LIVER MYOFIBROBLASTS

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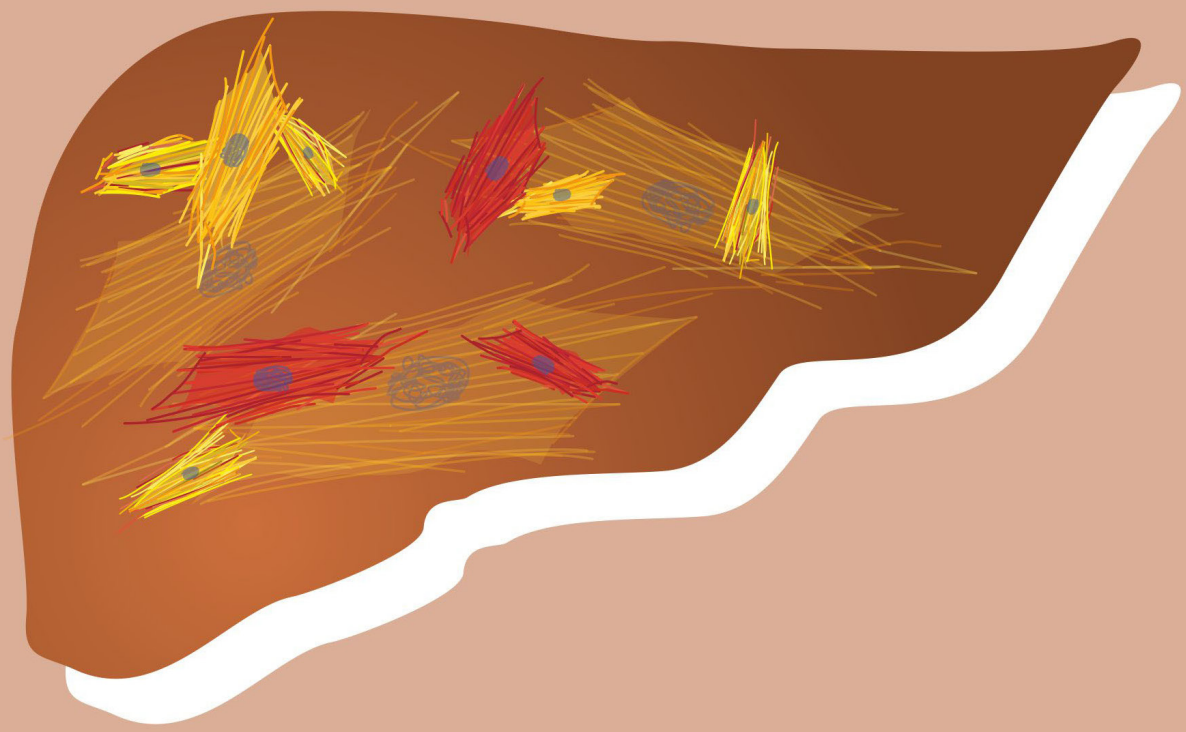

frontiers Research Topics

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Jiri Kanta

Dr. Kanta is an emeritus Associate Professor at the Department of Medical Biochemistry, Faculty of Medicine, Charles University, in Hradec Kralove, Czech Republic. He studied at the Faculty of Sciences, Charles University, in Prague, and graduated in 1965. After joining the Faculty of Medicine he obtained RNDr. and CSc. degrees in biochemistry. His scientific interests were wound healing and liver regeneration and fibrosis. After 1989 he began to build a new lab oriented on molecular biology. His research was positively influenced by contacts with several outstanding scientists in the field of hepatology. The work of Dr. Kanta was focused on hepatic stellate cells and myofibroblasts, the cells that produce collagen and other connective tissue components in the liver. In accord with the current trends to better reproduce conditions in animal tissues he cultured the cells in 3-dimensional gels. He is the author of the research project Liver Myofibroblasts and a Guest Associate Editor.

Alena Mrkvicová

Alena Mrkvicová (born Jiroutová) graduated at the Faculty of Pharmacy and continued with her PhD study at the Faculty of Medicine in Hradec Králové, Charles University in Prague. She studied the role of liver myofibroblasts in liver fibrosis and their interaction with extracellular matrix. She finished her studies in 2011 under the supervision of Assoc. Prof. Jiri Kanta. Alena is currently working at the Faculty of Medicine, Department of Biochemistry as a senior lecturer. She focuses on epithelial-mesenchymal transition (EMT), a biologic process allowing a polarized epithelial cell to undergo multiple changes that enable it to assume a mesenchymal cell phenotype. She works as a Young Associate Editor of the present Research Topic.

