Cellular basis of hepatic fibrosis and its role in inflammation and cancer

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1. ABSTRACT

Multiple etiologies of liver injury can lead to fibrosis, which results from an imbalance between production and resorption of extracellular matrix. Hepatic stellate cells (HSCs), resident vitamin A storing cells, play a vital role in the response to injury. Upon activation, HSCs orchestrate the responsiveness of the liver to different types of injury, leading to deposition of excessive scar matrix into the interstitium as a wound-healing response. Quantitatively and qualitatively, the altered extracellular matrix (ECM) provides a permissive milieu for the development of cellular dysplasia and ultimately hepatocellular carcinoma (HCC). There is a range of underlying mechanisms that contribute to progression of fibrosis to HCC. As the functional complexity of HSC activation and its roles in inflammation, immune responses, angiogenesis, and proliferation are being clarified, new advances in therapeutic options for patients with chronic liver disease are emerging.

2. INTRODUCTION

Acute or chronic injury of the liver leads to accumulation of interstitial matrix, or 'scar' matrix, the hallmark of liver fibrosis. Fibrosis typically occurs after a period of months to years of ongoing liver injury, in which scar matrix accumulates. Once fibrosis is present due to chronic liver disease, the risk of HCC strongly increases.

On the cellular level, a key feature of fibrosis is the activation of hepatic stellate cells (HSC), which orchestrate the exuberant wound healing response to injury in the liver (1), including inflammation, secretion of extracellular matrix, growth factor signaling, and angiogenesis (2). HSCs, portal myofibroblasts, and bone marrow-derived fibrogenic cells converge with hepatocytes and immune cells to activate fibrosis progression (3).

There are a variety of liver insults that may provoke the progression of fibrosis, including alcohol

abuse, viral hepatitis (especially chronic hepatitis B and C), drugs, metabolic diseases (haemochromatosis), autoimmune attack on hepatocytes or bile duct epithelium, as well as congenital abnormalities (4). In addition, non-alcoholic fatty liver disease (NAFLD) and its more advanced consequence, non-alcoholic steatohepatitis (NASH), are emerging risk factors for fibrotic liver disease and HCC due to their rising prevalence (5). Indeed, 80 % of all HCC cases develop on an established background of cirrhotic liver disease (6).

HCC is the fifth most common neoplasm in the world, and the third most common cause of cancer related death (7). It is one of the few cancers where major risk factors have been well-defined (8). Cirrhosis, the most advanced stage of fibrosis, is the strongest predisposing factor for the development of HCC. Therefore, patients with cirrhosis are at a high risk for HCC and must be closely monitored. Unfortunately, most HCC patients are diagnosed at advanced stages where palliative treatments remain the only therapeutic option (9).

3. MECHANISMS OF HEPATIC STELLATE CELL ACTIVATION - OVERVIEW

Hepatic fibrosis is a reversible scarring response to chronic liver injury, which ultimately leads to cirrhosis, associated with inflammation, deposition of extracellular matrix, nodule formation and organ contraction. Despite the continuous insult to the liver over decades, in the majority of patients with chronic liver disease fibrosis progresses slowly, due to the compensatory and regenerative capacity of the liver (2). This characteristic ability to regenerate and resorb fibrosis relies on the contribution of different cell types, which include epithelial cells (hepatocytes), endothelial cells, and resident non-parenchymal cells, including HSCs and Kupffer cells.

HSCs reside in the subendothelial space of Disse, between sinusoidal endothelial cells and hepatocytes. Sinusoids represent the microvascular unit of the liver, and are lined by endothelial cells that are characteristically fenestrated, facilitating metabolic exchange between sinusoidal cells and the bloodstream (10). The HSC is the primary source of extracellular matrix in normal liver and hepatic fibrosis. In addition, other cell types have been identified contributing to the accumulating ECM during liver injury, including portal fibroblasts, bone marrow derived cells, and possibly fibroblasts derived from epithelial-to-mesenchymal-transition (EMT) (11-13) (Figure 1).

The stroma of solid tumors consists of non-neoplastic cells (as well as ECM), which are recruited to the tumor from either circulating tissue or liver tissue surrounding the neoplasm. In fact, the fibrous stroma can comprise a large proportion of the tumor mass, and in certain carcinomas may account for up to 90 percent. The tumor stroma can be divided into two cellular components, a vascular part, which consists of endothelial cells and

associated mural cells (tumor pericytes), and a fibrous component, consisting of mesenchymal cells that generate scar (tumor fibroblasts) (14).

HSCs also contribute to the formation of liver metastases of solid tumors, in part by building a permissive stroma within the liver. Based on *in vivo* and *in vitro* studies, HSCs support invasion and proliferation of tumor cells when grown in co-culture, and HSCs are transdifferentiated into myofibroblasts when micrometastases develop in the sinusoidal area of liver lobules (15). Once activated, HSCs may contribute to the poor outcome of HCC patients even after curative resection, since they independently promote early recurrence and higher death rates (16, 17). Activated HSCs and hepatocytes are recruited into avascular micrometastases, where they create a microenvironment that supports tumor development through the release of both proangiogenic factors and cancer cell invasion- and proliferation-stimulating factors. These include vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), prostaglandin E2 (PGE2) and transforming growth factor-beta (TGF-β).

