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Hepatic stellate cells as key target in liver fibrosis

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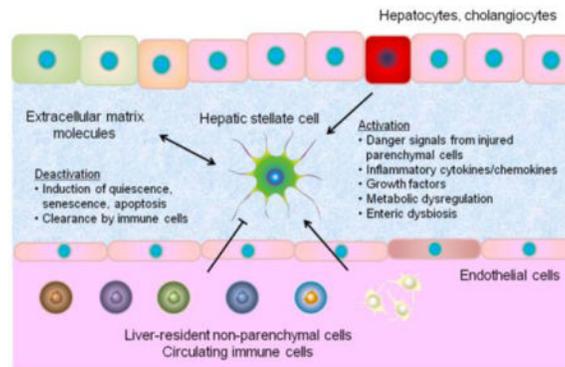
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Abstract

Progressive liver fibrosis, induced by chronic viral and metabolic disorders, leads to more than one million deaths annually via development of cirrhosis, although no antifibrotic therapy has been approved to date. Transdifferentiation (or “activation”) of hepatic stellate cells is the major cellular source of matrix protein-secreting myofibroblasts, the major driver of liver fibrogenesis. Paracrine signals from injured epithelial cells, fibrotic tissue microenvironment, immune and systemic metabolic dysregulation, enteric dysbiosis, and hepatitis viral products can directly or indirectly induce stellate cell activation. Dysregulated intracellular signaling, epigenetic changes, and cellular stress response represent candidate targets to deactivate stellate cells by inducing reversion to inactivated state, cellular senescence, apoptosis, and/or clearance by immune cells. Cell type- and target-specific pharmacological intervention to therapeutically induce the deactivation will enable more effective and less toxic precision antifibrotic therapies.

Graphical abstract



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Keywords

Myofibroblast; cirrhosis; hepatitis; alcoholic liver disease; non-alcoholic fatty liver disease; nonalcoholic steatohepatitis

1. Introduction

Chronic tissue injury leads to a sustained scarring response that gradually disrupts normal cellular functional units and eventually causes failure in multiple epithelial organs such as liver, lung, and kidney, which is estimated to account for one-third of deaths worldwide [1]. Progressive liver fibrosis can be caused by chronic infection of hepatitis B virus (HBV) or hepatitis C virus (HCV), alcohol abuse, non-alcoholic fatty liver disease (NAFLD)/non-alcoholic steatohepatitis (NASH), and other relatively rare conditions such as autoimmune hepatitis, hemochromatosis, Wilson's disease, and primary/secondary biliary cholangitis. Cirrhosis is the terminal stage of progressive liver fibrosis, which is estimated to affect 1% to 2% of global population and results in over 1 million deaths annually worldwide [2, 3].

Lethal complications of cirrhosis include functional liver failure, portal hypertension-induced variceal bleeding, ascites, and hepatic encephalopathy, systemic bacterial infection, and liver cancer, especially hepatocellular carcinoma (HCC) [3]. Annual direct and indirect costs for the care of cirrhosis exceed \$12 billion in the U.S. alone [4]. Although approved therapies directly targeting and reversing advanced fibrosis are still lacking, clinical studies have indicated that liver fibrosis and even cirrhosis can be regressed by therapeutic intervention aimed at the primary disease etiology [5]. In this review, we summarize recent findings relevant to direct therapeutic targeting of the major fibrogenic cell type in the liver, the activated hepatic stellate cell (HSC), as an antifibrotic strategy.

2. Cellular sources of matrix-producing myofibroblasts

2.1. Myofibroblasts: driver of tissue fibrogenesis in multiple organs

In chronic fibro-proliferative diseases, affecting multiple organs such as lung, kidney, and liver, presence of myofibroblasts is a key common feature [6]. The myofibroblast is a fibroblast-like cell with contractile properties, which is derived typically from cells of mesenchymal lineage via transdifferentiation, often referred to as "activation". Proliferating myofibroblasts are the key source of excess extracellular matrix (ECM) molecules such as collagen type I and III as well as other proteins that constitute pathologic fibrous tissues [7]. Paracrine factors such as platelet-derived growth factor (PDGF), transforming growth factor β (TGF β), and connective tissue growth factor (CTGF), together with other growth factors and cytokines/chemokines, activate cellular signaling pathways of cell proliferation, migration, ECM protein secretion, and contractility that promote transdifferentiation of precursor mesenchymal cells (e.g., pericytes and resident fibroblasts) into fibrogenic myofibroblasts and maintain their activated state [7]. PDGF receptor β (PDGFR β) and other transmembrane proteins such as integrins are induced on the surface of myofibroblasts and precursors to form positive feedback loops for their perpetuated activation.

2.2. Hepatic stellate cells (HSCs): precursor of myofibroblasts

HSCs are resident mesenchymal cells that retain features of resident fibroblasts (embedded in normal stromal matrix) and pericytes (attached to endothelial cells of capillaries), and comprise approximately one-third of nonparenchymal cells and 15% of total resident cells in normal human liver [7]. HSCs reside in a virtual subendothelial space between the basolateral surface of hepatocytes and the anti-luminal side of fenestrated sinusoidal endothelial cell layer (space of Disse) filled with thin permeable connective tissue, where exchange of biomolecules between portal blood flow from gastrointestinal tract and hepatocytes occurs. Intercellular communication via soluble mediators and cytokines/chemokines can occur between neighboring cell types such as hepatocytes, biliary epithelial cells, hepatic progenitor cells (oval cells), Kupffer cells (liver resident macrophages), bone marrow-derived macrophages, liver sinusoidal endothelial cells (LSECs), infiltrating immune cells, and nerve cells [7, 8]. In fibrogenic liver, quiescent HSCs transdifferentiate into proliferative, migratory, and contractile myofibroblasts, manifesting pro-fibrogenic transcriptional and secretory properties (so called “cell activation”), and secrete ECM molecules that accumulate and form scar tissue in the space of Disse that leads to sinusoidal capillarization characterized by loss of endothelial fenestrations [7] (Figure 1). Vitamin A (retinoid) storage in cytoplasmic droplets is a unique characteristic feature of quiescent HSCs, which is gradually lost during the process of transdifferentiation, although its causal relationship with HSC activation is still uncertain [7, 9]. Endothelin-1 (ET-1), a potent vasoconstrictor, is secreted by activated HSCs, and promotes cell proliferation, fibrogenesis, and contraction, which is plausibly linked to portal hypertension in cirrhosis [10].

A number HSC-specific marker genes and proteins, including glial fibrillary acidic protein (GFAP), nerve growth factor receptor (p75), desmin, lecithin-retinol acyltransferase (LRAT), integrin $\alpha_v\beta_3$, mannose 6-phosphate/insulin-like growth factor II receptor (M6P/IGF-II-R), collagen type I, collagen type VI receptor (CVIR), α -smooth muscle actin (α SMA), PDGFR β , vimentin, and cytoglobin, have been exploited for their histological detection, genetic targeting, cell fate tracing, therapeutic targeting, and imaging [7, 11–14]. In addition, by using genome-wide transcriptome profiling, HSC-specific 122-gene and 194-gene signatures, associated with poorer patient prognoses, have been identified [15, 16].

A widely used experimental culture model is primary human and rodent HSCs, isolated by discontinuous gradient ultracentrifugation with or without cell surface marker-based sorting, that undergo spontaneous fibrogenic activation when grown on a hard plastic dish [17, 18]. Although certain distinct differences in transcriptomic dysregulation have been noted between in vitro and in vivo settings, co-culture with other cell types such as Kupffer cells could partially restore more physiological molecular dysregulation [19]. Immortalized cell lines of human (LX-2 and TWNT-4), mouse (JS-1, GRX, and CoI-GFP), and rat (HSC-T6 and CFSC) HSCs, and rat portal myofibroblasts (RGF and RGF-N2) were established, and have been used for mechanistic analysis of antifibrotic targets and therapies [20–27].

2.3. Resident mesenchymal cells as major source of myofibroblasts

Rodent-based studies have elucidated several potential sources of myofibroblasts. Cell fate tracing, genetically labeling HSCs by using LRAT, involved in the cytoplasmic lipid droplet

formation, showed that 82% to 96% of myofibroblasts were derived from HSCs in mice treated with carbon tetrachloride (CCl₄), bile duct ligation (BDL), 3,5-diethoxycarbonyl-1,4-dihydrocollidin diet, and *Mdr2*-knockout mice [28]. Another study, labeling myofibroblasts using *Colla1* promoter in mice, reported that HSCs were the major source of myofibroblasts (>87%) in chemical injury (CCl₄), whereas portal fibroblasts were the major source (>70%) in an early stage of cholestatic injury (BDL) [29]. These studies collectively suggest that the liver resident mesenchymal cells, particularly HSCs, are the major source of fibrogenic myofibroblasts, although these rodent-derived, single gene marker-based findings need to be verified in humans. Furthermore, it is still unknown whether cell(s) of origin is/are associated with response to antifibrotic therapies.