Ralf Weiskirchen

Prof. Ralf Weiskirchen was born on February 2, 1964 in Bergisch Gladbach (Germany). After finishing high school, he studied Biology at the University of Cologne (Germany) and made his PhD in 1993. Thereafter, he was PostDoc at the Institute of Biochemistry at the University of Innsbruck in Austria. He investigated differential gene expression induced by v-*myc*. In 1999 he returned to Germany and made his habilitation at the RWTH University Hospital Aachen. In Aachen he became a Professor assignment in 2007. Actually, he is the director of the Institute of Molecular Pathobiochemistry, Experimental Gene Therapy and Clinical Chemistry (IFMPEGKC) in Aachen. His group focuses on the pathogenesis of hepatic fibrosis, the involvement of TGF-β and PDGF signalling in organ disease, biomarker research, adenoviral expression technology, and aspects of translational medicine. He is an Associate Editor of Frontiers in Pharmacology and served as a Guest Associate Editor of the research topic "Liver myofibroblasts" published in Frontiers in Physiology.

LIVER MYOFIBROBLASTS

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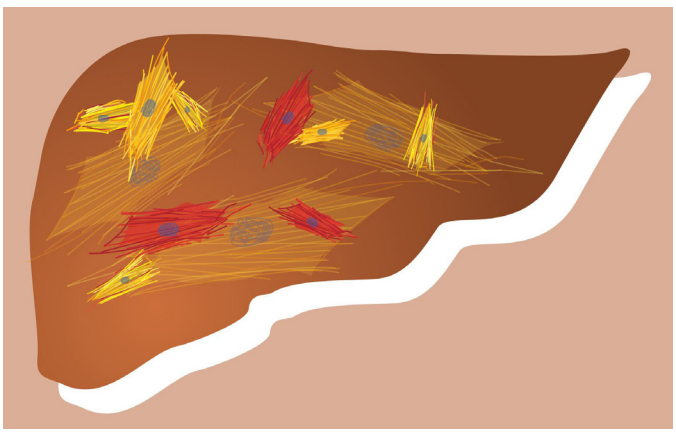
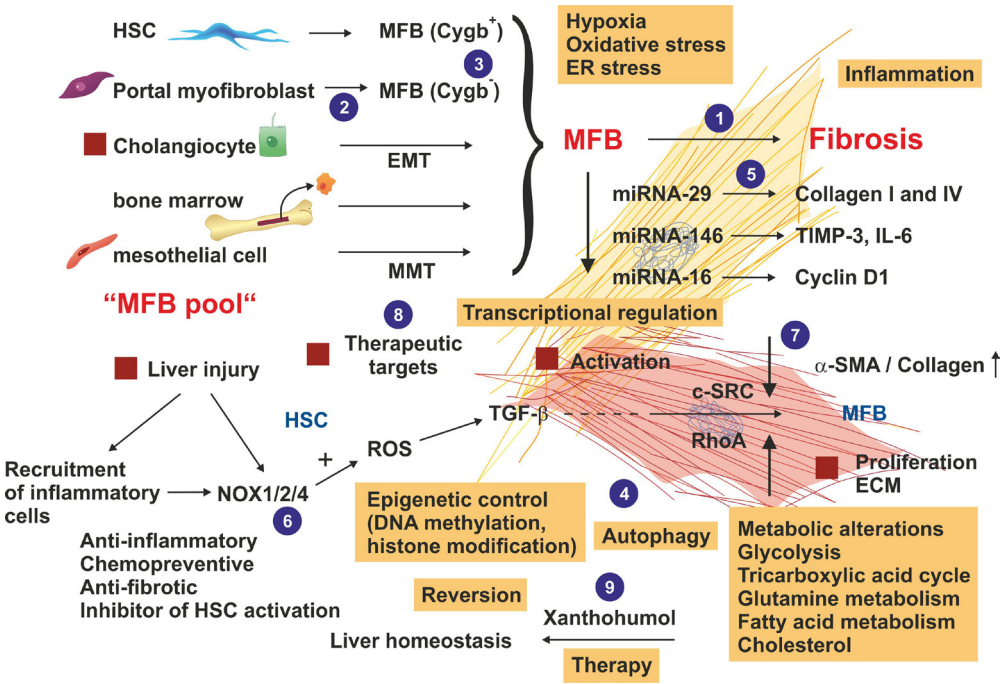


Cover image by Sabine Weiskirchen

Myofibroblasts (MFB) are found in most tissues of the body. They have the matrix-producing functions of fibroblasts and contractile properties that are known from smooth muscle cells. Fundamental work of the last decades has shed remarkable light on their origin, biological functions and role in disease. During hepatic injury, they fulfill manifold functions in connective tissue remodeling and wound healing, but overshooting activity of MFB on the other side induces fibrosis and cirrhosis. The present e-book "Liver myofibroblasts" contains 9 articles providing comprehensive information on "hot topics" of MFB.

In our opening editorial we provide a short overview of the origin of MFB and their relevance in extracellular matrix formation which is the hallmark of hepatic fibrosis. Thereafter, leading experts in the field share their current perspectives on special topics of (i) MFB in development and disease, (ii) their role in hepatic fibrogenesis, and (iii) promising therapies and targets that are suitable to interfere with hepatic fibrosis.



