liver fibrosis.11

Furthermore, cell fate mapping experiments that utilize the Cre-lox system in mice have identified a novel phenotype of HSCs. Thus, in addition to quiescent and activated phenotypes (aHSCs), HSCs can acquire an inactivated phenotype (iHSCs) during regression of liver fibrosis. Upon the removal of underlying etiological agents, liver fibrosis can regress. During fibrosis resolution, activated HSCs can either undergo apoptosis or become inactivated. Inactivation of HSCs is characterized by downregulation of fibrogenic gene mRNA, such as col1a1 and α-SMA, vimentin, Ssp1, and TIMP1, and partial re-expression of lipogenesis genes, such as PPARg, Insig1, Grap, IL-10RA, and BAMBI.4,5 It is unknown why some aHSCs undergo apoptosis, while others become inactivated. However, survival and inactivation of HSCs is associated with transient upregulation of heat shock proteins 1a/b (Hsp1a/b) that may protect these cells from apoptosis.5

Activation of HSCs is orchestrated by a complex mechanism that includes damage to hepatocytes, reactive oxygen species (ROS) formation, recruitment of inflammatory cells, and upregulation of proinflammatory and profibrogenic cytokines. Much less is known about the mechanism of iHSC inactivation. Some of the pathways that promote HSCs activation/inactivation are discussed below.

**TGF-β1 is a potent profibrogenic cytokine**

Activation of hepatic myofibroblasts occurs in response to increased levels of proinflammatory and profibrogenic cytokines. The fibrogenic cytokine TGF-β1 is considered the most potent activator of HSCs/myofibroblasts in the damaged liver (Figure 1). Kupffer cells (KCs) and recruited macrophages are a major source of TGF-β1 in fibrotic liver.8 In addition, other fibrogenic cytokines and growth factors, such as PDGF, CTGF, and FGF, contribute to HSC/myofibroblast activation. Furthermore, proinflammatory cytokines, IL-6, TNF-α, IL-1β, IL-17A, MIP-1, and MIP-2, also facilitate the activation of HSCs/myofibroblasts. They also mediate the recruitment of inflammatory cells to the site of injury, and mount a local and systemic immune response, including activation of BM-derived macrophages and KCs (liver-resident macrophages). IL-6, IL-17A, and leptin can directly activate hepatic myofibroblasts to produce collagen.

The binding of TGF-β1 to its corresponding receptors, TGF-β1 receptors type I and type II (TGFβRI and II), results in receptor heterodimerization, and subsequently serine phosphorylation of Smad2 and Smad3 (that dock at the intracellular domain of TGFβRI) at the serine residue (SSXS motif). Phosphorylation of cytoplasmic Smad2/3 (pSmad3) at the docking site of TGFβRI is the major event that triggers TGF-β1 signaling in aHSCs (Figure 1). Phosphorylated Smad2/3 form a complex with Smad4, a transporter molecule that facilitates nuclear translocation of Smad2/3/4 complex, where it binds the cis-regulatory elements of the target genes and triggers their transcription. Transcription of Col1a1, Smad7,
and PAI-1 is directly regulated by the Smad2/3/4 complex, and therefore these genes are considered as TGF-β1-target genes.\textsuperscript{12,13} Smad7 forms a stable complex with TGFβRI to block further Smad3 phosphorylation and prevent TGF-β1 signaling.\textsuperscript{14} Remarkably, the transcriptional activity of Smad2/3/4 complex is negatively regulated by Smad7 in quiescent HSCs, but such an inhibitory effect has not been observed in HSC-derived myofibroblasts. Loss of inhibitory function of Smad7 may also contribute to “sensitization” of aHSCs to TGF-β1 signaling, causing their activation.\textsuperscript{15–17} The middle-linker region of Smad3 is phosphorylated by mitogen-activated protein kinase (MAPK), which activates an alternative pathway of TGF-β1-induced Smad3 phosphorylation and subsequent activation of collagen Type I expression.\textsuperscript{18} The Smad3 and MAPK pathway produce an additive effect on collagen Type I expression. Inhibition of either Smad3 or MAPK can only partially reduce collagen Type I expression in TGF-β1 aHSCs, but inhibition of both pathways significantly reduces ECM expression in aHSCs.\textsuperscript{19} TGF-β1 signaling is also regulated by other mechanisms during HSCs activation. The TGF-β1 pseudoreceptor BMP and activin membrane bound inhibitor (BAMBI) exhibits similarity to the TGF-β1 receptor type II, but lacks the intracellular serine threonine-kinase domain. BAMBI interferes with TGF-β1 signaling by blocking the ability of TGFβRI to form heterodimers with the TGFβRII.\textsuperscript{20} Therefore, repression of BAMBI expression during HSC activation augments TGF-β1 signaling. During regression of liver fibrosis, BAMBI is one of the factors that is re-expressed in inactivated human (h) HSCs to block TGF-β1 signaling. Inactivated HSCs slowly regain BAMBI expression.\textsuperscript{4} Regulation of BAMBI expression in HSCs is under the control of toll-like receptor (TLR)4 receptor signaling and NFκB signaling.\textsuperscript{21,22} aHSCs express a high level of TLR4 and its coreceptors MD2 and CD14.\textsuperscript{23} In response to LPS, TLR4 signaling promotes the translocation of NFκB into the nucleus. The p50 subunit recognizes the cis-regulatory element of NFκB, which locates within the 5′ promoter region of BAMBI locus, and facilitates the recruitment of histone deacetylase 1 (HDAC1) responsible for epigenetic changes within the BAMBI promoter region that results in suppression of BAMBI transcription in the activated HSCs.\textsuperscript{22}