3.1. Hepatic stellate cell activation

HSCs are mesenchymal cells that respond to liver injury in a stepwise manner, thereby transitioning their phenotype from a quiescent vitamin A-rich, non-proliferative cell to an activated contractile myofibroblast that is highly proliferative and fibrogenic. This activation process consists of an initiation stage (also called 'preinflammatory stage') and perpetuation stage, followed by a resolution phase, when liver injury resolves (18).

3.1.1. Initiation

The earliest changes in HSC phenotype and gene expression occur initially in response to paracrine signaling, cytokines and other stimuli. These include altered composition of the extracellular matrix and exposure to lipid peroxides and products of damaged hepatocytes. Neighboring cell types, including sinusoidal endothelial cells, hepatocytes and platelets also contribute to these changes. Hepatocytes, upon membrane injury and lipid peroxidation, are a potent source of fibrogenic reactive oxygen species. As a result, these non-cytotoxic, low levels of O_2^- can up-regulate procollagen type 1 expression, stimulate migration of human HSCs in a Ras/extracellular regulated kinase (ERK) dependent, antioxidant-sensitive manner, without affecting basal or platelet derived growth factor (PDGF)-stimulated cell proliferation (19).

Another mechanism, which promotes the initiation of HSC activation is hepatocyte apoptosis. In primary or immortalized HSCs, incubation with apoptotic bodies leads to increased alpha-smooth muscle actin (α-SMA), TGF-β1, and collagen alpha-1(I) mRNA. This profibrogenic response is dependent upon apoptotic body engulfment (20). The connection between apoptosis and fibrogenesis has been investigated in a bile duct ligation mouse model, confirming that the process is mediated by Fas up-regulation in hepatocytes in this model (21).
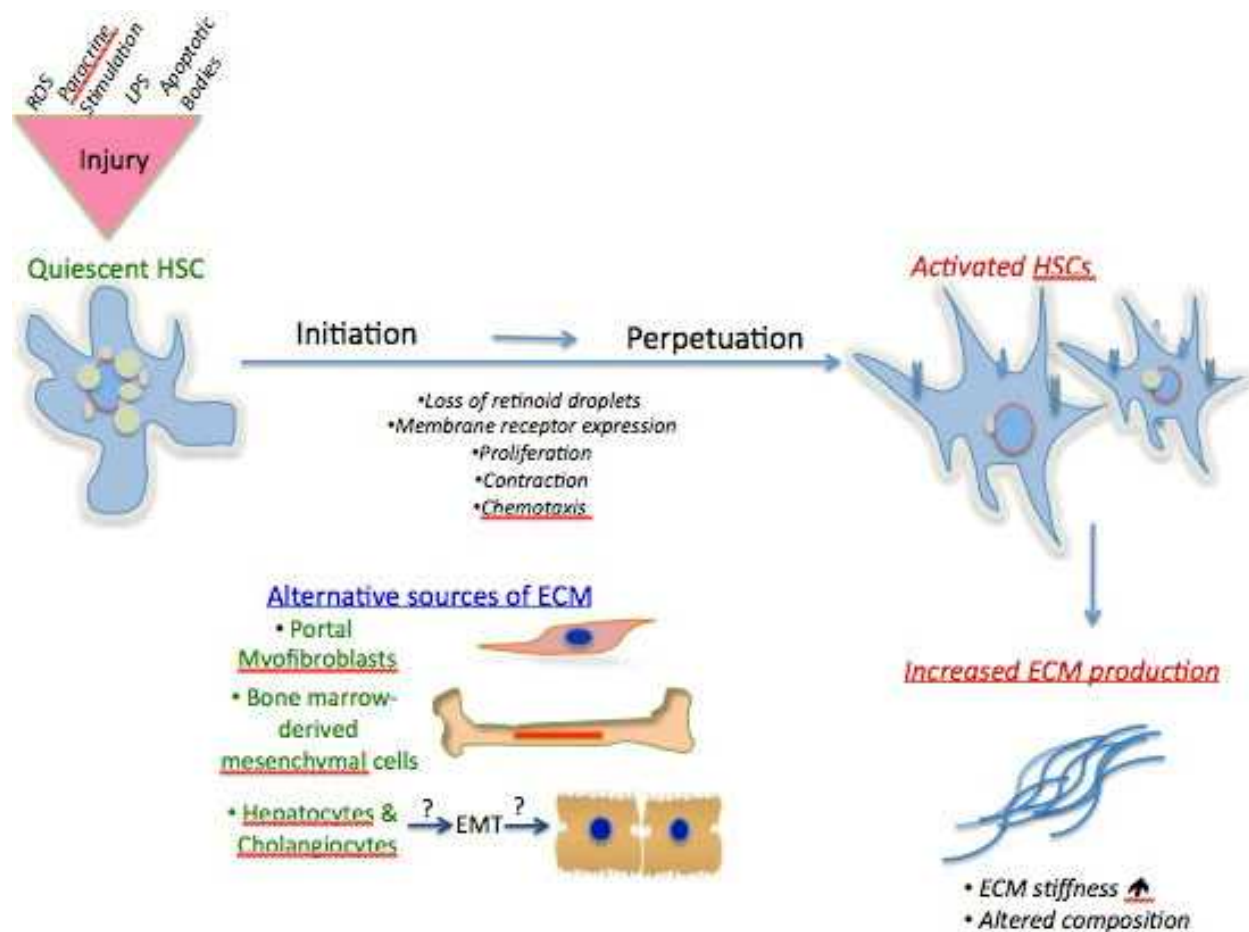
Figure 1. Mechanisms of hepatic stellate cell activation. The hepatic stellate cell (HSC) is the central mediator of hepatic fibrosis and undergoes activation through a two-phase process. During liver injury, HSCs are exposed to reactive oxygen species (ROS), released by hepatocytes undergoing apoptosis, paracrine stimulation by neighboring cells and lipopolysaccharide (LPS). Once activated, HSCs release a number of pro-inflammatory ligands and growth-promoting factors that are bound to the extracellular matrix (ECM). The ECM stiffens, also through a contribution by alternative sources of ECM: portal myofibroblasts, bone marrow derived cells, and from possible epithelial-to-mesenchymal transition (EMT) of hepatocytes and cholangiocytes into mesenchymal cells.

