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Minireview

Mechanisms of liver fibrosis and its role in liver cancer

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Impact statement

Advanced liver fibrosis results in cirrhosis, portal hypertension, and liver failure and often requires liver transplantation. Advanced liver fibrosis and cirrhosis are also major risk factors for hepatocellular carcinoma (HCC). Hepatic stellate cells (HSCs) play a pivotal role in the pathogenesis of liver fibrosis. In this review, we summarize the basic mechanisms that influence liver fibrosis development and how oxidative stress, mitochondrial dysfunction, and metabolic remodeling modulate HSC activation and indicate areas of potential therapeutic intervention.

Abstract

Hepatic fibrogenesis is a pathophysiological outcome of chronic liver injury hallmarked by excessive accumulation of extracellular matrix proteins. Fibrosis is a dynamic process that involves cross-talk between parenchymal cells (hepatocytes), hepatic stellate cells, sinusoidal endothelial cells and both resident and infiltrating immune cells. In this review, we focus on key cell-types that contribute to liver fibrosis, cytokines, and chemokines influencing this process and what it takes for fibrosis to regress. We discuss how mitochondria and metabolic changes in hepatic stellate cells modulate the fibrogenic process. We also briefly review how the presence of fibrosis affects development of hepatocellular carcinoma.

Keywords: Liver fibrosis, fibrosis regression, liver cancer, hepatic stellate cells, mitochondria, metabolic pathways, PNPLA3

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Introduction

Liver fibrosis is a consequence of sustained wound-healing response to chronic liver injury.[1] The main causes include chronic HCV and HBV infection, exposure to toxins (e.g. alcohol liver disease), non-alcoholic steatohepatitis (NASH), and autoimmune diseases such as primary biliary cirrhosis, primary sclerosing cholangitis, and autoimmune hepatitis. Morphologically, liver fibrosis is characterized by accumulation of extracellular matrix (ECM), followed by formation of fibrous scar and subsequent cirrhosis which is defined by the presence of nodules of regenerating hepatocytes with decreased blood supply to the liver.[2,3] Hepatic stellate cells (HSCs) are the main ECM-producing cells in the injured liver.[4] In healthy livers, HSCs localize in the space of Disse, where they are in a quiescent state and store vitamin A (Figures 1 and 2). However, following continuous liver injury, HSCs activate into myofibroblasts, start expressing alpha-smooth muscle actin (α-SMA), migrate at the site of tissue repair, and secrete large amount of ECM (Figures 1 and 2). Interestingly, when the liver injury is removed, myofibroblasts may undergo apoptosis and inactivation,[5] and therefore clearance of causative etiologies underlying liver fibrosis can slow down the process and lead to fibrosis regression. Despite extensive knowledge on liver fibrosis mechanisms, antifibrotic therapies effective in human have not been developed so far.[6] In this review, we highlight recent data of molecular mechanisms of liver fibrosis and summarize the current knowledge of targeting each pathway of the pathogenesis process to relieve liver fibrosis.

Origin of fibrogenic myofibroblasts

The majority of the studies aiming at identifying the origin of myofibroblasts have suggested that most likely HSCs are the main source of myofibroblasts in the injured liver.[7–9] However, this topic is still controversial and other than HSCs several other cell types have been proposed to give rise to myofibroblasts. For example, epithelial cells which in physiological conditions are located on the surface of blood vessels and organs could potentially lose their polarity, migrate and originate myofibroblasts through a process called epithelial–mesenchymal transition (EMT).