**Figure 1** TGF-β1 signaling plays a critical role in activation of HSCs (hepatic stellate cells).
immediate immuno-inflammatory responses in the damaged liver. BM-derived macrophages express similar markers, and upon recruitment to the injured liver can be identified by F4/80<sup>hi</sup>CD11<sup>hi</sup> markers and increased expression of costimulatory molecule CD80 (B7-1) (reviewed by Sica et al<sup>24</sup>). An attempt has been made to determine whether KC and BM-recruited macrophages perform specialized functions, but owing to difficulties with distinguishing KC and BM-derived macrophages and their high plasticity, this question remains unresolved.

Macrophages are traditionally divided into two groups on the basis of the ability to produce specific subsets of cytokines. Chronic liver injury causes damage to hepatocytes, increased gut permeability, leakage of intestinal LPS into circulation, and disruption of the endothelial barrier.<sup>6</sup> In response to TLR ligands and IFN-γ or IL-4/IL-13, macrophages undergo differentiation (polarization) and acquire M1 (classical) or M2 (alternative) phenotypes.<sup>24</sup> The M1 phenotype is characterized by the expression of high levels of inflammatory cytokines, high production of ROS, promotion of Th1 response, and activity against microbes and tumors. In addition, M1 macrophages express CD14, CD11c, CD68, CD115, Marco, NFkB, STAT1, IRF3, IRF4, IRF5, and IRF8, Socs1 and Socs2 markers, TNF-α, IL-1β, IL-6, IL-12, IL-15, IL-18, IL-23 cytokines, CCL5 and 9, CXCL1 and 2 chemokines, secrete MMP1,3 and 9, activation of NFkB and AP-1.<sup>26</sup> In contrast, M2 macrophages are involved in the protection against parasites, promote tissue remodeling and tumor progression, and have immunoregulatory functions. They exhibit high phagocytic activity; express scavenging, mannose, and galactose receptors; produce ornithine and polyamines through the arginase pathway; and have an IL-12<sup>low</sup>/IL-10<sup>high</sup>/IL1-decoyRhiIL-1RAhi phenotype (reviewed by Sica et al,<sup>24</sup> Yi and Jeong,<sup>27</sup> and Heymann et al<sup>28</sup>). M2 macrophages upregulate Arg1, Mcr2, Mgl1, CD115, Kif4, Dectin-1, Chi3l2 markers, IL-4, IL-10, IL-13 cytokines, and express MMP2, 9, 12, and 13<sup>29,30</sup> with activation of Irf4, Socs3, Stat3, Stat5, Stat6, and PPARs.<sup>26</sup> Polarization of M2 macrophages requires M-CSF, IL-4, and IL-13, and is inhibited in the presence of TNF-α and INF-γ.