The link between activation of Kupffer cells and their contribution to HSC activation was established when isolated murine Kupffer cells were shown to efficiently engulf apoptotic bodies from mouse hepatocytes (22). Engulfment of these apoptotic bodies stimulated Kupffer cell generation of death ligands, including Fas ligand, and tumor necrosis factor alpha (TNF-alpha). Consistent with the role for Kupffer cells in liver inflammation and fibrosis, gadolinium chloride, a Kupffer cell toxicant, attenuates neutrophil infiltration and markers of HSC activation (23). Kupffer cells, when activated, release the pro-inflammatory cytokines TGF-β and TNF-α, and reactive oxygen species, which in turn initiate the activation and proliferation of HSCs (22). Crosstalk exists between sinusoidal endothelial cells and HSCs through the release of fibronectin by sinusoidal endothelial cells, an early event in the liver's response to injury, which may contribute to the conversion of quiescent to activated HSCs (24). The role of platelets is uncertain, since although they contribute to paracrine release of PDGF, TGF-β$_1$ and EGF, (25), a recent study suggest that they may reduce liver fibrosis and promote regeneration, even in cirrhotic liver (26).

3.1.2. Perpetuation

The second step of HSC activation comprises the many classic features that HSCs display following injury: HSCs proliferate, migrate towards cytokine chemoattractants, contract, degrade matrix, lose their characteristic perinuclear retinoid (vitamin A) droplets, as well as release cytokines and leukocyte chemoattractants. The outcome of these changes is the replacement of 'normal' extracellular matrix, with 'scar' matix (27). As a result, the liver becomes progressively fibrotic.

3.1.3. Resolution

It is increasingly clear that fibrosis in patients with chronic liver disease can be reversible once the underlying cause of disease is eliminated. This is true of

NASH, secondary biliary fibrosis, Hepatitis B, Hepatitis C and autoimmune hepatitis (2). In animal models, the administration of CCl_4 or bile duct ligation leads to development of hepatic fibrosis, which is reversible upon cessation of the etiological agent (28-32).

Two mechanisms of cellular fibrosis resolution have been proposed: the reversion of activated HSCs back into a quiescent phenotype, or apoptosis of activated HSCs. *In vitro,* culture-activated HSCs become quiescent when transferred to a basement membrane matrix (33, 34), but no *in vivo* study has yet confirmed this possibility. Apoptosis of HSCs during regression of liver fibrosis is supported by a large body of evidence (35). When culture-activated, HSCs are sensitive to CD95-L and TRAIL-mediated apoptosis, and NK cells can kill activated HSCs via two mechanisms: 1) a retinoic acid-early-inducible 1/NKG2D-dependent; and, 2) a tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-dependent mechanism, thereby ameliorating liver fibrosis (36, 37). NK cell function in this context is dependent on interferon-γ (IFN-γ), consistent with earlier findings implicating IFN-γ as an anti-fibrotic cytokine (38). Hepatocytes secrete nerve growth factor (NGF), leading to a relative reduction in NF-kappaB binding activity in HSCs, which in turn is associated with a significant increase in caspase-3 activity. Moreover, NGF is expressed during fibrotic liver injury and may regulate the number of activated HSCs via induction of apoptosis (39).

4. PERMISSIVE EFFECTS OF FIBROSIS AND THE MICROENVIRONMENT ON HCC DEVELOPMENT

There are a number of potential mechanisms that may contribute to the permissive effect of the microenvironment towards the development of liver tumors. These include: enhanced oxidative stress and DNA damage, increased matrix stiffness, matrix-bound growth factors, telomere shortening, enhanced inflammation, reduced NK-mediated killing of tumor cells, stromal hedgehog signaling and stem cell expansion. Evidence for each of these mechanisms is detailed in the following sections.

4.1. Enhanced oxidative stress and DNA damage

Reactive oxygen species (ROS) are released by hepatocytes, HSCs, macrophages, cholangiocytes and inflammatory cells, which in turn initiate and perpetuate fibrosis (40). The release of ROS is enhanced by ethanol, iron and polyunsaturated fatty acids. The accumulation of free fatty acids (FFA) results from ethanol metabolism and promotes oxidative stress, thereby leading to the activation of HSCs in culture (41).