New perspectives in liver myofibroblast research. The research topic focuses on the origin and function of hepatic myofibroblasts (MFB) in development and disease. It provides information about epigenetic alterations during activation, therapeutic targets, and novel drug strategies in the treatment of hepatic disease. The numbers indicate topics within this e-book in which details about liver myofibroblast biology are discussed (1 = Lepreux and Desmoulière; 2 = El Mourabit et al., 3 = Kawada, 4 = Nwosu et al., 5 = Lambrecht et al., 6 = Liang et al., 7 = Görtzen et al., 8 = Wang et al., 9 = Weiskirchen et al.). Abbreviations: α-SMA α-smooth muscle actin; Cygb cytoglobin b; ECM extracellular matrix; EMT epithelial-mesenchymal transition; ER endoplasmic reticulum; MMT mesothelial-to-mesenchymal transition; NOX NAPDH oxidase; TIMP tissue inhibitor of metalloproteinases.
Figure by Sabine Weiskirchen

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EDITORIAL
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Editorial: Liver Myofibroblasts

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Keywords: myofibroblasts, liver fibrosis, hepatic stellate cells, portal fibroblasts, transdifferentiation, TGF-β, therapy

The Editorial on the Research Topic

Liver Myofibroblasts

Myofibroblasts (MFB) were first identified in the granulation tissue of healing wounds. They have features of both fibroblasts and smooth muscle cells. In contrast to fibroblasts they contain cytoplasmic actin microfilaments (stress fibers) connected to the extracellular matrix (ECM) by focal contacts. MFB are connected to each other by adherens and gap junctions. Mechanical forces produced by their contraction facilitate wound closure. Prominent stress fibers can be used to identify MFB in the tissue. They are of mesenchymal origin and are produced by activation and transdifferentiation of quiescent cell precursors after tissue injury. They are not found in normal liver but they appear in large numbers in damaged liver and become a major source of ECM proteins that replace functional tissue. MFB precursors in the liver are hepatic stellate cells (HSC), portal fibroblasts, and circulating bone marrow-derived collagen-producing cells (fibrocytes). They may also arise in a process termed epithelial-to-mesenchymal transition in which epithelial cells acquire a mesenchymal phenotype. Based on the important role of MFB, the knowledge of the transdifferentiation process is critical to understanding the development liver fibrosis. Profibrogenic and proinflammatory cytokines produced by macrophages and T cells regulate fibrogenesis. TGF-β1, the main profibrogenic cytokine, is also produced by MFB and stimulates ECM production in an autocrine manner. Mechanical factors play a role in fibrosis development. Tissue tension facilitates TGF-β1 production and α-smooth actin expression, which in turn increases tension development. MFB are susceptible to apoptosis, their disappearance is important for fibrosis reversibility, and they may be a target of anti-fibrogenic therapy.

With the important role of MFB in the development of liver fibrosis in mind, leading experts in the field share their current perspectives on these cells in this Research Topic. As reviewed by Lepreux and Desmouliere, MFB are found in fetal liver and they reappear during liver injury. They are involved in tissue repair, in liver regeneration, and in liver cancer. HSC-derived MFB are studied in most cases. El Mourabit et al. have described a method to isolate MFB precursors from the rat biliary tree. These cells are highly proliferative and can be easily multiplied *in vitro*. Portal MFB differ in the expression of several genes from HSC-derived MFB, highlighting the distinct origin of the respective cell populations. Kawada concludes in his review that cytoglobin, a member of the mammalian globin family, is expressed in HSC and HSC-derived MFB but it is absent in MFB derived from portal fibroblasts. Therefore, cytoglobin may be used in future studies requiring the discrimination of both MFB subpopulations.

The review by Nwosu et al. shows that HSC transdifferentiation is accompanied by changes in the main metabolic pathways, glycolysis, tricarboxylic acid cycle, as well as in glutamine, fatty acid, and cholesterol metabolism. The authors demonstrate that the antioxidant defense system is also affected and that autophagy, the process of degradation of cellular organelles to generate energy, correlates with HSC activation. On the other hand, autophagy may protect from liver fibrosis in certain circumstances. Gene expression in HSC is also modulated by epigenetic mechanisms.

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Lambrecht et al. discuss a possible role of miRNAs in liver fibrosis. These short RNA molecules regulate gene expression both in normal and pathological conditions. Hypoxia in the liver cells that accompanies the development of fibrosis affects a number of miRNAs. Several miRNAs have been implicated in HSC activation.

Another review in the Research Topic highlights the role of NADPH oxidases in mediating activation of MFB and hepatic fibrogenesis. Chronic liver injury generates oxidative stress that leads to the damage of lipids, proteins, and DNA and necrosis and/or apoptosis of hepatocytes. Reactive oxygen species (ROS) stimulate the production of profibrogenic mediators by Kupffer cells and circulating inflammatory cells and activate HSC. NADPH oxidases are the source of ROS and thus play a role in HSC activation (Liang et al.).

Görtzen et al. have found that the activity of the GTPase RhoA increases in cirrhotic liver and in activated HSC which leads to decreased activity of the transmembrane protein c-SRC. As a consequence, HSC motility and migration is decreased in favor of contractility and ECM synthesis. The increasing tension of liver tissue in the proces of fibrosis can be mimicked by culturing cells on fibrinogen-coated polyacrylamide gels of variable stiffness.