2.4. Other potential cellular sources of myofibroblasts

Although relative contribution is plausibly minor or negligible, other cell types in addition to HSCs and portal fibroblasts have been proposed as alternative sources of myofibroblasts. Collagen-producing fibrocytes, distinct from HSCs, are recruited from bone marrow in BDL mice [30, 31]. Mesenchymal stem cells are also suggested as another bone marrow-derived source of myofibroblasts [32]. *Wtl*-expressing mesothelial cells (approximately 15% of the cell population) may be a source of myofibroblasts near the liver surface via mesothelial–mesenchymal transition (MMT) in CCl₄ mice [33]. Epithelial-mesenchymal transition (EMT) of liver parenchymal cells, i.e., hepatocytes and cholangiocytes, is less likely the source of myofibroblasts, although the parenchymal cells could express some of the mesenchymal genes without ECM protein secretion [32, 34–36]. Hedgehog signaling contributes to mesenchymal-to-epithelial transition (MET) of a subpopulation of HSC-derived myofibroblasts into hepatic progenitor cells in the process of liver regeneration in mice [37].

3. Mechanisms of HSC activation

3.1. Extracellular events that promote HSC activation

Several cellular events in injured/inflamed hepatic tissue microenvironment as well as extrahepatic factors are known to directly induce HSC activation in etiology-specific or independent manner [38] (Figure 1).

3.1.1. Epithelial cell injury—Progressive fibrotic liver diseases are accompanied with chronic damage to parenchymal epithelial cells, i.e., hepatocytes and cholangiocytes (biliary epithelial cells). According to the liver disease etiology (viral infection, hepatic toxins, metabolic disorders, and autoimmune diseases), various modes of cell death (e.g., apoptosis, necrosis, necroptosis, and autophagic cell death), trigger cell death responses [39]. Immune cell-mediated killing of hepatocytes is a common theme in HBV- and HCV-related liver fibrosis, which involves pro-fibrogenic inflammatory chemokines and their receptors such as C-C motif chemokine ligand 2 (CCL2), CCL21, interleukin-8 (IL-8), IL-17, IL-22, C-X-C motif chemokine ligand 9 (CXCL9), CXCL10, CXCL11, and C-X-C motif chemokine receptor 1 (CXCR1) [8]. Hepatic nuclear factor κ B (NF- κ B)-inducing kinase (NIK) activates bone marrow-derived macrophages, increases hepatic injury, and promotes HSC activation and fibrogenesis [40]. The dead or dying epithelial cells as well as leukocytes

phagocytosing the cells release inflammatory mediators, damage-associated molecular patterns (DAMPs) or danger signals, which initiate and perpetuate a non-infectious “sterile” inflammatory response. Among such mediators, tumor necrosis factor (TNF), IL-6, IL-1 β , reactive oxygen species (ROS), hedgehog ligands, and nucleotides can contribute to initiation of HSC activation [8, 41, 42]. Such host biomolecules from injured hepatocytes, “alarmins”, include high mobility group protein B1 (HMGB1) and IL-33 [43, 44]. IL-33 induces IL-13 production in liver resident innate lymphoid cells type II, enhances TGF β signaling through IL-4R α and signal transducer and activator of transcription 6 (STAT6) in HSCs, and promotes fibrosis [45]. Loss of the ROS-generating enzymes, NADPH oxidase 1 (NOX1), NOX2, or NOX4, attenuates hepatocyte injury and liver fibrosis in CCl $_4$ and BDL mice [46, 47]. A NOX1/NOX4 inhibitor, GKT137831, reduces hepatic apoptosis and fibrosis in CCl $_4$ and BDL mice [48]. Specific modes of hepatocyte and cholangiocyte death may be monitored by circulating cytokeratins as biomarkers [49]. Apoptosis inhibitors such as a pan-caspase inhibitor, emricasan (IDN-6556), aiming to reduce hepatocyte death, are under evaluation in clinical trials in post-transplant HCV and NASH patients (ClinicalTrials.gov: NCT02138253, NCT02686762, NCT02960204; ENCORE trials). Apoptosis signal-regulating kinase 1 (ASK1) is a mitogen-activated protein kinase (MAP3K) that activates c-Jun N-terminal kinase (JNK) and p38 MAPK in response to various cellular stresses. A phase 2 trial of ASK1 inhibitor, selonsertib (GS-4997), resulted in more than 1 stage of liver fibrosis improvement in 43% of enrolled NASH patients (NCT02466516).

3.1.2. Altered extracellular matrix (ECM)—Progressive deposition of ECM proteins in the space of Disse leads to increased density and stiffness of ECM. In addition, matrix composition shifts from collagen type IV, heparan sulfate proteoglycan, and laminin to fibrillar collagen type I and III. These changes serve as mechanical stimuli to activate HSCs at least partially via integrin signaling pathways, forming positive feedback loops [50, 51]. LX-2 cells cultured on acrylamide gels with 12 kPa pressure, mimicking increased tissue stiffness in fibrotic liver, show higher collagen I expression via *RHOA* upregulation, which is suppressed by c-Src [52]. Primary HSCs cultured in hydrogels stiffened or softening in situ over time revealed time-course dynamic transcriptional changes during the process of culture activation and regression, respectively [53, 54]. Expanded ECM also serve as reservoir by binding growth factors such as PDGF, hepatocyte growth factor (HGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF), which promote HSC proliferation [6, 55]. Reduced matricellular protein, CCN3/NOV, enhances fibrogenic gene expression in primary HSCs and CFSC cells [56]. Lysyl oxidase-like-2 (LOXL2) is a matrix enzyme expressed by HSC, which catalyzes crosslinking of collagens and elastins, and its inhibition by monoclonal antibody (AB0023) reduces liver and lung fibrosis in experimental models [57]. A humanized monoclonal LOXL2 antibody, simtuzumab (GS-6624), has been tested in phase 2 trials for patients with HCV (NCT01707472), primary sclerosing cholangitis (NCT01672853), and fibrotic (NCT01672866) or cirrhotic (NCT01672879) NASH.

3.1.3. Immune regulation—Macrophages represent a heterogeneous population (10–15% of liver cells), which can have both pro- and anti-fibrogenic effects through paracrine

regulation of HSC activation [41, 58]. Macrophage depletion in murine models has revealed their pro-fibrogenic role [59]. Polarized and plastic activation of macrophages is traditionally classified into classic M1 and alternative M2 activation associated with helper T (Th)1 (TNF α , IL-1, IL-12, and inducible nitric oxide synthase [iNOS]) and Th2 (IL-4, IL-10, and IL-13) responses, respectively [60]. p53 depletion in HSCs in mice leads to M2 activation that promotes HCC proliferation by affecting the tumor microenvironment [61]. Lymphocyte antigen 6 complex (LY6C)^{hi} monocyte-derived macrophages are recruited in C-C motif chemokine receptor 2 (CCR2)-dependent manner and secrete pro-fibrogenic mediators such as TGF β , PDGF, CCL2, CCL3, CCL5, CCL7, and CCL8 [8, 62]. Galectin-3 is a macrophage-derived lectin, which promotes HSC activation and can be pharmacologically inhibited by GR-MD-02, currently in a phase 2 trial in NASH patients (NCT02462967) [63, 64]. In contrast, LY6C^{lo} macrophages represent a fibrolytic subset that expands during fibrosis regression [62]. C-X3-C motif chemokine receptor 1 (CX3CR1) induces differentiation to LY6C^{lo} cells, which promote apoptosis of activated HSCs and secrete matrix metalloproteinase 12 (MMP-12) and MMP-13 to promote ECM degradation [65, 66].

Th17 cells secrete IL-17A and IL-22, and are increased in the liver and circulation in chronic liver diseases [67]. IL-17 receptor is expressed on the surface of macrophages and HSCs, and the signaling induces pro-inflammatory cytokines such as TNF, IL-1 β , IL-6, and IL-17A, and collagen 1 expression in HSCs by activating STAT3 signaling in mice [68]. IL-22 transgenic mice are protected from CCl₄-induced fibrosis through increased HSC senescence by binding to its receptors, IL-10R2 and IL-22R1, up-regulating STAT3 and increasing p53 expression [69]. Recombinant IL-22 also attenuates HSC activation and fibrosis [70]. On the other hand, IL-22 may have adverse effect in HBV infection [71]. Intrahepatic IL-8 producing Foxp3⁺CD4⁺ regulatory T cells (Tregs) activate HSCs and promote fibrosis in chronic hepatitis C [72]. $\gamma\delta$ T cells restrict liver fibrosis by inducing HSC apoptosis in CCR6-dependent manner [73].

B cells, which account for up to half of lymphocytes in the liver, contribute to fibrogenesis in CCl₄ mice [74]. HSC-mediated MyD88-dependent innate B cell activation promotes hepatic fibrosis [75].

Natural killer (NK) cells kill senescent HSCs and produce interferon γ (IFN γ) that induces apoptosis and cell cycle arrest in HSCs [76]. IL-15 increases NK cells and support their killing of HSCs [77]. Regulatory CD4⁺ T cells can suppress NK cells, and thereby support survival of activated HSCs [78]. Natural killer T (NKT) cells also secrete IFN γ and kill activated HSCs, whereas a subset of NKT cells produce IL-4, IL-13, osteopontin, and hedgehog ligands, and promote HSC activation and liver fibrosis via CXCR6-CXCL16 axis in chronic liver diseases including NASH [79–81].

3.1.4. Other cell types—Liver sinusoidal endothelial cells (LSECs) maintain HSC quiescence, thus inhibiting intrahepatic vasoconstriction and fibrosis development [82]. In pathological conditions, LSECs become capillarized with lost fenestrations, lose the hepatoprotective property, and promote angiogenesis and vasoconstriction. A soluble guanylate cyclase activator, BAY 60-2770, leads to reversal of the capillarization, restores

HSC quiescence without directly affecting HSCs, and reduces fibrosis in thioacetamide (TAA)-treated cirrhotic rats [83].