Oxidative stress and the release of reactive oxygen species (ROS) contribute to the development of NASH. Insulin-resistant, obese mice with fatty hepatocytes release more ROS from hepatocyte mitochondria than lean mice (42), which may enhance hepatic hyperplasia (43). Oxidative stress can increase the tumor risk by enhanced inflammation, dysplasia and hyperplasia through increased

proliferation, or directly induce tumor growth by favoring mutations. For example, lipid peroxidation produces trans-4-hydroxy-2-nonenal (4-HNE), which provokes mutations in the p53 tumor suppressor gene, favoring the development of HCC (44).

The risk of HCC from cirrhosis is present regardless of the underlying etiology of liver disease. Obesity and diabetes mellitus have been established as independent risk factors in the development of HCC (45). These factors also enhance the risk of nonalcoholic steatohepatitis (NASH), the advanced pro-fibrotic and pro-inflammatory stage of nonalcoholic fatty liver disease (NAFLD) that is a precursor to cirrhosis. There are several mechanisms implicated in the deleterious effect of the metabolic syndrome and its impact on liver injury, as well as the progression to HCC. Insulin resistance, a key feature of the metabolic syndrome, is followed by the release of free fatty acids (FFA) from adipose tissue, the release of pro-inflammatory cytokines and peptide hormones such as tumor necrosis factor-alpha (TNF-α), IL-6, leptin, resistin, and a reduced release of adiponectin (46, 47), together leading to hepatic steatosis and inflammation (48).

Adiponectin regulates lipid metabolism and exhibits insulin-sensitizing, anti-inflammatory and anti-fibrogenic properties (44). In responsive cells, adiponectin's effects are mediated through adiponectin receptor-dependent activation of activated protein kinase-activated protein kinase (AMPK), enhancing catabolic pathways such as glycolysis and lipid peroxidation, and peroxisome proliferator-activated receptor-α (PPAR-α), through which energy partitioning and lipid metabolism are organized. These events in turn favor lipid burning, fructose utilization and prevention of hepatic fat accumulation. Adiponectin also directly counteracts TNF-α, which has been implicated as a mediator of insulin resistance and progression to NASH. Thus, adiponectin provides anti-inflammatory activity in liver. Adiponectin can also directly antagonize fibrogenesis (49). Additionally, hypo-adiponectinemia plays a role in the development of HCC, among various other cancer types (50). *In vitro* studies have shown that adiponectin suppresses HSC activation (51).

Hyperinsulinemia promotes the synthesis and biological activity of insulin-like growth factor 1 (IGF1), a peptide hormone that regulates growth through cellular proliferation and inhibition of apoptosis within the liver (52). The liver is the main source of IGF1, contributing > 80 percent of circulating IGF1. Over-expression of IRS-1 (insulin receptor substrate-1) in human hepatoblastoma cells in culture leads to constitutive activation of the mitogen-activated protein kinase (MAPK) cascade. Here, IRS-1 acts as a dominant oncogene and induces neoplastic transformation of NIH 3T3 cells. The biologic effects of hIRS-1 over-expression in the liver have also been analyzed in human tumor samples, where approximately 40 percent of 22 human hepatocellular carcinoma (HCC) tumors displayed enhanced (> 200 %) hIRS-1 gene expression compared with adjacent non-involved liver tissue (53).

Mice with somatic inactivation of nuclear respiratory factor-1 (Nrf1) in the liver, an essential transcription factor mediating activation of oxidative stress response genes through the antioxidant response element (ARE), have a higher rate of hepatic cancer. These mutant livers exhibited steatosis, apoptosis, necrosis, inflammation, and fibrosis, emphasizing the protein's protective function against oxidative stress (54).

Together, mitochondrial damage and increased ROS production through steatohepatitis lead to cellular death, inflammation, and induction of cytokines such as TNF- , thereby perpetuating the pathogenic process (55). This results in sustained hepatocyte death and a compensatory proliferative response, which may contribute to carcinogenesis in the liver (56).

4.2. Increased matrix stiffness promotes cancer

The balance of matrix production and degradation is precisely regulated in the normal liver. In the setting of fibrosis, normal extracellular matrix (ECM) is degraded by matrix-degrading proteases, which facilitates replacement by scar matrix, and in turn has deleterious effects on cell function (48). Thus, hepatic fibrosis develops as a result of the progressive thickening of fibrotic septae and chemical cross-linking of collagen (2). The disruption of normal liver matrix is a requirement for tumor invasion and desmoplasia (10). Although the degradation of ECM in this context is considered pathologic, its resorption in patients with chronic liver diseases serves as an opportunity to reverse hepatic malfunction, organ stiffness and portal hypertension.

4.2.1. Composition of ECM in the setting of fibrosis

In the setting of hepatic fibrosis, both quality and quantity of hepatic ECM are affected. Collagens, proteoglycans, laminin, fibronectin and matricellular proteins are the most important structural elements of the ECM. Collagen IV and VI are the main components of the low-density basement membrane-like matrix of the space of Disse in normal liver, which are replaced upon fibrosis progression by fibrillar collagens (collagens I and III), as well as fibronectin (57). The degradation of collagen during liver injury is mediated by calcium-dependent enzymes, the matrix-metalloproteinases (MMP) (35, 58), of which MMP-2, MMP-9 and MMP-13 are secreted by HSCs (37). Collagen type I is mainly degraded by MMP-1. MMP activity is potently regulated by their inactivation through binding to tissue inhibitors of metalloproteinases (TIMPs) (59-62). During liver injury, these proteins can inhibit the activity of collagenases, thereby leading to reduced degradation of the accumulating scar matrix during liver injury. During liver injury, TIMP-1 also supports HSC survival (63).