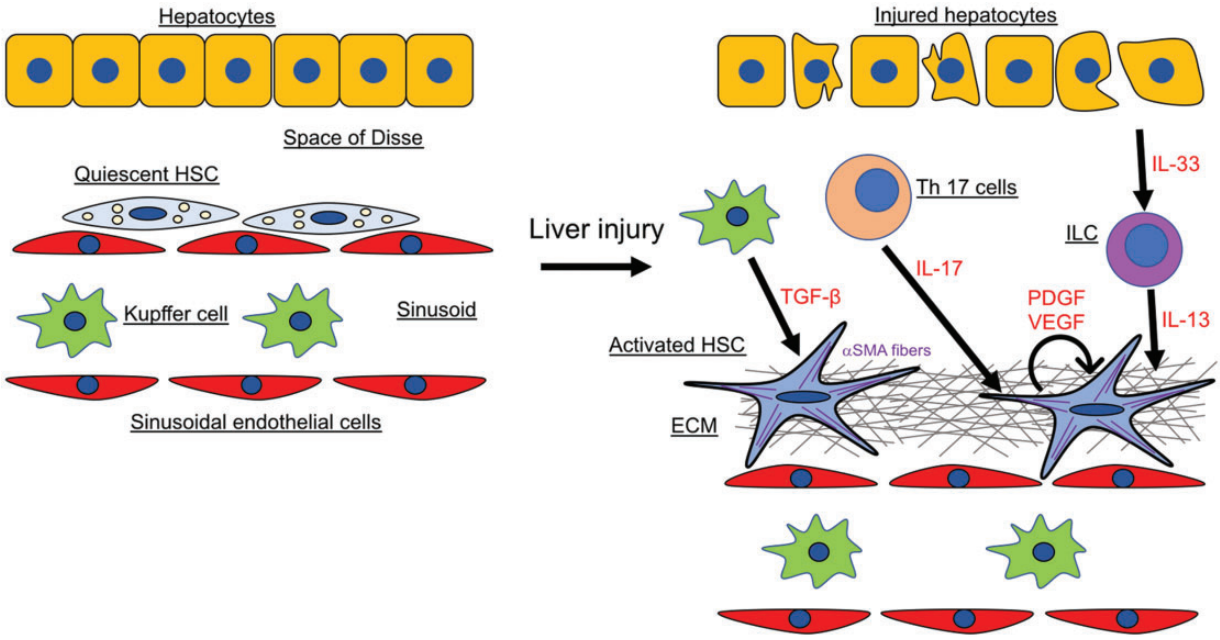

Figure 1. Cell types in liver fibrosis. Quiescent HSCs in healthy livers are localized in the space of Disse. Following chronic liver damage, injured hepatocytes, immune cells and activated Kupffer cells release pro-fibrogenic molecules which activate HSCs. Activated HSCs upregulate α-SMA expression, secrete growth factors, and produce large amounts of ECM. The figure depicts the different cell types and fibrogenic mediators involved in the fibrotic process and the crosstalk between them (see text for details).

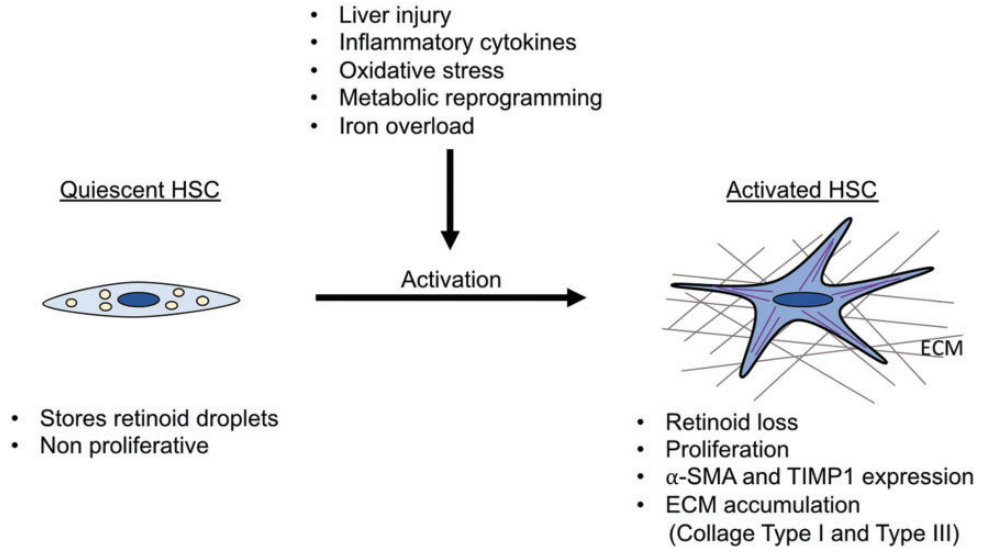

Figure 2. Features of quiescent and activated HSCs. Quiescent HSCs store retinoid droplets, proliferate slowly, and express high levels of GFAP and LRAT. Upon liver injury, increase of inflammatory cytokines, ROS production, metabolic reprogramming or iron overload, HSCs become activated. Activated HSCs are characterized by loss of retinoid droplets, increased proliferation, and expression of fibrogenic markers (α-SMA, TIMP1, Lox and LoxL2). Moreover, activated HSCs produce ECM proteins such as collagen type I and collagen type III which are hallmarks of liver fibrosis.

Interestingly, some studies have shown that cholangiocytes and hepatocytes under prolonged culturing conditions increase the expression of α-SMA and downregulate epithelial markers.[10,11] Nonetheless, lineage-tracing experiments that permanently label cholangiocytes, hepatocytes, and epithelial precursor cells have shown that epithelial cells do not give rise to hepatic myofibroblasts.[12–14] These observations therefore suggest that EMT is not crucial for liver fibrogenesis *in vivo*.[15]

Bone marrow (BM)-derived cells like mesenchymal stem cells (MSCs) and fibrocytes have also been proposed to originate myofibroblasts. MSCs are multipotent cells that differentiate into osteoblasts, adipocytes, myocytes, and chondrocytes. Surprisingly, recently it has been reported that MSCs may protect the tissue in which they are recruited from developing fibrosis.[16]

Fibrocytes are cells with a spindle-like shape which were first described in 1994.[17] They express fibroblast markers

such as fibronectin, vimentin, collagen type I as well as hematopoietic cell markers like CD45, MHCII, CD34, CD11b, Gr1, CD86, CCR2, Ly6c, CD54, CD80, CCR1, CCR7, and CCR5.[18,19] Studies have reported that both cholestatic and CCl_4-induced liver injury can trigger the recruitment of fibrocytes into the injured liver where they can start expressing α-SMA. However, their contribution to liver myofibroblasts range between 3% and 50%.[20–22]

Mesothelial cells are similar to epithelial cells, and they can be found in internal organs and serous cavities where they are organized into cell monolayers.[23] They originate from the embryonic mesoderm layer and some studies have suggested that both portal fibroblasts (PFs) and HSCs may derive from mesothelial cells.[24,25] Interestingly, it has been shown that after CCl_4-induced liver injury, both HSCs and myofibroblasts originate from mesothelial cells; however, in cholestatic liver injury, mesothelial cells give rise only to HSCs, but not myofibroblasts.[26,27] Therefore, some controversies remain whether mesothelial cells can differentiate into myofibroblasts in fibrotic livers. Nonetheless, a study has suggested that mesothelial cells may be involved in fibrosis of the liver capsule.[22]