The classical activated M1 macrophages are the major hepatic ROS source by expression of induced nitric oxide synthase (iNOS). The iNOS metabolizes L-arginine to nitric oxide (NO), which could directly activate HSCs, and promotes collagen expression.<sup>31</sup> In turn, M2 macrophages exhibit stronger phagocytic activity, express Argenase-1, which competes with iNOS for L-arginine, but metabolizes L-arginine into urea and L-ornithine.<sup>32</sup> M1 macrophages also secrete inflammatory cytokines, IL-1β, IL-6, and TNF-α that directly regulate HSCs’ activation. Macrophage-derived IL-1β promotes the expression of intercellular adhesion molecule 1 (ICAM-1) and TIMP-1 in aHSCs. Macrophage IL-1β triggers the secretion of MIP-2 by BM-derived cells, which further promotes recruitment and activation of inflammatory cells.<sup>33,34</sup> Macrophage-derived IL-6 plays a critical role in the pathogenesis of liver fibrosis. IL-6 signal pathway is mainly mediated by gp130 and IL-6R, which are ubiquitously expressed in HSCs, hepatocytes, KCs, and sinusoid endothelial cells. Upon activation, Janus kinases (JAKs) are recruited to the IL-6/receptor complex and activated (phosphorylated). Activated JAKs phosphorylate STAT3. p-STAT3 dissociates from the receptor complex and forms a homodimer, which is then translocated to the nucleus, binds to DNA, and facilitates transcription of its target genes. IL-6 is a potent cytokine for activation of TGF-β1 production in KCs. In addition, IL-6 is one of the few inflammatory cytokines that can directly activate HSCs to synthesize collagen Type I. Interestingly, upon activation, HSCs start producing IL-6, which further promotes their activation via a paracrine mechanism.<sup>35</sup> IL-17A, a cytokine produced by T helper 17 cells (Th17), stimulates IL-6 production by aHSCs. Both IL-6 and TGF-β1 regulate differentiation of Th17 from naive T-cells. Elevated levels of IL-17A are linked to liver fibrosis. IL-17A is also implicated in direct induction of collagen Type I production in aHSCs. Finally, macrophage-derived TNF-α mediates HSCs activation mainly via p38 MAPK and NFkB pathways;<sup>36,37</sup> TNF-α signaling in aHSCs results in inhibition of BAMBI expression, and subsequent augmentation of TGF-β1 signaling.<sup>22</sup> TNF-α also increases production of ROS in aHSCs.<sup>38</sup>

**T cell cytokines regulate liver fibrosis**

In addition to macrophages, T-cells, eosinophils, and plasma cells are recruited to the injured liver (reviewed by Wynn<sup>39</sup>). T-cells regulate recruitment and activation of macrophages by releasing unique cytokines that sustain liver fibrosis during chronic liver injury. Th2 and Th17 cells promote liver fibrosis by secreting cytokines IL-4/IL-5/IL-13 and IL-17A/IL-17F separately, while the interferon-γ-producing Th1 and IL-10-producing Treg cells inhibit fibrosis progression.<sup>35,40–46</sup> T-cell-derived cytokines can directly regulate activation of HSCs. IL-4 and IL-13 exhibit about 30% similarities in amino acid (AA) sequences,<sup>47</sup> and their cellular effects are mediated by the type II IL-4 receptor, following phosphorylation of STAT6 and expression of pSTAT6 downstream genes.<sup>48</sup> HSCs express type II
IL-4RA and IL-13RA1.49 Neutralization of IL-4 by anti-IL-4 antibodies (Abs) reduces collagen deposition in mouse models of schistosomiasis-induced liver fibrosis.50 Administration of IL-4 to cultured fibroblasts stimulates collagen synthesis.51 IL-13 is a more potent fibrogenic cytokine than IL-4. Neutralization of IL-13 with anti-IL-13 Abs attenuates schistosomiasis-induced liver fibrosis (specifically reduces collagen deposition around granulomas).52,53 Furthermore, administration of IL-13 to HSCs induces STAT6 phosphorylation, collagen expression and proliferation.49 IL-17A and IL-17F are secreted by Th17 cells. In patients with liver fibrosis, and mouse models of liver fibrosis, hepatic IL-17A, IL-17F, and IL-17RA are elevated. Disruption of IL-17 signaling protects IL-17RA−/− mice from liver fibrosis.35,54

The role of B cells in liver fibrosis of different etiologies has been suggested. B cell activation, differentiation, and proliferation occurs in the lymphoid follicles of secondary lymphoid organs, such as germinal centers of spleen. Intrahepatic B cells (IHB cells) are phenotypically similar to splenic B2 cells but express lower levels of CD23 and CD21 and lack of vitamin A. Gene expression profiling of isolated PFs identified new markers (calcitonin α, mesothelin, uroplakin 1β, basomucin 1, aspirin, proteoglycan 4, glipican, and CD200) that distinguish BDL-derived activated PFs from activated HSCs.64