In most tumors MMPs are abundantly and sometimes exclusively expressed by normal host-derived cells including fibroblasts, vascular endothelial cells, myofibroblasts, pericytes or inflammatory cells that contribute to the tumor microenvironment. *In vivo* experiments in animals have revealed that in mice deficient in specific MMPs, host-derived MMPs play a critical role not only in tumor cell invasion, but also in carcinogenesis, angiogenesis, vasculogenesis and metastasis. Therefore, enhanced matrix metalloproteinase (MMP) activity and collagen turnover have been implicated in tumor progression (37).

Increased expression of collagen is associated with an elevated incidence of metastasis of solid tumors (64). In fact, in breast, a higher mammographic density, which is characterized by higher collagen I tissue content, increases breast cancer risk (65). Importantly, the cross-linking of collagen promotes tissue fibrosis (66), which in turn increases the risk of malignancy (67). In tumors, the copper-dependent amine oxidase, lysyl oxidase (LOX) (68), which initiates the process of covalent intra- and intermolecular cross-linking of collagen (69), is frequently elevated (70). Active LOX stiffens tissues and can compromise their function (71). Reducing LOX activity tempers tissue stiffness and prevents fibrosis (72).

The crosstalk between HSCs and the ECM is bi-directional. The ECM itself can modulate the activation and proliferation of HSCs, promote angiogenesis and store growth factors and MMPs. The interaction between ECM and neighboring cells is mainly regulated by certain membrane adhesion receptors, among which the integrins, ADAMs (a disintegrin and metalloproteinase domain molecules) and discoidin domain receptors play an important role (73). Integrins can interfere with TGF-β1, PDGF, and hedgehog signaling pathways, and therefore contribute to cancer biology, including HCC (10).

Cell adhesion is mediated by integrin receptors, heterodimeric transmembrane receptors composed of α- and β-subunits, which have a critical function in organizing cells in tissues. However, the function of integrin adhesion receptors regulates cell behavior through their ability to transduce bi-directional signals into and out of the cell (74-76). Human solid tumors such as breast cancer tumors express high levels of integrins and focal adhesions, and display increased integrin signaling (77). ECM stiffness could therefore regulate malignancy by enhancing integrin-dependent mechano-transduction. Here, lysyl oxidase-mediated collagen cross-linking and tissue stiffening promoted focal adhesions and tumor progression *in vivo*. Collagen cross-linking and tissue stiffness promoted integrin clustering and could also enhance PI3K signaling, which can regulate invasion of a premalignant mammary epithelium *in vitro* and tumor progression *in vivo* (78). These events may be present in a range of tumors, including HCC, but must be validated directly.