PFs consist of heterogeneous cell populations which are localized underneath the bile duct epithelium. PFs were first described in cholestatic liver disease by immunohistochemistry, histology, and electron miscroscopy.[28–30] During biliary fibrosis, PFs start expressing α-SMA and produce ECM.[31–33] However, identification or purification of quiescent PFs is challenging due to the lack of markers which can efficiently discriminate fibroblasts from other mesenchymal cells. Nevertheless, markers such as Ntpdas2, elastin, and Thy1 were recently reported to be expressed specifically by PFs, but not by HSCs.[34] Intriguingly, by using transgenic mice, two collagen-producing cell populations were identified in the injured liver: Vitamin A-positive HSCs and Vitamin A-negative PFs.[35] In CCl_4-induced liver fibrosis, myofibroblasts originate mainly from HSCs, whereas PFs are the major source of myofibroblasts in early biliary fibrosis. However, as cholestatic disease progresses, HSCs contribute to the majority of myofibroblasts. More recently, it was reported that activation of PFs during cholestatic liver fibrosis is mediated by TGF-β1 and involves the interaction of mesothelin with a MUC16-Thy1-TGFβRI complex.[36]

In summary, current studies regarding the origin of hepatic myofibroblasts indicate that, depending on the type of liver injury, they arise mostly from liver-resident HSCs, mesothelial cells, and activated PFs. The contribution of BM-derived cells to hepatic fibrosis is quantitatively small, suggesting that these cells may be important in the modulation of other myofibroblast populations.

Key cytokines and chemokines involved in liver fibrosis

In liver fibrosis, death of hepatocytes and cholangiocytes causes activation of HSCs directly or through several cytokines which are released by immune cells including innate lymphoid cells (ILC), Kupffer cells, Th17 cells, and bone marrow-derived monocytes (Figure 1). Those inflammatory cytokines have been reported to affect liver fibrosis *in vivo* and *in vitro*.[37] Interestingly, while it has been shown that monocyte chemotactic protein type I (MCP-1) and CCL5 promote fibrogenesis, IL-10 and IFN-γ have the opposite effect.[38,39] IL-17 is secreted by Th17 cells and its levels are elevated in hepatitis B and C, alcoholic liver disease (ALD), and autoimmune hepatitis.[40] Neutrophils and mast cells can also be a major producer of IL-17 in fibrotic liver.[41] IL-17 is a profibrogenic cytokine which stimulates HSCs to increase levels of collagen type I, α-SMA, and TGF-β by activating NF-κB and STAT3.[42] Deletion of IL-17 or IL-17RA protects mice from cholestatic and toxin-induced liver fibrosis.[42] High blood levels of IL-22, another cytokine produced by Th17 cells, are observed in cirrhotic patients.[43,44] Although IL-22 was shown to promote human hepatocellular carcinoma,[45] other studies demonstrated the protective effects of IL-22 in murine models of ALD, acetaminophen-induced liver injury, and T-cell mediated hepatitis.[46–48] Accordingly, it has been shown that IL-22 induces HSC senescence and inhibits liver fibrosis in mice.[49] However, in contrast to these findings, other studies have suggested that IL-22 is proinflammatory and profibrogenic in hepatitis B patients and hepatitis B mice model.[50–52] Additionally, increased IL-17 and IL-22 is a common signature for advanced liver fibrosis and pharmacological inhibition of IL-22 and IL-17 reduced hepatic fibrosis in murine models.[41]

IL-33 and its receptor ST2 are elevated in cirrhotic patients.[53,54] In experimental models of mouse liver fibrosis, IL-33 is released from injured hepatocytes that stimulate ILC to produce IL-13 which in turn stimulates HSCs via the IL-4Rα and STAT6 axis.[53] Liver sinusoidal endothelial cells and HSCs can also be a potential source of IL-33 in fibrotic liver.[55] Mice lacking IL-33 or ST2 are protected from liver fibrosis.[53,54]

Chemokines are small chemotactic cytokines that direct chemotaxis and recruitment of leukocytes into the injured liver.[56] Within the chemokine family, C-C motif chemokine (CCL2), also known as monocyte chemoattractant protein 1 (MCP-1), is released by activated macrophages, fibroblasts, and parenchymal cells during injury.[57] CCL2 stimulates the recruitment of monocyte-derived macrophages in the injured liver causing activation of myofibroblasts.[58] Therefore, the CCR2-CCL2 axis can potentially be an excellent target for therapeutic interventions.[59–61]

Among growth factors, transforming growth factor-β (TGF-β) plays a central role in liver fibrogenesis.[62] Liver macrophages including Kupffer cells are the main producer or TGF-β, but it can also be secreted by HSCs. TGF-β binds to the TGF-β receptor type II which phosphorylates TGF-β receptor type-I which follows the activation of both Smad-dependent and Smad-independent pathways.[62] In HSCs, TGF-β activates Smad2/3 stimulating the synthesis of ECM proteins such as type I and II collagen and inhibiting their degradation. Overall, TGF-β promotes activation of HSCs into myofibroblast and liver fibrosis through various mechanisms. For example, it has been shown that the Rho GTPase signaling mediates the TGF-β-induced HSCs migration.[63] MicroRNAs (miRNA) have also been suggested to be involved in the regulation of HSCs by TGF-β.