Anti-fibrotic therapy may include protection of hepatocytes from apoptosis as the dying cells release signals recruiting immune cells to the sites of injury. According to the review by Wang et al., the blocking of TGF-β action, the elimination of activated HSC, and the inhibition of cholangiocyte proliferation are possible ways of anti-fibrotic treatment. Various chemicals of plant origin are tested as anti-fibrotic drugs. The hop constituent xanthohumol has been shown to inhibit HSC activation and hepatic carcinoma cell growth *in vitro*. However, as Weiskirchen and his associates discuss in their review, relatively large doses of xanthohumol need to be applied in animal experiments to inhibit pro-fibrogenic gene expression.

In conclusion, the articles in this Research Topic deal with the current topics of myofibroblast research—the origin of MFB in the fibrotic septa of cirrhotic liver, the regulation of the cell transdifferentiation and possible ways of fibrosis treatment. The editors hope that the Research Topic will help researchers to solve these problems.

AUTHOR CONTRIBUTIONS

JK and RW contributed equally to the design of the manuscript, drafted the manuscript, and revised it. AM read the manuscript critically and approved it.

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MINI REVIEW
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Human liver myofibroblasts during development and diseases with a focus on portal (myo)fibroblasts

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Myofibroblasts are stromal cells mainly involved in tissue repair. These cells present contractile properties and play a major role in extracellular matrix deposition and remodeling. In liver, myofibroblasts are found in two critical situations. First, during fetal liver development, especially in portal tracts, myofibroblasts surround vessels and bile ducts during their maturation. After complete development of the liver, myofibroblasts disappear and are replaced in portal tracts by portal fibroblasts. Second, during liver injury, myofibroblasts re-appear principally deriving from the activation of local stromal cells such as portal fibroblasts and hepatic stellate cells or can sometimes emerge by an epithelial-mesenchymal transition process. After acute injury, myofibroblasts play also a major role during liver regeneration. While myofibroblastic precursor cells are well known, the spectrum of activation and the fate of myofibroblasts during disease evolution are not fully understood. Some data are in accordance with a possible deactivation, at least partial, or a disappearance by apoptosis. Despite these shadows, liver is definitively a pertinent model showing that myofibroblasts are pivotal cells for extracellular matrix control during morphogenesis, repair and fibrous scarring.

Keywords: portal fibroblast, myofibroblast, hepatic stellate cell, alpha-smooth muscle actin, liver development, fibrosis, tumoral stroma

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Introduction

In homeostatic state, myofibroblasts are absent from the normal adult liver. Myofibroblasts are stromal cells showing myoid features and involved in production or remodeling of the extracellular matrix (ECM) scaffold. Myofibroblasts are recruited from the transdifferentiation of local stromal cells. Historically, in the liver, it has been postulated that the hepatic stellate cell, also named Ito cell or lipocyte, was the provider of myofibroblasts: in this logic, myofibroblasts were called also transitional cells, activated stellate cells or myofibroblastic cells (French et al., 1988; Mak and Lieber, 1988; Bachem et al., 1989). But other studies have shown afterwards, that fibroblasts located in the connective tissue of the portal tracts are important providers of myofibroblasts (Tang et al., 1994; Tuchweber et al., 1996). This implication of portal fibroblasts as precursor cells of myofibroblasts was observed in human obstructive biliary diseases as well as in animal models (Desmoulière, 2007; Wells, 2014). Indeed, portal fibroblasts are involved as hepatic stellate cells in liver repair after injury

Abbreviations: SM, smooth muscle; ECM, extracellular matrix; WD, week of development; CCl_4, carbon tetrachloride; EMT, epithelial-mesenchymal transition.

and in tumoral reaction. Portal fibroblasts are also an important stromal cell playing a major role during the fetal liver morphogenesis.

Myofibroblast Definition

Since their first description in granulation tissue (Gabbiani et al., 1971), numerous studies have been published leading to remarkable progresses in the understanding of myofibroblast biological characteristics and of their participation in physiological and pathological situations (Hinz et al., 2012). Myofibroblasts exert traction forces by expressing α-smooth muscle (SM) actin and are able to participate in connective tissue remodeling by synthesizing ECM components, matrix metalloproteinases and their inhibitors. When the repair process is completed, in normal situations, myofibroblasts disappear by apoptosis (Desmoulière et al., 1995). Although presenting SM cell features, myofibroblasts however do not express h-caldesmon (150 kDa caldesmon) and smoothelin which seem to be specific for SM differentiation last step (Frid et al., 1992; van der Loop et al., 1997; Ceballos et al., 2000).

Liver Myofibroblast during Liver Morphogenesis

Liver mesenchyme deriving from the mesoblast of the septum transversum is invaded by the epithelial part of the entodermal hepatic diverticulum during the 4th week of development (WD) of normal human embryo (Roskams and Desmet, 2008). The lobulation of the fetal liver begins near the liver hilum at the 9th WD, and continues with a centrifugal pattern in the liver until about 1 month *post-partum*. Mesenchymal part of the liver gives birth to the sinusoid and perisinusoidal space in the future lobules and future portal tracts at the edge of the lobules, including finally portal fibroblasts (Asahina et al., 2011). Each mesenchymal compartment shows a specific maturation pattern (**Figure 1**).