4.3. Matrix bound growth factors promote cancer

Apart from structural changes of ECM, a range of growth factors are incorporated into the ECM during liver fibrosis (Figure 2). Not only are HSCs a source of growth factors, but they can also respond to these factors, which results in chemotaxis and proliferation of epithelial cells, angiogenesis, chemotaxis and proliferation of ECM-producing cells as well as chemotaxis and proliferation of inflammatory cells.
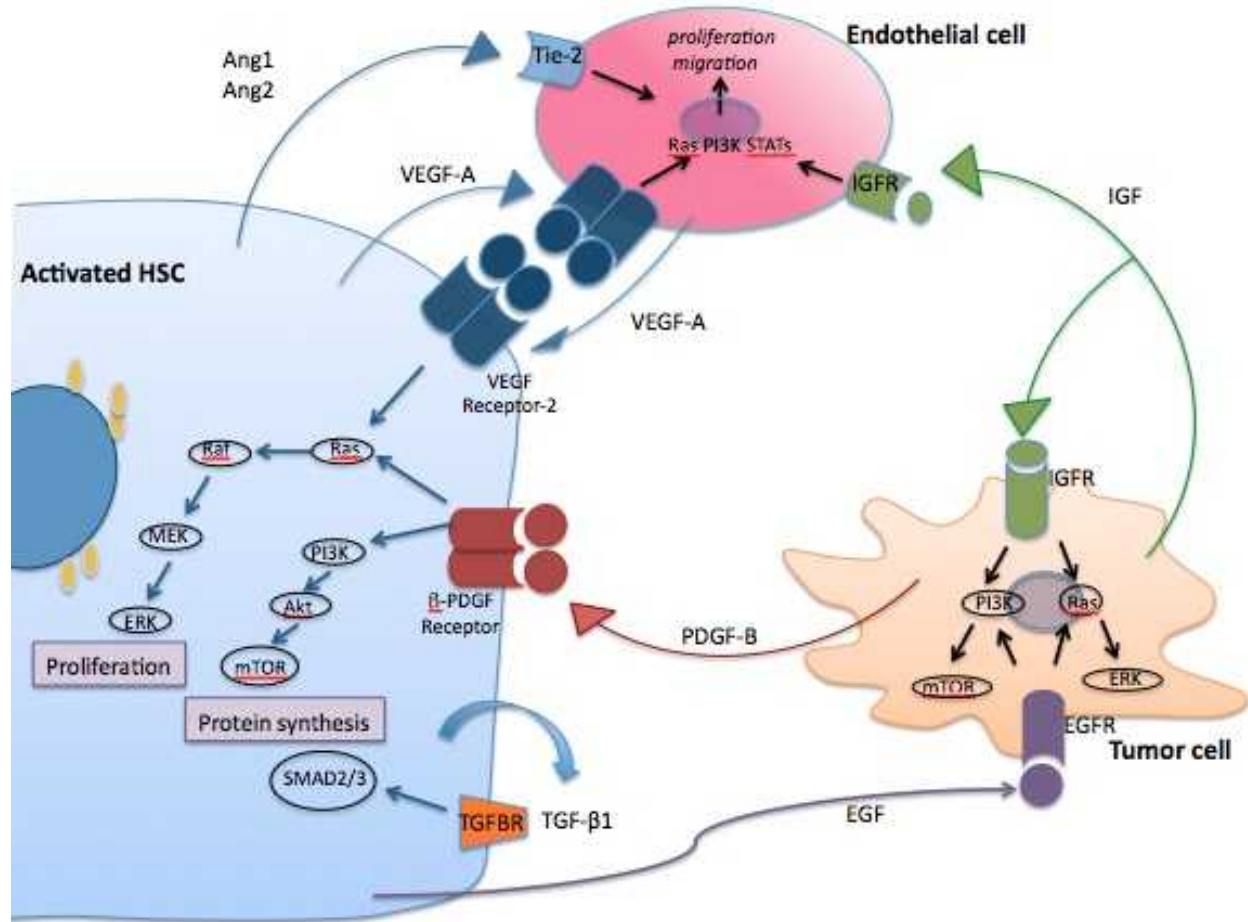
Figure 2. Main interactive pathways during liver injury and carcinogenesis. During activation, HSCs release growth promoting factors, that are bound to ECM and effectively promote proliferation and migration of sinusoidal endothelial cells and tumor cells. Abbreviations: Ang1, Ang2 (Angiopoietin1 & 2), IGF (insulin-like growth factor), PDGF (platelet-derived growth factor), PI3K (phosphatidylinositol-3-kinase), RAS (prototypical member of the RAS superfamily of proteins), VEGF (vascular endothelial growth factor), EGF (epidermal growth factor), ERK (extracellular signal-regulated kinase), mTOR (mammalian target of rapamycin), TGF- (transforming growth factor-beta).

Activated HSCs display increased expression of several receptors for soluble cytokines, including PDGF. In cultured HSCs, PDGF is the most potent proliferative cytokine (79). Responsiveness to PDGF requires the expression of the specific dimeric transmembrane receptor (PDGFR), which is composed of either $\alpha\alpha$, $\alpha\beta$, or $\beta\beta$ subunits (80). Upon PDGF binding, the receptor subunits dimerize, with subsequent phosphorylation of the tyrosine residues in the intracellular domain, which in turn leads to activation of the Fas-MAPK pathway, signaling through the PI3K-AKT/PKB pathway to release intracellular calcium ions that activate PKC family members (81). Mesenchymal expression of PDGF receptors by HSCs is low in normal tissues but dramatically increases during inflammation and in culture. Since induction of β-PDGF receptor leads to a contractile, fibrogenic phenotype of HSCs after their activation, the antagonism of its ligand, PDGF, is an appealing therapeutic option to treat hepatic fibrosis (82, 83).

Activated HSCs induce angiogenesis by the release of vascular endothelial growth factor (VEGF) as well as angiopoietin-1 or -2, which have a direct effect on endothelial cell (EC) function through binding of their specific receptors on EC surfaces (84). VEGF expression in tumor-activated HSCs may create a pro-angiogenic microenvironment, facilitating endothelial cell recruitment and survival during the transition hepatic metastases from an avascular to a vascular stage (85, 86). Receptors for VEGF are also induced during HSC activation, which results in increased mitogenesis (87).

Another member of the receptor tyrosine kinases is the discoidin domain receptor 2 (DDR2), which is primarily activated by collagen type I, and secondarily by collagens II, III and IV. DDR2 is widely expressed in postnatal tissues. DDR2$^{-/-}$ mice exhibit reduced proliferative skin healing compared with wild-type mice, and in culture, fibroblasts derived from DDR2-deficient mice proliferate more slowly than wild-type fibroblasts

(88). The receiver is also expressed during epithelial-to-mesenchymal transition (EMT), a process that comprises cellular transformation during many stages of embryonic development as well as in disease. The inhibition of collagen alpha-1(I) or DDR2 with siRNA leads to a disruption of EMT and cell migration induced by TFG-β, a common stimulus of EMT (89). The expression of DDR2 is elevated in activated HSCs, is induced upon endogenous or exogenous collagen type I exposure and can be down-regulated during cellular quiescence. Over-expression of the receptor on HSCs is followed by growth stimulation and invasion as well as an increase in MMP-2 production (35). Higher expression levels of DDR2 have also been demonstrated on small bile ducts of patients with primary biliary cirrhosis (90).