In particular, miR-29 is downregulated by TGF-β in HSCs with the consequent upregulation of ECM proteins.[64] In addition, TGF-β-dependent downregulation of miR-30c and miR-193 in HSCs has an effect on ECM remodeling.[65] Interestingly, it has been shown that the TGF-β pathway is inhibited by BAMBI and Smad7 which interact with and negatively regulate the TGF-β type I receptor.[66] Consequently, it was demonstrated that inhibiting TGF-β synthesis or overexpressing BMP-7, which is an opposing factor of TGF-β, resulted in reduction of liver fibrosis.[67,68]

Platelet-derived growth factor (PDGF) is produced by platelets, macrophages, myofibroblasts, and HSCs. PDGF is the most potent mitogen for HSCs and its levels are elevated in fibrotic livers where, synergistically with TGF-β, it acts as a fibrogenic factor.[69,70] PDGF is present in four isoforms (PDGF-A, B, C, and D) which interact with the PDGFR-α and -β, a receptor with intrinsic tyrosine kinase activity. In a rat model of biliary injury by BDL, the expression of PDGF-B, PDGF-D, and PDGFR-β was elevated compared to the other isotypes.[71] Another study showed that overexpression of PDGF-B in transgenic mice increased liver fibrosis and HSCs activation.[72] These results were confirmed by a study which reported that the PDGFR-β-mediated PDGF-B and PDGF-D signaling pathway are critical in the development of liver fibrosis.[73] Therefore, strategies aimed at inhibiting both TGF-β and PDGF signaling can potentially result in promising antifibrotic therapies.

Oxidative stress in liver fibrosis

In the injured liver, apoptotic or necrotic hepatocytes, activated Kupffer cells, activated HSCs, and neutrophils produce reactive oxygen species (ROS) which facilitate HSC activation and migration.[74] ROS are produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) by transferring electron from nicotinamide adenine dinucleotide phosphate to molecular oxygen.[75] Mammalian cells have seven NOX isoforms: NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1, and DUOX2. HSCs express NOX1, NOX2, and NOX4.[76,77] Mice lacking a regulatory component of NOX are not able to produce ROS in response to angiotensin II, PDGF, leptin, or apoptotic bodies. Consequently, those mice show less liver fibrosis after BDL or CCl_4 treatment.[78,79] In agreement with this result, deficiency of NOX1 and NOX4 protected mice from developing liver inflammation and fibrosis by inhibiting hepatic stellate cells activation.[80] Recently, it was also reported that NOX1 expression in macrophages promotes hepatic tumorigenesis by inducing the production of inflammatory cytokines.[81] Promising *in vivo* studies using the dual NOX1/NOX4 inhibitor GTK137831 have demonstrated that the compound can suppress ROS production, inhibit HSCs activation, and improve liver fibrosis in CCl_4, BDL, and fast-food diet mouse model of liver injury.[77,82–84] Interestingly, the cross talk between ROS and TGF-β is now very well recognized. On one hand, TGF-β inhibits the expression of several antioxidant enzymes, and on the other hand, it has been reported that ROS increases the expression of TGF-β in different organs.[85,86]

Role of mitochondria and metabolic reprogramming of HSC in fibrosis

Mitochondria play critical roles in energy and ROS production and homeostasis. About 90% of cellular ROS is produced in the mitochondria[87] and as mentioned above, has important roles in hepatic fibrogenesis. Although many studies have delineated the role of mitochondria in hepatocytes during disease progression,[88] very little is known about mitochondrial status in HSCs and how it plays a role during fibrogenesis. ECM production and secretion by activated HSCs demand abundant supply of intracellular ATP. Mild mitochondrial uncouplers that reduced ATP and ROS levels were able to prevent activation and proliferation of both mouse and human HSCs.[89]

Moreover, a recent study indicated an increase in mitochondrial respiration and membrane potential in fibrogenic HSCs.[90] The elevated mitochondrial membrane potential in activated HSCs (compared to quiescent HSCs) sensitized them to mitochondria delivery of doxorubicin (Dox) via triphenylphosphonium (TPP) conjugation (TPP-Dox). While activated HSCs were killed by TPP-Dox, non-fibrogenic HSCs were spared.[90]

The activation of HSCs is accompanied by metabolic reprogramming similar to the Warburg effect in tumor cells, in which the cells tend to favor energy production via glycolysis rather than the much more efficient oxidative phosphorylation pathway. The metabolic switch to glycolysis during HSCs activation happens rapidly within the first 48 h of activation. Inhibition of glycolysis by 2-Deoxy-D-glucose (2DG) converts the activated HSCs to a more quiescent state.[91] Pyruvate, the end product of glycolysis, is converted to lactate by the enzyme lactate dehydrogenase. Lactate accumulated in activated HSCs, and pharmacologic inhibition of lactate dehydrogenase suppressed HSCs activation.[91]

The amino acid glutamine is the most abundant circulating amino acid in blood and has important metabolic functions. Glutamine acts as a building block for several molecules such as for the synthesis of non-essential amino acids, synthesis of metabolites that are important in maintaining mitochondrial metabolism, and generation of antioxidants that counteract ROS.[92] Similar to the reliance of cancer cells on glutamine metabolism (glutaminolysis),[92] HSCs activation also depends on it.[93,94] Expression of genes that regulate glutaminolysis increased during activation of HSCs that result in increased glutamine consumption. Consequently, either depletion of glutamine or inhibiting enzymes involved in glutaminolysis disrupted transdifferentiation of HSCs.[93,94] Strategies that are targeted at lactate and glutamine metabolism may represent novel therapeutic approaches for the treatment of liver fibrosis. Hedgehog and YAP signaling were identified as important regulators of HSC metabolic switch during activation[91,93,94] and also provide potential therapeutic avenues to explore.