Close proximity between hepatic progenitor cells and HSCs is often observed at the site of ductular reaction, a pathological feature accompanying fibrotic liver diseases, which may have functional implication in HSC activation and/or liver regeneration possibly via contribution of HSCs to progenitor cell pool [84, 85].

Osteopontin, a multifunctional secreted phosphoprotein, induces collagen I expression in HSC by affecting biliary epithelial cells and promoting ductular reaction [86, 87]. Platelets are a source of pro-fibrogenic cytokines and growth factors such as PDGF, TGF β , and EGF, and they can also suppress collagen expression via HGF-Met signaling pathway [7, 88].

3.1.5. Metabolic dysregulation and enteric dysbiosis—A so-called Western diet can alter the intestinal microbiome (intestinal dysbiosis), and lead to release and increased exposure to pathogen-associated molecular patterns (PAMPs) that can activate HSCs through toll-like receptors (TLRs) [89]. A high-fat diet increases the proportion of intestinal Gram-negative endotoxin-producing bacteria, resulting in higher rates of bacterial translocation, and accelerated fibrogenesis by enhancing HSC activation in CCl₄ and BDL mice [90]. Transplantation of the fibrogenic microbiome to control diet-fed mice exacerbates liver damage and fibrosis, suggesting the causal role of microbiome in fibrogenic HSC activation. In contrast, commensal microbiota is hepatoprotective and prevents liver fibrosis by mitigating HSC activation in mice [91]. Lipotoxicity caused by free intracellular cholesterol accumulation sensitizes HSCs to TGF β -induced activation in NASH mouse model [92].

3.1.6. Chronic infection of hepatitis virus—Chronic infection by hepatotropic viruses, HBV and HCV, has been one of the major risk factors for fibrotic liver diseases worldwide [93]. Given that population-level viral eradication will not be achieved in the next several decades despite the development of highly effective antivirals, directly targeting fibrosis pathways irrespective of viral clearance will remain a viable antifibrotic strategy. Viral genes and proteins can directly or indirectly promote HSC activation. HBV e antigen directly induces activation and proliferation of rat HSCs in vitro via the TGF β pathway, and core and X proteins similarly activate human LX-2 cells via PDGF β signaling [94, 95]. Although HCV cannot infect HSCs, viral core and non-structural proteins directly induce inflammatory and pro-fibrogenic pathways in HSCs [96, 97]. HCV core protein may promote EMT of infected hepatocytes through TGF β signaling [98]. HCV core also increases ECM protein, thrombospondin-1 (TSP-1) and transcription factors, which increase bioavailability and expression of TGF β [99, 100]. Elevated expression of IL-34 and macrophage colony-stimulating factor (M-CSF) from HCV-infected hepatocytes stimulates maturation of peripheral monocytes into macrophages, which contributes to HSC activation by enhancing TGF β and PDGF β signaling, blocking antifibrotic NK cell-derived IFN γ , and inducing MMP-1 [101]. Human immunodeficiency virus (HIV) co-infection is clinically known to accelerate fibrosis progression in HCV-infected individuals, and it can be slowed down by antiretroviral therapies [102, 103]. HIV can directly infect HSCs, and viral envelop

protein, gp120, promotes collagen I expression via CXCR4 [104, 105]. Exposure to HIV induces TGF β , ROS, and NF- κ B in a hepatocyte/LX-2 co-culture reporter cell model [106].

3.2. Molecular dysregulation in activated HSCs

Functional studies have elucidated complex dysregulation of molecular pathways in HSCs/myofibroblasts for their perpetuated activation through multiple signaling pathways and other mechanisms as summarized below (Figure 2).

3.2.1. Membrane receptor signaling pathways

3.2.1.1. TGF β : Activated HSCs secrete latent TGF β in response to liver injury, which forms an autocrine positive feedback loop driving fibrogenesis via SMAD2/SMAD3, whereas SMAD7 suppresses the activation [7, 107, 108]. Apoptotic body-engulfing macrophages produce TGF β and activate HSCs [30, 109–111]. Liver-targeted TGF β 1 expression induces fibrosis and chemical carcinogenesis in mice [112, 113]. Therapeutic strategies, e.g., blocking circulating TGF β 1, antagonizing its receptors, or blocking its activation at the cell surface, have been challenging due to adverse effects of systemic TGF β antagonism, including inflammation and cancer [6, 7, 114]. Knockdown of transient receptor potential melastatin7 (TRPM7) attenuates TGF β 1-induced expression of myofibroblast markers, increases the ratio of MMPs/tissue inhibitor of metalloproteinases (TIMPs), and decrease SMAD2/SMAD3-associated collagen production [115, 116]. Ca(2+)/calmodulin-dependent protein kinase II (CaMKII) mediates TGF β -induced HSC proliferation, but not collagen production [117]. CUG-binding protein 1 (CUGBP1), induced by TGF β , binds the 3'-untranslated region (UTR) of antifibrogenic IFN γ mRNA and promotes its decay [118].

3.2.1.2. PDGF: Cell proliferative/inflammatory cytokines, PDGF, FGF, TGF α , p38 MAPK, and JNK, converge on MAPK/extracellular signal-regulated kinase (ERK) pathway activation [6, 119]. PDGF is a potent chemoattractant for HSCs and myofibroblasts [7]. PDGF β receptor (PDGFR β) expression is induced during initiation of HSC activation, and enhances inflammatory and fibrogenic responses to chemical injury via ERK, AKT, and NF- κ B pathways, without affecting hepatocarcinogenesis in mice [120, 121]. Small molecule tyrosine kinase inhibitors, including imatinib, sorafenib, nilotinib, and sunitinib, suppress proliferative and fibrogenic properties of activated HSCs in organotypic ex vivo culture of fibrotic human liver tissues [122–125]. CD248/endothelial cell protein receptor 2 is a PDGF downstream regulator of HSC proliferation during chronic liver injury [126]. A tyrosine phosphatase SHP-1 inhibits proliferation of activated HSCs via PDGF signaling [127]. Knockdown of protein tyrosine phosphatase receptor type O (PTPRO) can suppress PDGF β -induced HSC proliferation and activation by reducing phosphorylation of ERK and AKT in mice [128]. PDGF-C transgenic mice exhibit HSC activation and develop hepatic steatosis, fibrosis, and cancer despite limited hepatocyte injury and lack of extensive fibrosis [129]. Pigment epithelium-derived factor (PEDF)-derived 34-mer peptide (Asp44-Asn77) down-regulates PDGF receptor expression and blocks HSC activation [130].

3.2.1.3. CTGF: CTGF/CCN2 is a central fibrogenic effector of TGF β pathway, and its knockout suppresses fibrogenesis in CCl₄-treated mice [131]. A human monoclonal antibody to CTGF, FG-3019, was tested in HBV fibrosis patients, although its clinical utility

as antifibrotic therapy was not clear due to prominent effect of anti-HBV drug, entecavir (NCT01217632) [132, 133].

3.2.1.4. EGF: EGF receptor is overexpressed and phosphorylated in activated HSCs, and its pharmacological inhibition reduces hepatic fibrosis and HCC nodules in rodent models, although EGF receptor expression is not restricted to HSCs [134]. Genetic knockout of EGF receptor in macrophages reduces HCC development in mice [135].

3.2.1.5. Rho kinases (ROCKs): ROCKs and family proteins, including Rac1, promote reactive oxygen species (ROS) production and enhance contractility of HSCs, which can be inhibited by kinase inhibitors and other measures such as adenosine [136–139]. HSC-specific inhibition of Rho-kinase reduces portal pressure in cirrhotic rats without major toxicity [140].

3.2.1.6. Integrins: Integrins are heterodimeric transmembrane proteins composed of α/β subunits that sense cell adhesion, transmit signals from ECM, and promote fibrogenesis by activating latent TGF β [6, 7]. Genetic deletion of integrin αv as well as its pharmacological blockade by a small molecule, CWHM 12, in activated HSCs reduces CCl₄-induced hepatic fibrosis, whereas no such effect is observed for integrins $\beta 3$, $\beta 5$, $\beta 6$, and $\beta 8$ [51]. Integrin $\beta 1$ induces PAK proteins and YAP signaling in myofibroblasts and promote liver fibrosis [141]. Integrin $\alpha 1\beta 6$ induces ROS accumulation through Rac1-NADPH oxidase 1 enzyme complex in HSCs [142].

3.2.1.7. Renin-angiotensin system: Angiotensin II, secreted by HSCs, binds to angiotensin II type 1 receptor (AT1R) and promotes liver fibrosis via JAK2, which is associated with portal hypertension [143, 144]. Blocking the renin-angiotensin system by angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARBs) such as losartan may be an effective antifibrotic therapy as suggested by experimental and clinical studies [145, 146]. The AT1R pathway has a synergistic activity with cannabinoid signaling through receptor heterodimerisation during HSC activation, suggesting that combination of ACE/ARB and CB1 receptor antagonist may be an option of antifibrotic therapy [147]. Swertiamarin (Swe), an extract of *Gentiana manshurica* Kitag, significantly inhibits angiotensin II-induced HSC proliferation and activation via AT1R [148].