Transforming growth factor α (TGF-α) and epidermal growth factor (EGF) are important epithelial factors that are secreted by HSCs, but can also stimulate proliferation of HSCs in an autocrine manner (83). Their release furthermore promotes hepatocyte proliferation through paracrine stimulation (91, 92). Additionally, TGFβ, which is secreted by HSCs, has a direct effect on tumorigenesis by inducing autocrine TGF-beta signaling and nuclear beta-catenin accumulation in neoplastic hepatocytes (91).

Thrombin stimulates proliferation of liver HSCs and expression of monocyte chemotactic protein-1 (MCP-1), which increases HSC proliferation and monocyte chemotaxis during human liver disease (93). Other mitogenic pathways include keratinocyte growth factor (KGF), which increases upon liver injury, and has a proliferative effect on hepatocytes (94), and FGF-1 and -2, which are up-regulated upon liver injury and contribute to fibrogenesis (95).

Hepatocyte growth factor (HGF) is released by several organs and tissues; in the liver it contributes to HCC development and fibrosis. In clinical studies with non-cancerous tissues of livers with HCC, HGF is localized in cells of mesenchymal origin and in atypical hepatocytes. In cancerous regions, HGF is present in infiltrating mesenchymal cells and in the cytoplasm of cancer cells (96). HGF has been detected in adenomas, in 80% of HCCs, and in cirrhotic tissue. Tissue localization studies of HGF and its receptor c-met protein support the existence of both autocrine and paracrine mechanisms of action of HGF in HCC, whereas its activity is primarily paracrine in normal liver (97).

4.4. Telomere shortening

Telomeres consist of repeat DNA sequences (TTAGGG) together with a protein complex, the telosome or shelterin. They stabilize chromosome ends in order to protect them from chromosomal instability and prevent activation of a DNA damage response. Shortening of telomeres limits the regenerative capacity of hepatocytes during chronic liver injury and promotes cirrhosis development. A reactivation of telomerases, in turn, may reduce cellular damage and organ dysfunction. Telomerases are enzymatic protein complexes that consist of two components, the telomerase reverse transcriptase (hTERT) and the telomerase RNA component (hTERC). In the setting of HCC, telomerases are reactivated, whereas during chromosomal instability and tumor initiation telomere shortening proceeds (98). Telomere shortening occurs during fibrosis development and its progression to cirrhosis (99). In fact, 80 percent of human HCCs express high levels of telomerase activity (100, 101). In the setting of hepatocarcinogenesis, the activation of telomerase has been linked to the re-expression of human telomerase reverse transcriptase (hTERT), which is usually suppressed after birth (102). The activation of telomerase and re-expression of hTERT occurs at an early premalignant stage of hepatocarcinogenesis, in regenerative nodules and liver cirrhosis (103). Other findings suggest that the activation of telomerase and hTERT occurs in nodules with high-grade dysplasia, or even when the carcinoma is evident; as a result telomerase and hTERT activation have been proposed as markers for HCC (104-106).

4.5. Enhanced inflammation associated with fibrosis

During chronic liver injury chemokines directly modulate the function of resident liver cells and the positioning of immune cells within the injured liver (107-109). Chemokines are soluble mediators that accumulate in the liver upon acute or chronic liver injury, and are expressed by both resident and infiltrating cells. They are a class of small chemotactic molecules known to orchestrate the infiltration of immune and stem cells in the organ and affect proliferation of resident cells, especially HSCs, hepatocytes and endothelial cells. Chemokines can be divided into different groups, and ligands of any given group can only bind receptors of the same family. The predominant groups are the CC-chemokine ligand family (CCL) and the CXC-chemokine ligand family (CXCL), as well as the C-chemokine ligand family (CL) and the CX_3C-chemokine ligand family (CX_3CL). Chemokines promote the migration of fibrogenic cells to the site of injury, thus determining the local concentration of fibrotic changes upon persistent injury and inflammation. HSCs express several chemokine receptors, including CXCR2, CCR5, and CCR7 and are a major source of chemokines upon activation, including CCL2, CCL3, CCL5, CXCL1, CXCL8, CXCL9 and CXCL10 (110, 111). Chemokines not only stimulate HSCs, but also induce further release of chemokines. The intracellular signaling cascades following binding to chemokine receptors involve the Ras/ERK, PI3K/Akt and Src pathways. In HSCs, for example, the activation of these pathways leads to cell migration, cell proliferation, collagen secretion and production of reactive oxygen species (110). During chronic liver damage, either by CCl_4 administration or bile duct ligation, mice genetically lacking CCR1 or CCR5 develop less fibrosis than wild type mice (110, 112). The platelet-derived chemokine (C-X-C motif) ligand 4 (CXCL4) is also a mediator of fibrotic liver damage. Patients with chronic liver disease as well as mice upon toxic liver injury display elevated serum concentrations and intra-hepatic mRNA levels of CXCL4, whereas its genetic deletion reduces liver damage significantly, with decreased infiltration of neutrophils and CD8^{+} T cells into the liver. In addition, CXCL4 stimulates HSC proliferation, chemotaxis and

chemokine expression (112). Together, these factors promote HSC migration and proliferation. and the crosstalk between HSCs and leukocytes during fibrogenesis (113).