Adenosine 5′ monophosphate-activated protein kinase (AMPK) is a master regulator of metabolism that maintains cellular energy homeostasis.[95] Absence of adiponectin (an AMPK activator) in mice augmented CCl_4-induced

fibrosis.[96] Moreover, activation of AMPK in human HSCs by adiponectin or AICAR inhibited HSCs activation and migration in response to PDGF.[97] Similarly, AMPK silencing increased PDGF-induced HSCs proliferation, migration, and activation.[97] It was indicated that AMPK activation can inhibit HSCs activation by dampening the activation of either NFkB or mTOR signaling.[97]

Angiogenesis and liver fibrosis

During chronic liver injury, two distinct pathways are responsible for the functional changes in liver vascular architecture. First, inflammation and fibrosis cause hypoxia which promotes angiogenesis.[98,99] Second, the wound-healing process that is typical of chronic liver disease promotes the release of proangiogenic cytokines and growth factors.[98–101] HSCs function as the pericytes of the liver because of their anatomical location and their ability to regulate sinusoid contraction.[102] Once activated, HSCs promote angiogenesis by producing vascular endothelial growth factor (VEGF) and angiopoietin-1[103,104] and increasing the expression of their receptors VEGFR-2 and Tie-2. Interestingly, it has been shown that VEGF directly stimulates HSCs proliferation, migration, and chemotaxis.[105] In support to these findings, blocking VEGF or angiopoietin-1 reduces liver fibrosis.[104,106] In addition to VEGF, PDGF can also contribute to the angiogenic phenotype of HSCs.[107] Moreover, HSCs can secrete several CXC chemokines such as CXCL8, CXCL9, CXCL10, and CXCL12 which have been shown to stimulate angiogenesis during liver fibrosis.[108,109]

Role of iron overload in liver fibrosis

Iron is one of the most abundant element on Earth and is essential for all living organisms as it plays a critical role in various processes, such as oxygen transport, various enzymatic reactions including DNA synthesis and electron transport chain.[110] The human body lacks proper physiological mechanisms to excrete iron[110] and therefore needs to be closely regulated. On one hand where iron deficiency can cause anemia, excess iron can lead to severe oxidative stress and cellular damage by fueling the "Fenton reaction" that generates ROS and hydroxyl free radicals.[111] In the human body, iron is primarily found bound to hemoglobin or stored in the liver by hepatocytes or Kupffer cells, which are especially prone to the toxic effects of iron overload. The clearest example is the toxic effect of iron on hepatocytes in hereditary hemochromatosis, which eventually leads to cirrhosis and HCC.[111–113] Iron overload is a risk factor and might play a causal role in the development and progression of metabolic syndrome, type-2 diabetes, non-alcoholic fatty liver disease (NAFLD), ALD, NASH, and fibrosis.[111,114]

Hepcidin, a 25 amino acid polypeptide hormone produced by hepatocytes, is a central regulator of iron homeostasis.[115] Hepcidins also have anti-microbial properties.[115] Binding of hepcidin to the transmembrane iron exporter ferroportin on the iron-storing cells such as hepatocytes and Kupffer cells leads to ferroportin internalization and degradation and thereby prevents release of stored iron into the circulation.[115,116] Similarly, ferroportin degradation by hepcidin in intestinal epithelial cells results in reduction of iron transfer into circulation.[115] Although ferroportin expression was reported in both murine[117] and human[118] HSCs, very little is known about the role of ferroportin and hepcidin on HSC activation and fibrosis. Emerging evidence indicate that hepcidin has anti-fibrotic effects and its expression is inversely related to the extent of fibrosis in patients.[118] Adenoviral-mediated hepcidin overexpression inhibited fibrosis in CCl4 and bile duct ligation models of mice.[118] Ferroportin expression increases in activated HSC and the anti-fibrotic action of hepcidin in HSC is primarily mediated by degradation of ferroportin.[118] Mechanistically, hepcidin inhibits TGF-β-mediated Smad3 activation which is linked to ferroportin-mediated regulation of AKT signaling. Ferroportin is a negative regulator of AKT activation. Therefore, hepcidin-mediated ferroportin downregulation increases P-AKT levels. Activated AKT sequesters unphosphorylated Smad3 in the cytosol and prevents heterodimerization with Smad4 and subsequent nuclear translocation thereby blunting the TGF-β-mediated signaling.[118,119] Consequently, treatment of mice with either hepcidin or deletion of ferroportin inhibited experimental fibrosis in mice.[118] Interestingly, bone morphogenic protein 6 (BMP6), a known inducer of hepcidin, which belongs to the TGF-β superfamily of growth factors, also inhibits hepatic fibrosis.[120] Compared to WT mice, *Bmp6*$^{-/-}$ mice are prone to hepatic inflammation and fibrosis when fed MCD diet and recombinant BMP6 prevented HSC activation.[120] Taken together, excessive iron can induce HSC activation and fibrosis by inducing oxidative stress in hepatocytes and Kupffer cells, thereby inducing inflammation and fibrosis.