3.2.1.8. Wnt/ β -catenin signaling: The Wnt pathway regulates a variety of cellular processes such as development, differentiation, and proliferation, and can have both activating and inhibitory effects on HSCs [149]. siRNA-mediated β -catenin knockdown reduces collagen I and III expression, inhibits cell proliferation, and induces apoptosis of HSCs in vitro [150]. Wnt signaling inhibition by Dickkopf-1 (DKK1) abrogates epigenetic repression and restores peroxisome proliferator-activated receptor- γ (PPAR γ) activity, and reduces fibrosis [151]. Conversely, HSC-derived delta-like homolog 1 (DLK1) activates HSCs via epigenetic PPAR γ repression and promote liver regeneration [152]. A selective inhibitor of the cAMP-response element-binding protein (CBP)/ β -catenin interaction, PRI-724, inhibits HSC activation and reduces liver fibrosis in mice [153]. In contrast, β -catenin-dependent canonical Wnt activation is required to maintain quiescent state of HSCs in vitro [154]. Roof plate-specific spondin (RSPO) proteins, potent Wnt pathway agonists, suppressed HSC

activation, which is impaired by DKK1 [155]. In activated rat HSCs, non-canonical Wnt pathway activation dominates and confers HSC survival, accompanied with Wnt5a over-expression [156]. A natural Wnt5a inhibitor, secreted frizzled-related protein 5 (Sfrp5), decreases HSC activation and liver fibrosis [157]. These findings collectively suggest that involvement of Wnt pathway in HSC activation is highly complex and biological context-dependent.

3.2.1.9. Hedgehog (Hh) signaling: Hh ligand expression from hepatocytes induces HSC activation and promotes liver fibrosis and hepatocarcinogenesis in a rodent model [158, 159]. Smoothed (Smo), an obligate intermediate in Hh signaling pathway, is required not only for fibrogenic activation of HSCs, but also for epithelial regeneration in mice [37]. Hh signals transduced via activated Smo are transmitted to Gli transcription factors that induce target gene expression [160]. Gli1-positive perivascular mesenchymal cells give rise to 39% of α SMA-positive myofibroblasts after tissue injury [161]. Forskolin, a hedgehog signaling inhibitor, attenuates CCl₄-induced liver fibrosis in rats [162]. Pharmacological inhibition of Hh signaling by vismodegib (GDC-0449) attenuates liver fibrosis in BDL and NASH models [163, 164]. Inhibition of Notch or Hh signaling in myofibroblasts leads to mesenchymal-to-epithelial like transdifferentiation with reduced fibrosis in mice [165].

3.2.1.10. Neurochemical receptors: The cannabinoid system is involved in neuro/immune-modulatory function, and has two G protein-coupled receptors, CB1 (pro-fibrogenic) and CB2 (hepatoprotective) [166]. Systemic antagonism of CB1 can induce serious psychiatric adverse effects as seen during trials of rimonabant. Newer generation antagonists with improved target specificity and limited central nervous system penetration such as AM4113 and AM6545 have been developed based on the crystal structure of CB1 [167, 168]. A germline genetic variant in the *CNR2* gene (encoding CB2), CB2-63QQ, was found to be associated with more severe necroinflammation in patients with chronic hepatitis C [169]. A selective CB 2 agonist, JWH-133, reduces fibrosis by inducing HSC quiescence/apoptosis and reducing IL-17 production by Th17 cells [166, 170]. Nutritional products such as curcumin (diferuloylmethane), a bioactive polyphenolic ingredient of turmeric, may have potential to inhibit CB1 as well as virtually all cellular pathways involved in HSC activation [171, 172]. Antagonism of a serotonin receptor, 5-hydroxytryptamine 2B receptor (5-HT_{2B}), on HSCs attenuates fibrosis and enhances hepatic regeneration [173]. Inhibition of 5-HT_{2A} similarly suppresses fibrogenic gene expression and induces apoptosis in HSCs [174].

3.2.1.11. Toll like receptors (TLRs): TLRs are pattern recognition receptors (PRRs) that detect microbial infection and exposure as PAMPs, including bacterial protein products, transcripts, and genomic DNA [8]. Liver is consistently exposed to PAMPs from gastrointestinal and oral microflora, and it is known that plasma and portal level of endotoxin such as lipopolysaccharide (LPS), a Gram-negative bacteria constituent, is elevated in cirrhotic patients due to bacterial dysbiosis and overgrowth, as well as gut leakiness [175]. The composition of the gut microbiome from cirrhosis patients was highly comparable across different populations, suggesting its influence on pathogenesis of fibrotic liver diseases via TLRs [176]. HSCs express TLR4 and TLR9, and TLR2 is induced by

TLR4, upon activation by pro-inflammatory and pro-fibrogenic pathways and cytokines including NF- κ B, JNK, TGF β , IL-6, IL-8, CCL2 (MCP-1), MIP-2, intercellular cell adhesion molecule 1 (ICAM1), vascular cell adhesion molecule 1 (VCAM1), and E-selectin [177]. LPS-induced TLR4 activation downregulates Bambi (a TGF β pseudoreceptor) and sensitizes quiescent HSCs to TGF β activation mediated by MyD88-NF- κ B pathway, and enhances liver fibrogenesis in CCl₄ and BDL mice [178]. TLR4 activation is required for HCC promotion, but not initiation in mice [179]. TLR9 senses DNA from apoptotic hepatocytes to stop chemotaxis at the site of dying hepatocytes and stimulate collagen synthesis [180]. Bacterial DNA activates TLR9 on resident and recruited macrophages to release IL-1 β , which activates HSCs and enhances lipid accumulation, insulin resistance, and injury in hepatocytes, and collectively promotes fibrogenesis and hepatic inflammation [181]. TLR2 stimulates TNF α production from intestinal monocytes that activates the RhoA pathway in intestinal epithelial cells to increase intestinal permeability and bacterial translocation to the liver [182]. TLR2 deficiency leads to attenuated liver fibrosis in choline-deficient amino acid-defined (CDAA) diet-fed NASH mice [183]. Exosome-mediated TLR3 activation in HSCs promotes liver fibrosis by inducing IL-17A production by $\gamma\delta$ T cells in mice [184].

3.2.1.12. Lysophospholipid pathway: Lysophosphatidic acid (LPA) is a pleiotropic growth factor-like lysophospholipid, involved in lipid homeostasis. Hepatocyte-derived autotaxin (ATX) catalyzes production of LPA that activates LPA receptor 1 (LPAR1) on HSCs, and increased ATX in various chronic liver diseases has been association with poorer prognosis and cancer risk, [185, 186]. Pharmacological and/or genetic inhibition of ATX and/or LPAR1 attenuates HSC activation and reduces liver fibrosis and cancer in rodent models [185, 186].

3.2.1.13. Adipokines: Adipokines, e.g., leptin and adiponectin, are soluble factors secreted by adipose tissue, which contribute to the pathogenesis of obesity- and metabolic disorder-related complications through their pro- or anti-inflammatory properties [187]. Leptin and adiponectin exert pro- and anti-fibrogenic function, respectively, via their receptors on HSCs [188, 189]. Leptin reduces expression of sterol regulatory element-binding protein-1c (SREBP-1c) and activates HSCs through involvement of β -catenin pathway [190]. Adiponectin attenuates liver fibrosis by inducing nitric oxide (NO) production via adiponectin receptor 2-AMP-activated protein kinase (AMPK)-JNK/ErK1/2-NF- κ B pathway and TIMP-1, and inhibits proliferation and migration of activated HSCs [191, 192]. Adiponectin induces expression of aquaglyceroporin (known to be down-regulated in association with obesity) and inactivates HSCs [193]. Adiponectin-derived synthetic short peptide, ADP355, suppresses focal adhesion kinase (FAK) activity and TGF β 1/SMAD2 signaling and promotes AMPK and STAT3 signaling, and inactivates HSCs in mice [194, 195].

3.2.1.14. Vascular adhesion protein 1 (VAP-1): VAP-1 (encoded by *AOC3*) is a membrane sialoglycoprotein expressed by LSECs that promotes lymphocyte recruitment [196]. VAP-1 is also expressed on activated HSCs and associated with hepatic inflammation and fibrosis in NASH patients and mouse models [197]. A selective VAP-1 inhibitor, PXS-4728A, was tested in a phase 1 study.

3.2.1.15. Tumor necrosis factor-like weak inducer of apoptosis (TWEAK):

TWEAK (encoded by *TNFSF12*) is secreted from macrophages, monocytes, and T lymphocytes upon liver injury, and signals through its receptor, FGF-inducible 14 (Fn14), to activate liver-resident progenitor cells, while hepatocyte replication is impaired in chronic liver diseases [198]. TWEAK also leads to liver fibrosis progression by directly promoting HSC proliferation. TWEAK, and by enhancing *SIRT1* expression and decreasing p53 acetylation, which assumedly inhibits HSC senescence [199, 200].

3.2.1.16. G protein-coupled receptor 91 (GPR91): Sirtuin 3 (SIRT3), an NAD(+)-dependent protein deacetylase decreased in NAFLD, suppresses α SMA expression via GPR91 on HSCs in methionine- and choline-deficient (MCD) diet-fed NAFLD mice [201].