- The portal tract maturation follows a sequence classically divided in three stages. At the first stage, *the ductal plate stage*, a mesenchymal future portal tract containing a large branch of portal vein and limited stroma is surrounded by segments of double-layered cylindrical or tubular structures. At the second stage, *the ductal plate remodeling stage*, the future portal tract incorporates the tubular structures into the stroma and branches of hepatic artery develop. At the last stage, *the remodeled stage*, the portal tract is mature and is characterized by a normal connective tissue containing a branch of portal vein, two branches of the hepatic artery and two bile ducts (Crawford et al., 1998).
- The mesenchyme of the septum transversum framework gives rise to sinusoidal compartment, which comprises endothelial and mesenchymal cells entrapped in the perisinusoidal space (future Disse's space). Endothelium of the sinusoid is continuous in the beginning of development and discontinuous after the 12th WD (Enzan et al., 1997). Perisinusoidal mesenchymal cells contain the developing hepatic stellate cells (Wake, 2006), which the embryonic origin is controversial (for review, see Geerts, 2004).

Other mesenchymal cells in the septum transversum can differentiate into fibroblasts, which occupy the subcapsular connective tissue of the liver (Enzan et al., 1997).

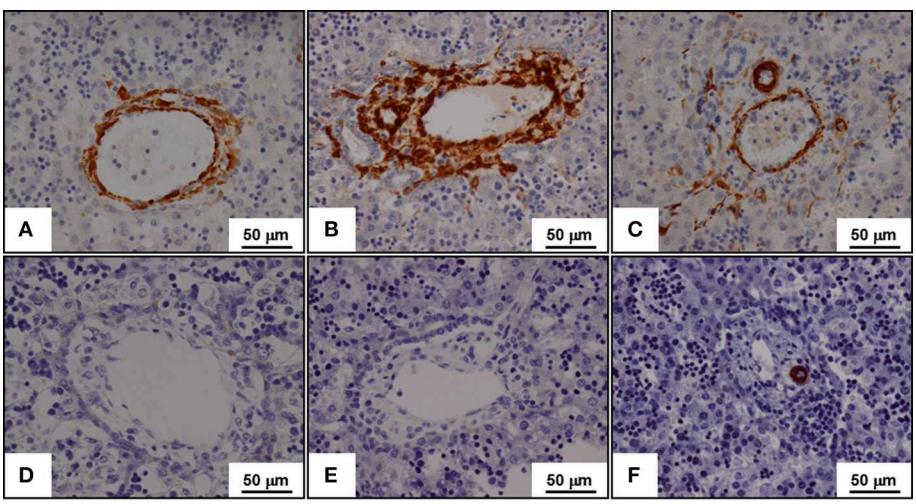

FIGURE 1 | Expression of α-smooth muscle actin (A,B,C) and of h-caldesmon (D,E,F) in fetal liver tissues during the lobulation of the fetal liver. α-Smooth muscle (SM) actin is expressed by myofibroblasts while h-caldesmon is expressed by SM cells. The lobulation of the fetal liver begins near the liver hilum at the 9th week of development, and continues with a centrifugal pattern in the liver until about 1 month *post-partum*. Three stages of the portal tract maturation are described. At the ductal plate stage, the portal vein is surrounded by myofibroblasts that express α-SM actin **(A)**; SM cells expressing h-caldesmon are not yet present **(D)**. At the ductal plate remodeling stage, α-SM actin expressing myofibroblasts surround the biliary tubular structures from the ductal plate, which were incorporated in the portal stroma **(B)**; again, h-caldesmon is not still present **(E)**. At the remodeled stage, α-SM actin expressing myofibroblasts disappear; only arterial tunica media SM cells expressed both α-SM actin **(C)** and h-caldesmon **(F)**.

Stromal cells with myoid features, called myofibroblasts by Libbrecht et al. (2002), are specially implicated in the maturation of the future portal tract (Villeneuve et al., 2009). During the first stage, future portal tract stroma contains myofibroblasts, which surround also portal vein branch. At the second stage, myofibroblasts surround developing bile ducts, developing arterial branches and portal vein. Outside these areas, portal myofibroblasts give place to fibroblastic cells, which do not express α-SM actin. During the maturation of the arterial branches, the tunica media myofibroblasts are replaced by SM cells, which express h-caldesmon. At the last stage, myofibroblasts have disappeared from the portal tract. On these morphological data, we suggest as other a potential role of the portal myofibroblasts during the maturation of biliary tree (Libbrecht et al., 2002; Villeneuve et al., 2009).

Quite the opposite, myofibroblasts are poorly implicated in perisinusoidal maturation. Numerous cellular retinol-binding protein-1 positive hepatic stellate cells extend cytoplasmic processes from the 13th WD, but only few of them also express α-SM actin (Geerts, 2001; Villeneuve et al., 2009).