Fibrosis regression

For long time, scientists believed that liver fibrosis is an irreversible process. However, studies performed in patients and rodent models have indicated that fibrosis can regress when the fibrotic insult (e.g. HBV, HCV, alcohol, chemicals, biliary obstruction, and obesity) is removed.[121–124] The time needed to obtain a significant regression varies according to the cause and severity of the liver disease. However, whether cirrhosis, characterized by the most advanced stage of fibrosis, vascularized septae, and nodular parenchymal regeneration, can be reversed is still a matter of debate.[125] Maturation of ECM scar makes it less accessible and resistant to proteases.[122] The presence of non-reducible cross-linked collagen and changes in hepatic vasculature suggested that the cirrhotic process is irreversible.[125,126] However, it is now clear that fibrosis and even cirrhosis may regress. When risk factors for chronic liver diseases are no longer present or the underlying conditions are successfully treated, hepatic reparative mechanisms can slowly improve the hepatic architecture.[127–130] During the regression process, fibrous septa of cirrhotic livers may become thinned and perforated and the characteristic parenchymal nodularity can disappear with time.[127]

Increased collagen degradation is the main mechanism of fibrosis resolution.[121] During fibrosis resolution, the loss of TGF-β signaling is critical. Then the reduction in the expression of tissue inhibitor of metalloproteinases 1 (TIMP-1) enables matrix metalloproteinases (MMPs) secreted by Kupffer cells/macrophage to degrade fibrillar collagen types I and III.[131] Partial degradation of collagen in turn triggers HSCs apoptosis by several mechanisms, such as activation of death receptor-mediated pathways (Fas or TNFR-1 receptors), caspase 8 and 3, upregulation of pro-apoptotic proteins (e.g. p53, Bax, caspase 9), and downregulation of anti-apoptotic proteins (e.g. Bcl-2).[132] Overexpression of peroxisome proliferator-activated receptor γ (PPARγ) or treatment with a PPARγ agonist reverted activated HSCs to a quiescent phenotype.[133] Additionally, during liver fibrosis resolution, about 50% of activated HSCs reverted to a more quiescent state.[5,134] These inactivated HSCs are more sensitive to further fibrogenic stimuli than quiescent HSCs. However, it is still not very well understood whether inactivation is a common feature of all activated myofibroblasts.

Liver fibrosis and HCC

HCC is the most common type of liver cancer and the second leading cause of cancer deaths worldwide with very limited treatment options.[135] Furthermore, HCC is the fastest growing cancer in the United States.[136] The main risk factors for HCC are chronic hepatitis B virus (HBV) or hepatitis C virus (HCV), excessive alcohol consumption, diabetes, and NASH.[137] Advanced liver fibrosis and cirrhosis are major risk factors for HCC, with up to 90% of cases occurring on the background of a cirrhotic liver.[138,139] Rarely, HCC can also develop in the absence of cirrhosis and advanced fibrosis such as those induced by inherited metabolic disorders, e.g. hemochromatosis, porphyria, and type-1 glycogen storage diseases.[139,140] Compared to HCV-induced HCC that relies more on the cirrhotic microenvironment and the necroinflammatory response, HBV-induced HCC can progress more frequently in the absence of cirrhosis, most likely due to the differential ability of HCV and HBV to integrate into the host genome and directly modulate cellular oncogenesis.[140,141] Nevertheless, HBV patients with cirrhosis more frequently progresses to HCC compared to non-cirrhotic HBV patients.[142]

Although the present main risk factors for HCC are HBV and HCV infections, their relative contribution, especially in developed countries, is rapidly declining due to the effectiveness of HBV vaccines and anti-HCV drugs.[143–145] Non-alcoholic fatty liver disease (NAFLD) is now the most common cause of chronic liver disease in the United States.[146] About 30% of the US adult population has NAFLD that increases to 90% in morbidly obese individuals.[147] NAFLD is characterized by the histological presence of >5% macrovesicular steatosis of hepatocytes in an individual without significant alcohol use or other known causes of chronic liver disease. About 10–20% of NAFLD patients exhibit NASH, which is a severe and chronic liver inflammation that includes ballooning degeneration of hepatocytes, fibrosis, and liver damage.[146,148] Quite a few of the patients suffering from NASH progress to end-stage liver disease (cirrhosis), liver failure, and HCC. Although NAFLD is currently the major cause of HCC without advanced fibrosis or cirrhosis in the US population,[149] the risk of NAFLD patients for severe liver disease and mortality increased with the stage of fibrosis.[150]

Fibrosis and cancer-associated fibroblasts (CAF) can influence liver cancer development by modulating the tumor microenvironment.[151,152] Fibrosis is a dynamic process. Deposition of ECM in fibrotic liver not only alters the mechanical properties of the liver but also has the ability to modulate and co-ordinate multiple signaling networks in epithelial, endothelial, stromal, immune, and tumor cells by either directly binding specific receptors such as integrins and growth factor receptors or by forming complexes with ligands that augment their activity and promote binding to their receptors.[153] For example, fibronectin and vitronectin derived from ECM can either bind their cognate integrin receptors[154] or can also bind hepatocyte growth factor (HGF) and modulate signaling by complexing c-Met (HGF receptor) and integrin receptors thereby stimulating the growth and survival of transformed cells.[155]