3.2.1.17. Axl/growth arrest specific 6 (Gas6): Axl is a receptor tyrosine kinase, activated by its ligand, Gas6. Serum Gas6 level is increased as alcoholic liver disease or chronic hepatitis C progresses [202]. Axl knockout as well as inhibition by BGB324 result in attenuated HSC activation and reduced fibrosis in CCl₄ mice.

3.2.2. Nuclear receptor signaling pathways

3.2.2.1. Farnesoid-X-receptor (FXR): FXR signaling, activated by bile acids, enhances insulin sensitivity, and fatty acid beta-oxidation, and reduces gluconeogenesis and lipogenesis in hepatocytes [89]. A synthetic lipophilic bile acid, obeticholic acid (OCA), has an FXR agonistic activity stronger than the chemically similar ursodeoxycholic acid, and reduced liver fibrosis in non-cirrhotic NASH patients in a phase 2 trial (FLINT trial) [203]. OCA is currently being evaluated in a phase 3 trial in fibrotic NASH patients (REGENERATE trial, NCT02548351). In addition, the following agents targeting FXR signaling are being evaluated in clinical trials: GS-9674, synthetic non-steroidal FXR agonist; INT-767, bile acid analogue agonizing FXR and TGR5 (transmembrane G-protein bile acid receptor expressed on sinusoidal and bile duct epithelial cells) [89, 204]. Although FXR expression and OCA response are limited in HSCs, induction of FXR signaling and resulting suppression of sphingosine-1-phosphate receptor 2 (S1PR2) signaling by dihydroartemisinin (DHA), an artemisinin derivative, restricts HSC contraction and fibrogenesis, and improves portal hypertension in rodent models [205–207].

3.2.2.2. PPARs: PPARs, consisting of α , β/δ , and γ isoforms that share common target DNA sequences but with distinct ligand selectivity and tissue distribution, serve as receptors for a wide range of fatty acids and derivatives; they transcriptionally regulate various metabolic processes to maintain lipid and energy homeostasis [208]. PPAR γ has been extensively studied as a potential antifibrogenic target in HSCs [209]. PPAR γ suppresses angiogenic PDGFR β signaling and TGF β 1 via the β -catenin pathway in HSCs [210, 211]. Lipid-induced hepatocyte-derived extracellular vesicles regulate HSCs via miR-128-3p targeting PPAR γ [212]. Histone H3K9 demethylase JMJD1A modulates HSCs activation by epigenetic regulation of PPAR γ [213]. Leptin inhibits PPAR γ expression via GATA binding protein 2 in HSCs [214, 215]. Adiponectin regulates HSC activation via PPAR γ -dependent and independent mechanisms [216]. Curcumin regulates PPAR γ coactivator-1 α expression by AMPK pathway in HSCs in vitro [217]. Activation of PPAR γ /p53 signaling is required

for curcumin to induce HSC senescence [218]. Thiazolidinediones (TZDs), synthetic insulin sensitizing PPAR γ agonists such as pioglitazone and rosiglitazone, improved steatosis and lobular inflammation but not fibrosis in non-cirrhotic NASH patients in a phase 3 trial (PIVENS trial) [219]. Safety concerns about TZDs such as the cardiovascular toxicity of rosiglitazone and weight gain due to redistribution of body fat have led to suboptimal acceptance of these agents as NASH treatments [89]. A dual PPAR α/δ agonist, elafibranor (GFT-505), improved histological NASH with no worsening of fibrosis in non-cirrhotic NASH patients in a phase 2 trial, which may suggest indirect suppression of hepatocyte lipotoxicity-driven fibrogenesis (GOLDEN-505 trial) [220]. A follow-up phase 3 trial is currently recruiting patients with fibrotic NASH patients (NCT02704403). A dual PPAR α/γ agonist, saroglitazar, is being evaluated in a phase 3 trial for no worsening fibrosis in NASH patients (Indian trial registry, CTRI/2015/10/006236). Novel selective agonists of PPAR α (pemafibrate, K-877), PPAR γ (INT-131), PPAR δ (HPP-593), PPAR α/γ (DSP-8658) and a pan-PPAR agonist (IVA337) are under evaluation.

3.2.2.3. Liver X receptors (LXRs): LXRs are lipid-activated nuclear receptors involved in cholesterol transport, lipogenesis, and anti-inflammatory signaling. LXR activation suppresses HSC activation in mice [221]. Leptin-induced activation of p38 MAPK leads to inhibition of LXR activity and downregulation of SREBP-1c expression, and increases collagen type I expression in HSCs [222]. The therapeutic benefit of LXR agonism may be limited due to induction of de novo lipogenesis by this mechanism [5].

3.2.2.4. Vitamin D receptor (VDR): In HSCs, TGF β 1 causes a redistribution of genome-wide VDR-binding sites (VDR cistrome) at SMAD3 profibrotic target genes via chromatin remodeling, and VDR ligands reduce TGF β /SMAD3 target gene expression and inhibit fibrosis [223]. p62/SQSTM1 suppresses fibrogenic and carcinogenic HSC activation by promoting VDR signaling, which is abrogated by VDR knockout in mice [224]. In human primary HSCs, vitamin D treatment similarly suppressed TGF β -induced fibrogenic transcription, but A1012G single nucleotide polymorphism (G allele) within the VDR gene reduces VDR expression and abolishes the vitamin D effect [225].

3.2.2.5. Retinoid receptors: Retinoic acid, an active metabolite of vitamin A, regulates various physiological processes such as energy metabolism in metabolically active cells through retinoic acid receptors (RARs) and retinoid X receptors (RXRs) that form homo/heterodimers [226]. Retinoid receptor signaling in hepatocytes is implicated in liver carcinogenesis, and a synthetic acyclic retinoid, peretinoin, has been assessed as a potential cancer chemoprevention measure [227, 228]. In HSCs, all-trans retinoic acid (ATRA) (an RAR ligand) moderately inhibits expression of procollagen I, III, IV, fibronectin, laminin, α SMA, TGF β , and IL-6, but with no effect on cell proliferation, whereas 9-cis (RXR ligand) moderately inhibits proliferation without affecting the fibrogenic gene expression in rodents [229, 230]. These receptors, also together with PPAR γ signaling, have additive inhibitory effects on HSC activation [231, 232]. RXR can form heterodimer with VDR to transmit signal from p62 in HSCs to promote fibrogenesis and inflammation, and support carcinogenesis [224].

3.2.2.6. Rev-Erb receptors: Rev-Erbs, consisting of Rev-Erb α and Rev-Erb β , are heme-sensing receptors involved in regulation of metabolism, development, immunity, and circadian rhythm, and are potentially druggable [233]. Rev-Erb α is up-regulated in both nucleus and cytoplasm in activated HSCs in association with increased contractility and fibrogenic gene expression, which can be antagonized by a synthetic ligand, SR6452 [234]. Another synthetic Rev-Erb α ligand, SR9009, similarly reduces fibrogenic gene expression and proliferation in HSCs, accompanied by decreased autophagosome formation [235].

3.2.3. Transcription factors

3.2.3.1. Sex-determining region Y-box 9 (SOX9): SOX9 has been implicated in control of liver progenitor cells and organ development [236]. A recent study reported that leptin stimulates SOX9 expression, and SOX9 suppresses PPAR γ expression by binding to its promoter region to stimulate HSC activation [237]. SOX9 induces osteopontin, and contributes to liver fibrosis in human [238].

3.2.3.2. GATA binding protein 4 (GATA4): Conditional knockout or haploinsufficiency of a zinc finger transcription factor, GATA4, in hepatic mesenchymal cells leads to fibrogenic HSC activation by directly regulating an antifibrogenic transcription factor, *Lhx2*, and accelerates fibrosis in mice, and GATA4 expression is reduced in human cirrhotic livers [239, 240].

3.2.3.3. Myocardin-related transcription factor A (MRTF-A): MRTF-A induces histone H3K4 di/tri-methylation, recruits histone methyltransferase complex (COMPASS), and recruits and interacts with regulatory epigenetic factors, including ASH2, WDR5, and SET1, and activates expression of fibrogenic genes, including TGF β target genes, which can be suppressed by estradiol [241, 242].

3.2.3.4. Nuclear receptor subfamily 4 group A member 1/2 (NR4A1/2): NR4A1 and NR4A2 are orphan nuclear receptor family members that act as transcription factors, regulating cell differentiation, proliferation and apoptosis. NR4A1 suppresses TGF β target genes, and its small molecule agonist, Csn-B, reduces fibrosis in mice [243]. NR4A2 expression is reduced in fibrotic liver, and its genetic knockout in HSCs promoted ECM production and cell proliferation via MAPK pathway [217]. NR4A2 over-expression suppresses ECM production and invasive ability, and induces cell cycle arrest and apoptosis in vitro. Moreover delivery by adenoviral infection in dimethylnitrosamine (DMN)-treated fibrotic rats significantly reduces liver fibrosis [244].

3.2.3.5. Kruppel-like factors (KLFs): KLF6, a zinc finger transcription factor and tumor suppressor, directly represses collagen I and PDGFR β expression and increases apoptosis of HSCs, and KLF6 haploinsufficiency increases fibrosis in rats [245]. Statins up-regulates KLF2 expression in sinusoidal endothelial cells, and induces vasoprotective gene expression, and quiescence of HSCs through a KLF2-nitric oxide-guanylate cyclase-mediated paracrine mechanism [246].