4.6. Reduced NK-mediated killing of tumor cells

Natural killer (NK) cells attenuate liver fibrosis through the direct elimination of activated HSCs (114). In alcoholic liver disease, fibrosis is accelerated through inhibition of the anti-fibrotic effects of natural killer (NK) cells (115), and in patients with HCV impaired activity of natural killer (NK) cells may contribute to persistence of hepatitis C virus (HCV) infection. The function of NK cells is primarily regulated by NK cell receptors (NKR), and defective NKR expression is related to decreased NK cell function, which is evident in patients with chronic HCV (116). The activation of innate immunity (natural killer [NK] cells and interferon- [IFN-]) plays an important role in antiviral and antitumor defenses in the liver. However, as liver injury persists, IFN-γ-induced NK cell activation and NK cell-mediated killing of HSCs are significantly reduced in mice after administration of CCl$_4$ for 10 weeks, compared to a 2-week challenge. In culture, early culture-activated HSCs (4 days) induce NK cell activation via an NK group 2 member D/retinoic acid-induced early gene 1-dependent mechanism, which is reduced after a longer duration of culture-activation, paralleling the production of transforming growth factor-β (TGF-β) by HSCs. IFN-γ also can inhibit proliferation of early activated HSCs, whereas HSCs that are culture-activated are resistant to this mechanism (117, 118). In addition, activated Kupffer cells can also induce HSC apoptosis by a caspase 9- and receptor-interacting protein-dependent mechanism (119). Consistent with the experimental data is the clinical observation of increased liver fibrosis in patients on immunosuppressive therapy, especially when corticosteroids and immunosuppressive agents are combined (120).

In aggregate, NK cells stimulate HSC apoptosis, thus limiting liver fibrosis. This feature highlights the importance of HSC apoptosis as a mechanism of regression of liver fibrosis.

4.7. Stromal Hedgehog signaling

HSC activation may be regulated by Hedgehog (Hh) signaling (121). HSCs express multiple components of the Hh pathway, indicating that the Hh pathway contributes to HSC activation and prevention of HSC apoptosis. The inhibition of the Hh pathway reduces HSC activation and decreases HSC cell survival *in vitro*, whereas pharmacologic inhibition *in vivo* in healthy adult mice reduces activation of HSC by at least 50 percent (122).

Hh has been linked to tumor growth in several human tissues, in which Hh has a cell-autonomous function in these tumors (122). However, Hh ligands may fail to activate signaling in tumor epithelial cells. Instead, these data implicate ligand-dependent activation of the Hh pathway in the tumor stromal microenvironment. Moreover, specific inhibition of Hh signaling leads to growth inhibition in xenograft tumor models (123, 124).

Here, the secreted proteins activate the hedgehog pathway in non-malignant stromal cells within the tumor microenvironment, which in turn supports tumor growth. In fact, the typical role of Hh in development is mediated by paracrine effects on mesenchymal cells (125). Still, the question remains how stromal cell types and mechanisms support tumor growth, and to what extent hedgehog influences several events in the tumor microenvironment. To date, one can conclude that paracrine signaling of Hh ligands is required during tumorigenesis of Hh-expressing cancers, which may be bidirectional between the tumor and its surrounding stroma.

4.8. Activated stellate cells may promote stem cell expansion

Recent studies indicate a potential association between fibrosis and progenitor cell activation. Although the mechanism by which this occurs is still unknown, several signaling pathways are implicated in the setting of liver regeneration and repair (126, 127).

HSCs express epithelial markers such as E-cadherin and cytokeratins, which indicates that they may be derived from epithelial cells or are epithelial progenitor cells themselves (128). Additionally, primary HSCs express the stem cell marker CD133 and can differentiate into endothelial cells and hepatocytes in culture, supporting a possible progenitor cell function (129-131). Caution is warranted in interpreting these results since they occurred in culture rather than *in vivo*, however. Recently, HSCs have been proposed as a type of oval cell that can differentiate into hepatocytes during injury (132). Taken together, these findings raise the prospect that HSCs may function as stem or progenitor cells. Alternatively, HSCs may function as a supporting cell type within the stem cell niche, therefore promoting liver progenitor cell expansion and differentiation (132). Co-culture experiments demonstrate that HSCs indirectly modulate the proliferation and differentiation of mesenchymal stem cells (128). It is unclear to what extent cell-cell-interactions are involved; nevertheless, sonic hedgehog and HGF might play a role (133). In general, activated rather than quiescent HSCs have been the source of soluble factors in these experimental models.

5. PERSPECTIVE

Fibrosis promotes the development of HCC through a range of mechanisms, and HSCs orchestrate many of the intertwining pathways. A body of knowledge has already accumulated that highlights the determinants of fibrosis progression from different perspectives. Genetic mouse models may help decipher this complicated network, and more *in vivo* studies are needed to dissect the pathways that directly link fibrosis to HCC. Elucidating the exact mechanisms that promote fibrosis progression and then cancer should be addressed by employing suitable animal models. These can include genetic models with specific deletion of key mediators of fibrogenesis that are also implicated in the development of HCC. Future studies should specifically consider the role of receptor tyrosine kinases, as these receptors are activated during HSC

activation and are also involved in vascularization and amplification of tumor mass and micrometastases. Early intervention with novel therapeutic strategies and development of sensitive diagnostic tools when fibrosis is developing will be essential to reduce the incidence of HCC.

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