HSCs play both direct and indirect roles in HCC development. Besides being a key cell type responsible for fibrosis development, activated HSCs can indirectly support hepatic tumorigenesis by secreting angiogenic factors such as VEGF and angiopoietin-1, and CXC chemokines that can influence tumor vascularization.[103,104,108,109] Additionally, HSCs can influence the immune landscape of the liver, tilting the scale towards malignant transformation. Besides being able to secrete pro-inflammatory cytokines, and stimulate Kupffer cells, activated HSCs express PDL-1 (B7-H1)[156] and B7-H4[157] that can induce either exhaustion or anergy of activated T-cells, respectively, in the liver thereby protecting malignant cells from the adaptive immune system and creating an environment conducive for survival and proliferation of pre-malignant cells. Additionally, HSCs may induce a tolerogenic environment in the liver by expanding immunosuppressive Treg cells through IL-2[158] or retinoic acid-dependent manner.[159]

Not only activation of HSCs but also HSCs senescence may strongly influence the tumor development. Senescence of activated HSCs has been shown to improve liver fibrosis,[160] that in effect would be against tumor development. Senescent cells develop a secretory profile composed mainly of inflammatory chemokines, cytokines, and proteases, which is known as senescence-associated secretory phenotype (SASP).[161] Senescent HSCs have an inflammatory phenotype,[162] and SASP from HSCs promotes HCC in obese mice treated with carcinogen.[161] HSCs SASP can skew macrophage polarization towards a tumor-inhibiting M1-state, and inhibiting the HSCs SASP by p53 deletion results in M2 macrophage polarization and HCC promotion.[163]

HSC activation can also directly influence HCC development by secreting critical cytokines and chemokines such as HGF, TGF-β, PDGF, IL-6, and Wnt ligands that can act directly on the tumor cells.[164–166] Cross-talk between HSCs and HCC cells has been shown in both *in vitro* and

in vivo experiments.[165,167] Co-culture with HSCs as well as conditioned media from HSCs culture promotes proliferation, migration, and inflammatory phenotypes of HCC cell lines,[165,167] and the cross-talk between HSCs and HCC cells is bidirectional.[167] Additional evidence of a tumor promoting role of HSCs is that HCC xenograft tumors grow better in the presence of HSCs.[165]

PNPLA3, fibrosis, and HCC

The patatin-like phospholipase domain-containing 3 (PNPLA3), also known as adiponutrin (ADPN), is a nutritionally regulated protein whose expression is controlled by the sterol regulatory element-binding protein 1c (SREBP-1c) and possess triglyceride (TG) hydrolase activity.[168,169] In a cell, PNPLA3 is found localized in the endoplasmic reticulum and lipid droplets.[170] The human PNPLA3 polymorphism (I148M), rs738409, has been strongly implicated in the development and progression of NAFLD, NASH, and fibrosis.[170,171] Genome-wide association studies indicate that homozygous PNPLA3 (I148M) mutation increases the risk of NAFLD-associated HCC by 12-fold.[172,173] However, whether PNPLA3 (I148) SNP promotes HCC development by triggering a specific oncogenic pathway or by creating a conducive microenvironment by promoting steatosis, inflammation, and fibrosis needs further investigation.[174]

The I148M SNP leads to a functional loss of the enzymatic activity and leads to TG accumulation in hepatocytes. However, the loss of PNPLA3 enzymatic activity is not sufficient to describe the functional consequence of PNPLA3 (I148M) mutation since *Pnpla3*$^{-/-}$ mice do not develop NAFLD.[175,176] PNPLA3 (I148M) mutant protein is resistant to proteasome-mediated degradation and accumulates on the surface of hepatic lipid droplets which might interfere with the activity of other triglyceride metabolizing enzymes.[170] Expression of an enzymatically active but ubiquitylation-resistant isoform of PNPLA3 accumulated on the lipid droplets and increased hepatic triglyceride levels when expressed in livers of mice.[177] Therefore, not the loss of enzymatic activity but rather the property of PNPLA3 (I148M) mutant protein to accumulate on the lipid droplets might explain the observed phenotype associated with it. Consequently, *Pnpla3* silencing reduces hepatic steatosis, NASH, and fibrosis.[177,178]

In the liver, PNPLA3 is expressed in both hepatocytes and HSCs and its expression is much higher in HSCs compared to hepatocytes.[179] While PNPLA3 modulates TG metabolism in hepatocytes, PNPLA3's primary role in HSCs is the hydrolysis and release of retinyl esters.[174,179] Therefore, loss of PNPLA3 function would lead to intracellular retention of retinol in HSCs. Consequently, *PNPLA3* 148 M carriers with fatty liver or obesity have lower fasting circulating retinol concentrations[180] and retinyl-palmitate was found elevated in the livers of homozygous *PNPLA3* I148M carriers.[181] HSCs are the primary storage depot of retinol (Vitamin A) in the body which are stored in HSCs lipid droplets.[182] Retinol (Vitamin A) is known to influence cell growth, apoptosis, and differentiation[183] and has also been associated with NAFLD.[184,185] Upon activation, HSCs lose their retinol content and activate into myofibroblasts[182]; however, it is not clear whether loss of retinol plays any role in HSCs activation. PNPLA3 was shown to increase during HSCs activation in a TGFβ-dependent manner[186] and may play a role during the activation process since PNPLA3 ablation may suppress HSCs activation.[187] Moreover, PNPLA3 (I148M) mutant HSCs have a more inflammatory and fibrogenic phenotype and