Liver Myofibroblast as a Repair Cell during Adult Liver Injury Response

Depending on the predominant tropism and the duration of the injury, different patterns of liver inflammation are described. First, two preferential tropisms of hepatitis can be separated in theory: hepatitis with lobular tropism such as viral hepatitis or hepatitis with portal tropism such as biliary obstruction diseases. On light microscopy, a liver zone was preferentially injured depending on the etiology but the other zone is often involved. Two, with the duration and the severity of the injury, the parenchyma architecture can become entirely modified.

In case of acute hepatitis, liver morphology shows inflammatory cell infiltration and hepatocellular damage. The regression is characterized by macrophage cleaning of the necrosis and regeneration. Gradually, these residual changes fade and with times, the liver architecture returns to normal (*restitutio ad integrum*). During this process, hepatic stellate cell play a major role (Kordes et al., 2014). In addition, after partial hepatectomy, incredible liver regeneration capacities are obvious and hepatic stellate cell-derived myofibroblasts can become progenitors, including epithelial progenitors, participating in this specific property of the liver (Swiderska-Syn et al., 2014).

In case of chronic hepatitis, the repetition and/or the persistence of the injury lead to extensive involvement of the inflammatory reaction in the organ. Then, a chronic scarring process destroys the normal architecture of the organ leading to fibrosis and finally cirrhosis. The scars are characterized by extensive fibrous septa due to accumulation of collagenous ECM. They surround regenerative nodules formed by hepatocyte hyperplasia. Profound disturbance of the liver vascular bed accompanying cirrhosis is characterized by venous thrombosis and anarchic angiogenesis within the fibrous scars, sinusoid remodeling and capillarization within the regenerative nodules (Bosch, 2007). Myofibroblasts are the producers of the ECM constituting the scars. But fibrosis is now not considered as a static state, because it can be modified in structure or remodeled in composition in regard to the extraordinary capacity of liver regeneration (Schuppan et al., 2001). Depending on the stimulus, myofibroblasts can contribute to fibrosis regression by releasing of ECM degrading proteases.

Origin of the Myofibroblast Involved in Adult Liver Repair Process

Depending on the duration of the injury, activated stromal cells in acute inflammation and myofibroblasts in sub-acute/chronic inflammation are recruited as a reaction to the lesion.

Local Production

Depending on the site of injury—portal, lobular or both—, the corresponding stromal cells can be activated into myofibroblasts (**Figure 2**).

Portal Fibroblasts

Fibroblasts maintain the connective tissue architecture *via* the ECM that they secrete, but because they are a heterogeneous population of connective tissue cells, they have specific functions depending on their embryological origin and depending *in fine* on their organic site (Rinn et al., 2006, 2008; Tschumperlin, 2013). The fibroblasts located within the connective tissue of the portal tract—the portal fibroblasts—give rise to portal myofibroblasts, which are involved in portal fibrosis, notably in congenital biliary malformations and acquired biliary diseases in human (Ozaki et al., 2005) or after common bile duct ligation in animal models (Tuchweber et al., 1996; Kinnman et al., 2003). It is well known that hepatic stellate cells are also activated when the peripheral lobular parenchyma is invaded by the inflammatory reaction (Tuchweber et al., 1996; Kinnman and Housset, 2002). However, data concerning the origin of the myofibroblasts during portal fibrosis, i.e., hepatic stellate cells or portal fibroblasts, as well as the kinetic of this cellular contribution are controversial: in murine models, for Mederacke et al. (2013), hepatic stellate cells are the principal providers at a late time point of the injury, while it was not the case for Beaussier et al. (2007). Nevertheless, portal fibroblasts and myofibroblasts definitively have an important role in the biliary patterning. They participate in the polarity maintenance and the proliferation regulation of the cholangiocytes (Jhandier et al., 2005; He et al., 2008; Tanimizu et al., 2012). In the same way, interactions between myofibroblasts and biliary cells are also important in the ductular reaction and fibrosis development during the chronic bile duct diseases. In rat model of biliary fibrosis, reactive ductules express growth factors such as platelet-derived growth factor, connective tissue growth factor, or transforming growth factor-β2, which activate portal fibroblasts and increase matrix deposition (Milani et al., 1991; Grappone et al., 1999; Sedlaczek et al., 2001). Accompanying these epithelial-mesenchymal interactions, myofibroblasts produce tenascin and type IV collagen, which play an important role in biliary development and activation (Terada