3.2.3.6. Aryl hydrocarbon receptor (AhR): AhR is a ligand-activated transcription factor involved in the regulation of xenobiotic-metabolizing enzymes such as cytochrome P450, and AhR-deficient mice develop liver fibrosis [247]. HSC activation, i.e., TGF β activation, PPAR γ reduction, and increased collagen secretion, induced by AhR deletion requires vitamin A in mice [248]. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) inhibits lipid droplet storage, and enhances cell proliferation and α SMA expression, but not collagen I expression, in the LX-2 human HSC line [249].

3.2.3.7. Ga-interacting vesicle-associated protein (GIV): GIV/Girdin serves as a cellular hub that transmits signals from multiple receptors such as PDGF and TGF β , promotes pro-fibrogenic signaling and inhibit anti-fibrogenic pathways [250].

3.2.3.8. Yes associated protein (YAP): YAP is a transcription co-activator, which is downstream and suppressed by Hippo pathway. YAP also transmits signal from integrin β 1 in myofibroblasts, and promotes fibrosis [141]. YAP knockdown inhibits HSC activation, and pharmacological inhibition of YAP suppresses fibrogenesis in mice [251]. YAP triggers accumulation of reactive-appearing ductular cells, increases myofibroblasts, and induces fibrosis in murine NASH model [252].

3.2.3.9. Embryonic stem cell-expressed RAS (ERAS): ERAS is a RAS family GTPase, which plays an important role in maintaining quiescent HSCs with correlated activation of AKT, STAT3, mTORC2, and Hippo signaling pathways and inactivation of FOXO1 and YAP. [253].

3.2.4. Epigenetic transcriptional dysregulation—Global epigenomic and local epigenetic transcriptional dysregulation in HSC can occur as response or adaptation to environmental and/or etiological stressors, and has been implicated in its fibrogenic activation [254].

3.2.4.1. Genomic DNA methylation, histone modification, and chromatin remodeling: Genome-wide characterization of DNA methylation (5-mC) and hydroxymethylation (5-hmC) of cytosine-phosphoguanine dinucleotides (CpGs) during HSC activation in rat and human has revealed modulation of regulatory enzymes, e.g., induction of DNA methyltransferase (DNMT) 3a/b and suppression of ten-eleven translocation methylcytosine dioxygenase (TET) 2 and 3, accompanied by decreased levels of 5-hmC [255]. HSC activation can be suppressed by inhibitors of DNMT1 (e.g., 5-Aza-2'-deoxycytidine) and enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2), a polycomb group-protein complex catalyzing trimethylation of histone 3 lysine 27 (H3K27me3) (e.g., 3-deazaneplanocin), supporting their regulatory role in the activation [256–258]. DNMT1-mediated DNA hypermethylation of phosphatase and tension homolog (PTEN) leads to HSC activation [259]. Methyl-CpG binding protein 2 (MeCP2), released from translational repression by miR-132, induces HSC activation and liver fibrosis by binding promoter of PPAR γ gene and suppresses its expression [256]. In parallel, MeCP2 stimulates EZH2 expression and H3K27 methylation to form a repressive chromatin structure in the 3' exons of PPAR γ , which can be pharmacologically inhibited. ASH1, a transcriptional repressor induced by MeCP2, binds regulatory regions of α SMA, collagen I,

TIMP1, and TGF β 1 in activated HSCs, and depletion of ASH1 suppresses fibrogenic gene/protein expression [260]. Histone H3K9 demethylase JMJD1A suppresses HSC activation and liver fibrosis via the H3K9me2 mark in the PPAR γ gene promoter [213]. Low-molecular-weight fibroblast growth factor 2 (FGF2(lmw)) attenuates hepatic fibrosis by epigenetic downregulation of Delta-like 1 [261]. The bromodomain-containing protein 4 (BRD4), a member of bromodomain and extraterminal (BET) proteins highly enriched at enhancers of pro-fibrogenic genes, can be pharmacologically inhibited by its inhibitor, JQ1 to suppress HSC activation [262]. The PPAR γ promoter methylation detected in plasma cell-free circulating DNA stratifies mild and severe fibrosis in patients with NAFLD and alcoholic liver disease, although it was more correlated with methylation in hepatocytes than in myofibroblasts [263].

3.2.4.2. Dietary factors: Ethanol induces an H3K4 methyltransferase, lysine methyltransferase 2A (KMT2A), and transcriptionally activates promoter of multiple ECM genes, including elastin [264]. Ethanol can also induce H3K9 acetylation in HSCs in time- and dose-dependent manner [265]

3.2.4.3. Inheritable epigenetic adaptation: Ancestral history of liver damage is associated with epigenetic suppressive adaptation that leads to higher PPAR γ expression, fewer myofibroblasts, and lower expression of profibrogenic factors such as TGF β in offspring rats [266].

3.2.4.4. Non-coding RNAs: Recent studies have elucidated dysregulation of non-coding RNAs, especially microRNAs (miRs), involved in HSC activation and inactivation by post-transcriptional regulation of mRNA degradation and translation. High-throughput miRNA profiling and other technologies have identified pro-fibrogenic miRNAs over-expressed in activated HSCs, including miR-9a-5p, miR-17-5p, miR-21, miR-27, miR-31, miR-33a, miR-34a/c, miR-125, miR-126, miR-130a/b, miR-181b, miR-214-5p, miR-195, miR-199a/b, miR-221, and miR-222, each of which induces proliferation, collagen secretion, and/or migration via a variety of pathways, and anti-fibrotic miRNAs, including miR-16, miR-19b, miR-29, miR-30, miR-101, miR-122, miR-133a, miR-144, miR-146a, miR-150, miR-155, miR-192, miR-195, miR-335, miR-454, and miR-483, although direction of change could depend on biological and/or experimental contexts [267–269]. miR-378 was recently shown to limit HSC activation by suppressing Gli3 [270]. miR-145 inhibits HSC activation and proliferation by targeting zinc finger E-box-binding homeobox 2 (ZEB2), a key mediator of epithelial-to-mesenchymal transition, via Wnt/ β -catenin pathway [271]. Interferon-induced miR-195 down-regulates cyclin E1 and suppresses HSC proliferation [272]. miR-200a over-expression inhibits α SMA expression and suppresses TGF β -induced proliferation through β -catenin, SIRT1/Notch1, and NRF2/KEAP1 pathways [273–275]. miR-214, delivered to HSCs by exosome, binds 3'-UTR of CTGF gene, and suppresses its expression [130]. The role of long non-coding RNAs (lncRNAs) is less studied compared to miRNAs. lncRNA, H19, which can be silenced by MeCP2, represses expression of insulin-like growth factor 1 receptor (IGF1R) and suppresses HSC proliferation [276]. Circulating cell-free miRNAs, e.g., miR-122, miR-138, miR-143, and miR-185, have been assessed as potential non-

invasive biomarkers of HSC activation, liver fibrosis, and/or prognosis in patients mostly affected with HBV or HCV [277–279].

3.2.5. Dysregulation of cellular homeostasis and stress

3.2.5.1. Autophagy, endoplasmic reticulum (ER) stress: Intracellular energy homeostasis is dysregulated during the process of HSC activation to meet the energy demand. Autophagy is a highly regulated stress response pathway induced by chronic liver injury that fuels HSC activation by providing critical ATP substrates [280, 281]. Inhibition of autophagy suppresses HSC activation and proliferation [282]. Autophagy in HSCs can be induced by oxidative stress and ER stress in response to chronic liver injury [283]. The unfolded protein response (UPR) is a highly conserved ER stress effector pathway transmitting signal through either of protein kinase R-like ER kinase (PERK) and inositol-requiring enzyme 1 (IRE1)/X-box transcriptional factor (XBP1) pathways that link ER stress, UPR, and fibrogenic HSC activation in distinct ways: the TGF β pathway is stimulated in the PERK-mediated, but not IRE1/XBP1-mediated, UPR during HSC activation [284–286]. Advanced glycation end product (AGE)-induced HSC activation requires autophagy in HCV-related fibrosis [287]. Transient receptor potential vanilloid 4 inhibits apoptosis in rat HSC line, HSC-T6, through induction of autophagy [288]. Rev-Erb-activating synthetic ligand, SR9009, and TGF β similarly affect autophagy, but differentially regulate HSCs [235]. Rev-Erba and LXR, along with PPAR γ , are critical to preserve adipogenic phenotype of HSCs [221, 234]. Deletion of a collagen-specific molecular chaperone, heat shock protein 47 (Hsp47), causes ER stress-mediated apoptosis of HSCs [289]. Vitamin A-coupled liposome nanoparticles loaded with Hsp47-targeting siRNA, ND-L02-s0201, resolves fibrosis in rodent models, and is now under clinical evaluation in a phase 1b/2 trial (NCT02227459) [290]. Reticulon 4B (RTN4B, Nogo-B), associated with ER, is over-expressed in activated HSCs [291, 292]. Hypoxia-inducible factor-1 α (HIF1 α) regulates autophagy to activate HSCs [293]. Dimethyl α -ketoglutarate reduces CCl $_4$ -induced liver fibrosis through inhibition of autophagy in HSCs [294]. Suppression of autophagy in hepatocytes by antagonism of IL-17A inhibits liver fibrosis by restoring the IL-10/STAT3 pathway in BDL and TAA-treated mice [295]. Potentiation of HSC activation by extracellular ATP is dependent on P2X7R-mediated NLRP3 inflammasome activation [296].