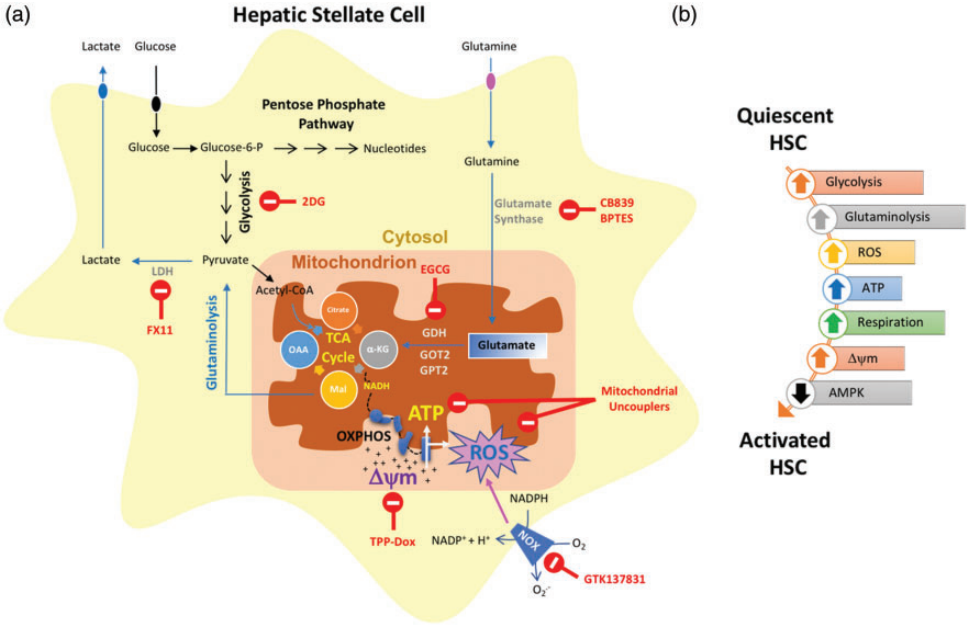

Figure 3. Metabolic reprogramming of HSC. (a) Multiple aspects of glucose and glutamine metabolism are altered during HSC activation that can provide potential therapeutic avenues (indicated by red font). (b) A simplified depiction of metabolic pathways altered during HSC activation. Up arrows indicate upregulated pathway and down arrows mean downregulated. $\Delta\psi$m: Mitochondrial membrane potential; LDH: lactate dehydrogenase; GDH: glutamate dehydrogenase; GOT2: glutamic-oxaloacetic transaminase 2; GPT2: glutamate pyruvate transaminase 2.

therefore can directly contribute to fibrosis development.[187] Increased activation of PNPLA3 (I148M) HSCs is attributed to the suppression of PPARγ activity by JNK-mediated phosphorylation of the inhibitory Serine 84 residue and increase in pro-fibrogenic AP-1 activity.[187]

Besides classical TG and retinol metabolism, PNPLA3 might also affect other metabolic pathways. Overexpression of PNPLA3 I148M in HCC cell line (Huh7) led to two-fold increase in lactic acid suggesting a shift to anaerobic metabolism and mitochondrial dysfunction[188] which, as discussed above, favors HSC activation. Additional studies are needed to confirm and better understand mechanisms of PNPLA3 (I143M) in HSC activation and whether the critical function of PNPLA3 (I148M) is in the hepatocyte, the HSCs, or both.

Conclusions and future prospects

Liver fibrosis is a major health problem that still lacks effective therapeutic strategies. Therefore, understanding the mechanisms underlying this process is crucial for translating basic research into new clinical therapies. In particular, translational research should focus on studying fibrosis in different types of human liver diseases such as ASH, NASH, and HCC in order to design targeted therapeutic interventions.

Since several liver cell types are involved in liver fibrosis, identifying the origin of myofibroblasts and a better understanding of how HSCs get activated and inactivated is of paramount importance. Moreover, uncovering the detailed crosstalk between hepatocytes, HSCs, and Kupffer cells during liver fibrosis progression and regression will definitely help to provide new therapeutic strategies.

How CAFs/HSCs, fibrosis, and the tumor microenvironment influence hepatocarcinogenesis also need to be fully elucidated. Although several studies have demonstrated that HSCs and fibrosis promote development of HCC, an alternative hypothesis is that CAFs may limit cancer progression. Therefore, future studies should aim at investigating whether HSCs and CAFs can also exert tumor-suppressive functions in the context of liver cancer. Answering this question may open new therapeutic paths for the treatment of HCC.

Metabolic reprogramming of HSCs during activation (Figure 3) is another area that needs further investigation. Metabolic addiction of HSCs to glycolytic and glutaminolytic pathways could be exploited to develop therapeutic strategies. Additionally, although the importance of PNPLA3 (I143M) in hepatocytes in promoting NAFLD and ALD is well established, its potential role in HSC activation remains enigmatic.

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DECLARATION OF CONFLICTING INTERESTS

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