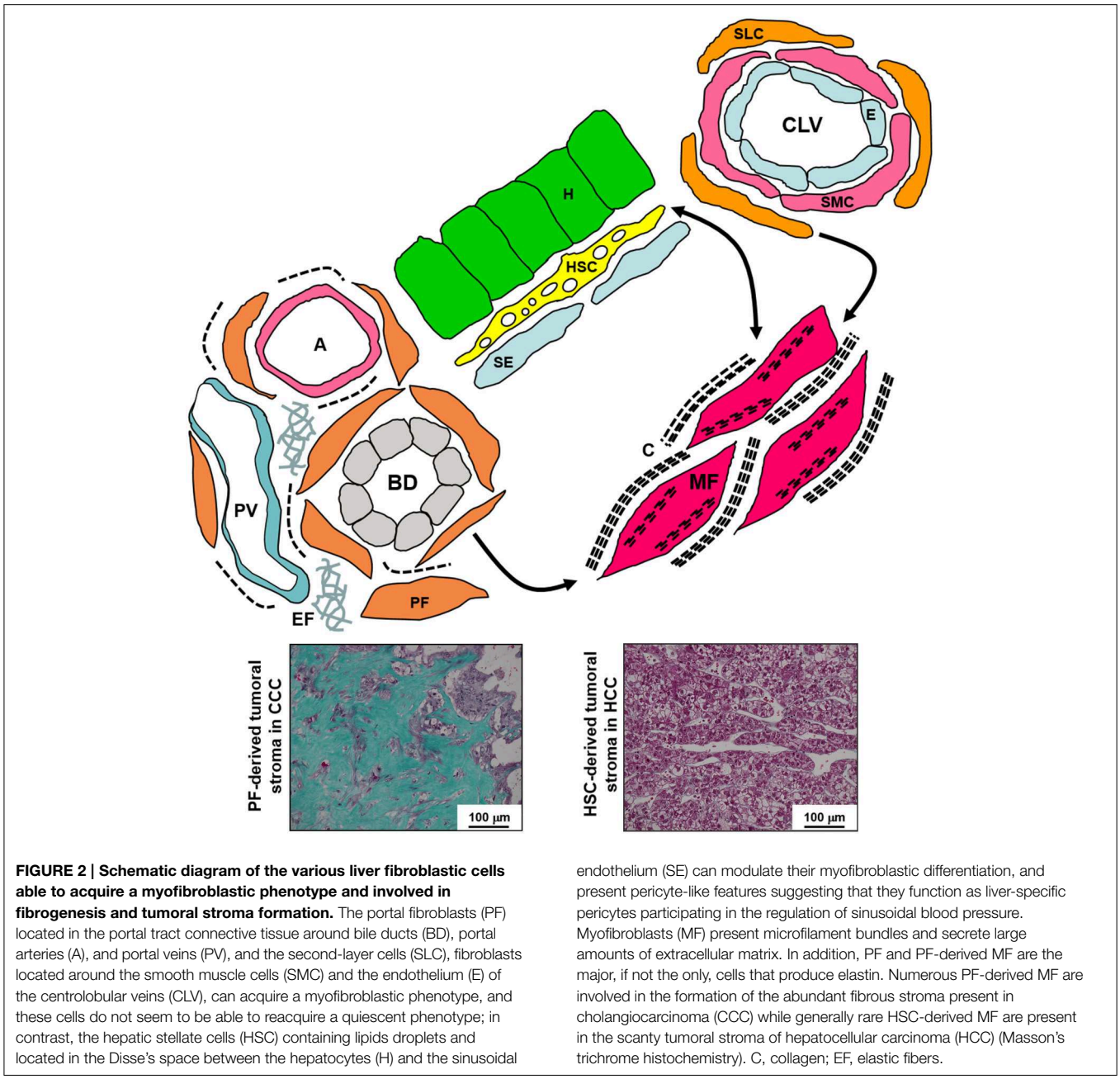

FIGURE 2 | Schematic diagram of the various liver fibroblastic cells able to acquire a myofibroblastic phenotype and involved in fibrogenesis and tumoral stroma formation. The portal fibroblasts (PF) located in the portal tract connective tissue around bile ducts (BD), portal arteries (A), and portal veins (PV), and the second-layer cells (SLC), fibroblasts located around the smooth muscle cells (SMC) and the endothelium (E) of the centrolobular veins (CLV), can acquire a myofibroblastic phenotype, and these cells do not seem to be able to reacquire a quiescent phenotype; in contrast, the hepatic stellate cells (HSC) containing lipids droplets and located in the Disse's space between the hepatocytes (H) and the sinusoidal endothelium (SE) can modulate their myofibroblastic differentiation, and present pericyte-like features suggesting that they function as liver-specific pericytes participating in the regulation of sinusoidal blood pressure. Myofibroblasts (MF) present microfilament bundles and secrete large amounts of extracellular matrix. In addition, PF and PF-derived MF are the major, if not the only, cells that produce elastin. Numerous PF-derived MF are involved in the formation of the abundant fibrous stroma present in cholangiocarcinoma (CCC) while generally rare HSC-derived MF are present in the scanty tumoral stroma of hepatocellular carcinoma (HCC) (Masson's trichrome histochemistry). C, collagen; EF, elastic fibers.

and Nakanuma, 1994; Lamireau et al., 1999). Portal fibroblasts and myofibroblasts could be also involved in portal vasculature and nerve development (for review, see Wells, 2014).