Origin of portal fibroblasts
Recent studies suggest that during embryonic development, PFs, HSCs, and vascular smooth muscle cells (VSMC) originate from the same precursor in the septum transversum.70,71 Lineage tracing of the mesothelial precursor cells using MesP1Cre × Rosa26-flox-Stop-flox-reporter mice provides evidence that the septum transversum originates from the mesoderm. At day 9.5 (E9.5) of embryogenesis, when the hepatic bile duct and sinusoids start to form, the septum transversum is invaded by the foregut endoderm. At E12.5, the perihepatic membrane, mesothelial cells, and submesothelial cells give rise to the mesenchymal cell precursors that invade the liver parenchyma.72,73 Several cell markers of these mesenchymal precursors have been identified, including mesothelin, desmin, p75NTR, Wt1, and ALCAM. These mesenchymal precursors interact with endodermal hepatoblasts to induce differentiation of the hepatic parenchyma. It remains unclear at which time point and specific stage of embryonic development the PF and HSC precursors diverge from each other and retain expression of cell-specific markers throughout adulthood. One explanation is that positioning during hepatic lobule formation may affect functional specialization of PFs and HSCs, leading to their divergence. In concordance, fetal PFs secrete BMPs, Jagged1, and Hedgehog ligands to...
They remain controversial. A significant population of fibrocytes of hepatic fibrocytes in the pathogenesis of liver fibrosis, lagen expressing myofibroblasts, and, therefore, the roles of hepatic fibrocytes in the pathogenesis of liver fibrosis remain controversial. A significant population of fibrocytes was shown to be recruited into the spleen in response to liver and kidney injury, LPS, bacterial infection, or TGF-β1. While splenic fibrocytes are capable of antigen presentation and differentiation into myofibroblasts (similar to hepatic fibrocytes), they exhibit unique functions. Gene expression profiling of splenic fibrocytes revealed that they also contribute to the innate immunity by mounting an elaborate immune response: splenic fibrocytes are not capable of phagocytosis, but can kill bacteria by the formation of DNA-based extracellular traps containing the antimicrobial peptide cathelicidin (CRAMP).}

**TGF signaling activates PFs**

In the cases of cholestatic liver injury, PFs respond rapidly to increasing levels of TGF-β1 by upregulation of fibrogenic genes, Col-α1( I), α-SMA, TIMP1, TGF-β2, PAI-1, elastin, fibronectin, and of CD73 ectoenzyme. At the same time, activated PFs give rise to myofibroblasts. BDL-activated PFs also secrete TGF-β1 and TGF-β2, which induce TGF-β1 signaling and transduction in nearby HSCs. FGF-2 facilitates the activation of PFs as well. Binding of FGF-2 to its tyrosine kinase receptors FGFRs and subsequent activation of Ras-MEK-Erk1/2 signaling cause proliferation and migration of αPFs. Proliferation and activation of αPFs are shown to be blocked by curcumin, a nonsteroidal yellow pigment found in rhizomes of the perennial herb Curcuma longa, which blocks ERK1/2 phosphorylation in αPFs, and thus has the potential to reduce cholestasis-induced fibrogenesis.

**Bone marrow-derived collagen-producing cells (fibrocyte)**

Fibrocytes are bone marrow-derived collagen-producing cells in the circulating blood with dual characteristics of both hematopoietic and stromal cells. They are derived from a subset of monocytes labeled with CD11b, CD115, and Gr1 in a T-cell-dependent manner. Outgrowth of fibrocytes from monocytes is controlled by T-cell-released cytokines. IL-4 and IL-13 from Th2 cells promote outgrowth of fibrocytes from CD14+ precursors, while interferon-γ and IL-12 from Th1 inhibited fibrocytes outgrowth. The circulating fibrocytes are multipotent and can differentiate into several cell types. In the injured organs, fibrocytes commit to the myofibroblast fate and contribute to the ECM deposition and tissue remodeling. At the same time, tissue fibrocytes are involved in adaptive immunity and serve as antigen-presenting cells. They express antigen-presenting membrane proteins MHC I, II, CD80, and CD86, and are able to recognize antigen and promote T-cell proliferation. Hepatic fibrocytes were implicated in collagen Type I deposition in fibrotic liver. However, they contribute only to 5%–6% of total collagen expressing myofibroblasts, and, therefore, the roles of hepatic fibrocytes in the pathogenesis of liver fibrosis remain controversial. A significant population of fibrocytes...


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