3.2.5.2. Retinoids/vitamin A: The loss of cytoplasmic retinoids in activated HSCs is linked to retinyl ester hydrolysis that generates fatty acids as part of the autophagic response, although a causal relationship between retinoid metabolism and HSC activation is not established [282]. Retinoids exacerbate rat liver fibrosis by activating latent TGF β in HSCs [297]. Conversion of retinol to retinoic acid leads to TGF β and collagen expression via alcohol dehydrogenase 3, and retinol released from HSCs suppresses NK cells and contributes to HSC survival [298]. Vitamin A is required in AhR deficiency-mediated murine HSC activation [248]. Murine HSCs, lacking retinoid droplets due to genetic knockout of LRAT, can be activated, suggesting that retinoid droplets are not indispensable for activation [299]. Vitamin A and insulin are required for the maintenance of HSC quiescence [300]. A retinoic acid receptor β 2 agonist reduces HSC activation in NAFLD [301]. Perilipin 2, adipose TG lipase (ATGL), and comparative gene identification-58 (CGI-58) are lipid droplet-associated proteins in rat HSC line, HSC-T6 [302]. LXRs balance

lipid stores in HSCs through Rab18, a retinoid responsive lipid droplet protein [303]. Autophagy regulates turnover of lipid droplets via ROS-dependent Rab25 activation in HSC [304]. Alcohol dehydrogenases (ADHs) oxidize retinol to retinaldehyde, which is metabolized to retinoic acid. Loss of ADH3 enhances NK cell activation and IFN γ production, increases HSC apoptosis, and attenuates liver fibrosis in CCl₄ and BDL mice [298]. Patatin like phospholipase domain containing 3 (PNPLA3) is highly expressed in HSCs and hepatocytes, and hydrolyzes retinol palmitate to retinol and palmitic acid with influence from retinol availability, and its over-expression is associated with reduced cytoplasmic lipid droplets. A single nucleotide polymorphism (SNP) in the PNPLA3 gene, I148M, is associated with its reduced expression, more advanced NAFLD fibrosis, and HCC, and may have functional implication in HSC activation [305].

3.2.5.3. Protein ubiquitination: Ubiquitination is a post-translational modification, ligating ubiquitin to a substrate protein to regulate its degradation, subcellular localization, and interaction with other proteins. Ubiquitin C-terminal hydrolase 1 (UCHL1), a deubiquitinase, is up-regulated following HSC activation, and promote their proliferation in CCl₄ and BDL mice [306]. Small molecule UCHL1 inhibitor, LDN-57444, attenuates PDGF-induced HSC proliferation by modulating phosphorylation of a cell cycle regulator, retinoblastoma protein (Rb).

3.2.5.4. Bone morphogenetic protein 6 (BMP6): BMP6 is an iron homeostasis regulator. BMP6 deficiency promotes hepatic inflammation and fibrosis in MCD plus high-fat diet-fed mice, but not in other non-NASH models, and recombinant BMP6 protein inhibits HSC activation [307].

4. Deactivation and elimination of fibrogenic HSCs

Termination of chronic liver injury results in regression of liver fibrosis accompanied with resolution of cytokine-rich inflammatory tissue microenvironment as well as reduced or loss of activated HSCs [5]. These observations suggest that deactivating and/or reducing fibrogenic HSCs could be an antifibrotic strategy irrespective of the cause of liver injury.

4.1. Induction of non-fibrogenic state

4.1.1. Reversion, transdifferentiation—Genetic cell lineage tracing studies in mice have showed that activated HSCs can be reverted to a quiescent-like state upon withdrawal of causative agents, although the reverted cells are sensitized to reactivation by exposure to fibrogenic stimuli [308, 309]. More recent study demonstrated that in vivo transcriptional reprogramming by ectopic expression of *FOXA3*, *GATA4*, *HNF1A*, and *HNF4A* transdifferentiates activated HSCs into hepatocyte-like non-fibrogenic cells, namely induced hepatocytes (iHeps), and reduces liver fibrosis [310].

4.1.2. Senescence—Cellular senescence is a genetically controlled physiological mechanism that restricts cell division within a finite proliferative capacity to prevent accumulation of genetically damaged cells. Senescence mediated by the p53/p21 pathway limits proliferation of activated HSCs, and reduces liver fibrosis in mice [311, 312]. Thus, induction of HSC senescence might serve as an antifibrotic strategy. A CCN family

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matricellular protein, cysteine-rich protein 61 (CCN1/CYR61), attenuates TGF β signaling and induces senescence and apoptosis of activated HSCs [142, 313, 314]. Proteomic analysis has revealed that CYR61 and Wnt5a are among several matrix proteins secreted from a human HSC line, LX-2 [315]. A celecoxib derivative, OSU-03012, inhibits the proliferation and activation of HSCs by inducing senescence [316]. Soluble egg antigens (SEA) of *Schistosoma japonicum* induce senescence of activated HSCs by activating FoxO3a/SKP2/p27 pathway [317]. HSC senescence can also be induced by phytochemicals such as curcumin by activating PPAR γ /p53 signaling [218]. RAR/RXR and PPAR γ may play a role in inducing HSC senescence as antifibrotic therapy [318].

4.2. Induction of HSC death or killing

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Activated HSCs are resistant to cell death stimuli by activating survival signals via the NF- κ B pathway and inducing anti-apoptotic proteins such as Bcl-2 [5]. Therapeutic induction of susceptibility to cell death signals may reduce the number of ECM-producing transdifferentiated HSCs and serve as an option of antifibrotic therapy. Indeed, liver fibrosis can be reduced in vivo by the NF- κ B inhibitor, BAY 11-7082, as well as by proteasome inhibitors, bortezomib and MG132, via NF- κ B target gene *A1* [319]. Inhibition of neurochemical receptors such as CB1 and 5HT can also induce HSC apoptosis. IFN γ is a hallmark of activated NK and NKT cells that kill senescent HSCs and limits liver inflammation and fibrosis [8, 320]. IFN γ delivery specifically targeting activated HSCs by PDGFR β -mediated uptake suppresses HSCs and reduces hepatic fibrosis [321]. Nilotinib induces apoptosis and autophagic cell death of activated HSCs via inhibition of histone deacetylases [322]. A multi-kinase inhibitor, sorafenib, similarly induces autophagic cell death and apoptosis in HSCs through the JNK and Akt signaling pathways [323].

5. Summary and future direction

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As summarized in this review, comprehensive characterization of molecular dysregulation of HSCs and their functional assessment in engineered animal models have elucidated multiple candidate therapeutic targets and strategies, many of which are currently under evaluation in patients with chronic liver diseases [324]. However, both fibrogenesis and fibrosis regression involve highly heterogeneous and evolving cell populations whose interactions have not yet fully characterized. Single cell/molecule-resolution genome-wide molecular characterization, combined with experimental perturbations and special reconstruction may overcome the limitations of the current strategies that rely upon single gene/protein marker-based cell type determination [325–327]. Newly emerging experimental systems such as multi-cell type and/or 3D culture will enable more physiological modeling of fibrogenic molecular dysregulation [328]. Animal models based on chemical, surgical, and/or genetic interventions have been criticized for their physiological and/or clinical irrelevance [329]. However, unbiased omics-based characterization could enable identification of models that recapitulate specific molecular dysregulation of interest [186]. In addition to these advancements, systematic strategies to translate the experimental model-based findings to clinically meaningful long-term consequences in human fibrotic liver diseases will substantially increase the chance of success in the clinical evaluation of candidate antifibrotic therapies [186].

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Abbreviations

| | |
|-----------------|---|
| 5-HT(2B) | 5-hydroxytryptamine 2B receptor |
| ACE | angiotensin-converting enzyme |
| AMPK | AMP-activated protein kinase |
| ARB | AT1R blocker |
| ASK1 | apoptosis signal-regulating kinase 1 |
| AT1R | angiotensin II type 1 receptor |
| ATX | autotaxin |
| BDL | bile duct ligation |
| CB | cannabinoid |
| CCL | C-C motif chemokine ligand |
| CCl4 | carbon tetrachloride |
| CCR | C-C motif chemokine receptor |
| CpG | cytosine-phosphoguanine dinucleotide |
| CVCR | C-X-C motif chemokine receptor |
| CXCL | C-X-C motif chemokine ligand |
| DAMP | damage-associated molecular pattern |
| DKK1 | Dickkopf-1 |
| DNMT | DNA methyltransferase |
| ECM | extracellular matrix |
| EGF | epidermal growth factor |
| ER | endoplasmic reticulum |
| ERK | extracellular signal-regulated kinase |
| EZH2 | enhancer of zeste 2 polycomb repressive complex 2 subunit |
| FGF | fibroblast growth factor |

| | |
|--------------------------------|--|
| FXR | farnesoid-X-receptor |
| HBV | hepatitis B virus |
| HCV | hepatitis C virus |
| HGF | hepatocyte growth factor |
| Hh | hedgehog |
| HMGB1 | high mobility group protein B 1 |
| HSC | hepatic stellate cell |
| IL | interleukin |
| iNOS | inducible nitric oxide synthase |
| JNK | c-Jun N-terminal kinase |
| LPA | lysophosphatidic acid |
| LRAT | lecithin-retinol acyltransferase |
| LSEC | Liver sinusoidal endothelial cell |
| LY6C | lymphocyte antigen 6 complex |
| MeCP2 | methyl-CpG binding protein 2 |
| MMP | matrix metalloproteinase |
| NAFLD | non-alcoholic fatty liver disease |
| NASH | non-alcoholic steatohepatitis |
| NF-κB | nuclear factor κ B, natural killer |
| NO | nitric oxide |
| OCA | obeticholic acid |
| PAMP | pathogen-associated molecular pattern |
| PDGF | platelet-derived growth factor |
| PPAR | peroxisome proliferator-activated receptor |
| PRR | pattern recognition receptor |
| RAR | retinoic acid receptor |
| ROS | reactive oxygen species |
| RXR | retinoid X receptor |
| SREBP | sterol regulatory element-binding protein |

| | |
|--------------|--|
| STAT | signal transducer and activator of transcription |
| TGF | transforming growth factor |
| Th | helper T |
| TLR | toll-like receptor |
| TNF | tumor necrosis factor |
| TWEAK | tumor necrosis factor-like weak inducer of apoptosis |
| TZD | thiazolidinedione |
| UPR | unfolded protein response |
| UTR | untranslated region |
| VAP-1 | vascular adhesion protein 1 |
| VDR | vitamin D receptor |
| VEGF | vascular endothelial growth factor |

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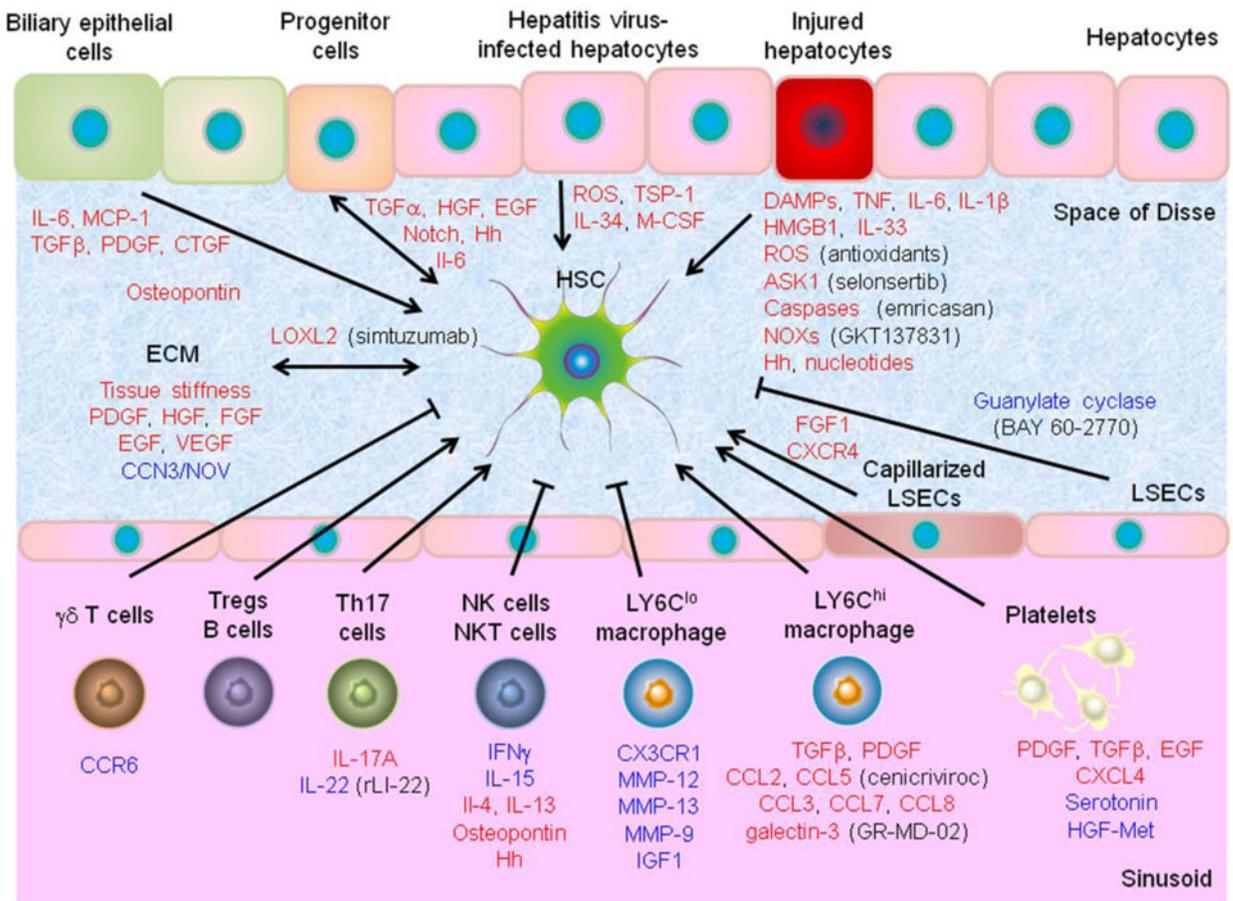


Figure 1. Extracellular regulation of HSC activation

HSC activation is promoted (sharp arrow) or inhibited (blocked arrow) by liver resident cells, ECM, and circulating cells via paracrine factors. Red and blue font colors indicate positive and negative regulators of HSC activation, respectively. Pharmacological intervention to each candidate target is shown in parenthesis.

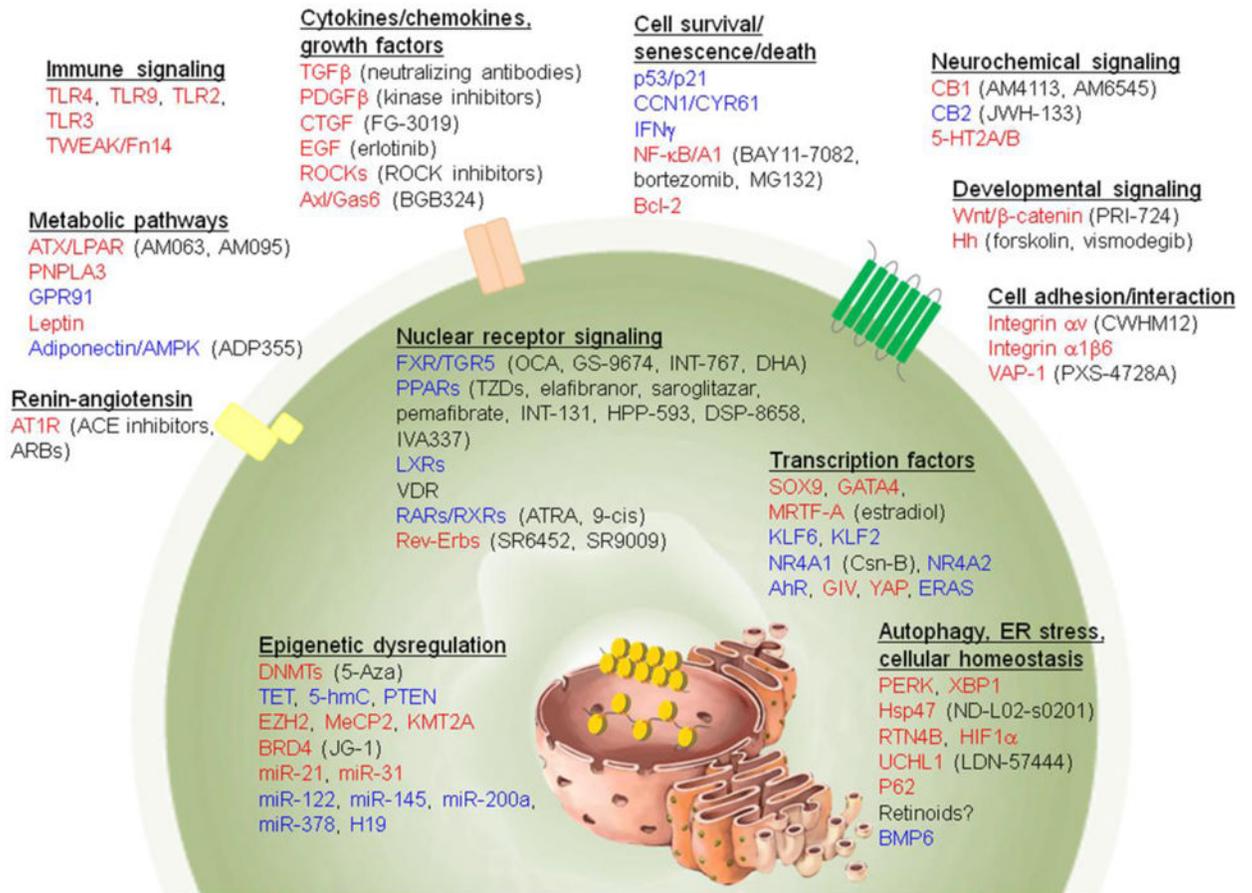


Figure 2. Cellular signaling pathways involved in HSC activation and deactivation

A variety of cell surface receptor-, nuclear receptor-, and intracellular signal transmitter-mediated mechanisms of HSC activation has been identified. Dysregulation of cellular homeostasis and epigenetic modifications also regulate HSC activation. HSC deactivation can be induced via specific targets. Red and blue font colors indicate positive and negative regulators of HSC activation, respectively. Black font color indicates involvement in both pro- and anti-fibrogenic functions depending on biological context or undetermined role in HSC activation. Pharmacological intervention to each candidate target is shown in parenthesis.