Hepatic Stellate Cells

Hepatic stellate cells, which account for about 5–8% of cells in the normal liver, are characterized by a perisinusoidal distribution in the Disse's space and long processes extending along and around sinusoids, between the hepatocyte plates (Lepreux et al., 2004). The close association of hepatic stellate cells with endothelial cells resembles that of pericytes in capillaries. However, in normal liver, the endothelium is discontinuous and presents multiple fenestrations without diaphragms, allowing the rapid transport of solutes to the subendothelial space. In the normal liver, a basal lamina-like structure separates the two cell types but there is no true basement membrane. Hepatic stellate cells secrete collagens but, contrary to portal fibroblasts, they seem to not produce elastin (Lorena et al., 2004; Perepelyuk et al., 2013) even if, at least *in vitro*, hepatic stellate cell-derived myofibroblasts secrete tropoelastin into the culture medium (Kanta et al., 2002). On activation, the hepatic stellate cells acquire a myofibroblastic phenotype, contributing to the excessive ECM deposition observed in the pathological conditions of fibrosis and cirrhosis. Capillarization of the sinusoids also occurs, with a continuous

endothelium formed, and the presence of a true basal lamina. The experimental model of carbon tetrachloride (CCl_4) treatment in rats has been extensively used to study the involvement of hepatic stellate cells in liver fibrogenesis (Sakaida, 2008). Following chronic injury induced by CCl_4 treatment, a large number of myofibroblastic cells accumulate around centrolobular veins; septa containing myofibroblastic cells expressing α-SM actin then develop between centrolobular areas, and large amounts of ECM are deposited (Reeves and Friedman, 2002). Elastin and α-SM actin are co-localized in septa developing after CCl_4 treatment, but activated α-SM actin-positive hepatic stellate cells in the parenchyma do not contain elastin. Thus, in the CCl_4 model, the typical activated hepatic stellate cells containing α-SM actin seem to play little or no part in elastin deposition (Lorena et al., 2004). These observations suggest that different liver fibroblast subpopulations are involved in deposition of the different ECM components.

Others Cells

Other quiescent fibrocompetent cells can be activated into myofibroblasts: vascular tunica media SM cells (Andrade et al., 1999), second layer cells around the centrilobular veins (Bhunchet and Wake, 1992), and capsular fibroblasts in the Glisson's capsule (Blanc et al., 2005). Recently, a process of mesothelial-to-mesenchymal transition has been mentioned as a novel source of myofibroblastic cells (for review, see Fausther et al., 2013).

Epithelial-Mesenchymal Transition (EMT)

EMT defines a process in which epithelial cells acquire mesenchymal features (Kalluri and Weinberg, 2009). EMT, as well the reverse process of mesenchymal-epithelial transition, occurs normally during the fetal development notably through Hedgehog and Notch signaling pathways. The exploration of Hedgehog signaling pathway in case of human or rat liver fibrosis secondary to biliary obstruction showed that cholangiocytes could undergo EMT (Omenetti et al., 2011). Choi and Diehl (2009) have suggested that some quiescent hepatic stellate cells are transitional cells which can differentiate into epithelial cells or myofibroblasts. But, particularly in this domain, the lack of specificity of lineage markers or tracers, the kinetic of their expression and the fact that the *in vitro* conditions do not reflect the *in vivo* situations, give rise to conflicting results (for review, see Xie and Diehl, 2013).

Systemic Contribution

Some studies, particularly in advanced stages of fibrosis and cirrhosis, have shown that myofibroblasts may originate from bone marrow. For example, in a mouse model of chronic alcohol consumption, bone marrow-derived cells contribute to the development of α-SM actin expressing cells (Fujimiya et al., 2009). However, the real contribution of bone marrow-derived fibrocytes as a source of myofibroblats during liver fibrosis and cirrhosis remains a question of debates (Kisseleva and Brenner, 2012).

Myofibroblasts in Tumoral Stroma

Hepatocellular carcinomas (liver-cell carcinoma) have numerous etiologies, notably chronic B and C virus infection or chronic alcohol abuse and often, hepatocellular carcinomas arise in livers showing cirrhosis, which is in itself a precancerous condition. Bile-duct carcinomas (cholangiocarcinomas) are less common than hepatocellular carcinomas.

During liver regeneration, myofibroblasts are involved in regenerative response, but they are also implicated in the tumoral stroma development (Lemoinne et al., 2013). These myofibroblasts derive locally from hepatic stellate cells and/or portal fibroblasts (**Figure 2**).

However, the nature of the tumoral stroma is totally different in hepatocellular carcinoma and in cholangiocarcinoma.

In hepatocellular carcinoma, except in rare forms of scirrhous or fibrolamellar hepatocellular carcinoma, tumoral stroma is scanty. Often, the tumoral stroma is mixed with the fibrous stroma of the surrounding cirrhosis. Interestingly, the vessels surrounding the tumoral plates are not sinusoids, but continuous capillaries with a true basement membrane. In contrast, in cholangiocarcinoma, the tumoral cells are surrounded by an abundant fibrous stroma containing numerous myofibroblasts (Darby et al., 2011). This stroma is sclerous, sometimes with calcification, may be extensive, and submerges the scanty tumoral tubules.

We suggest that in hepatocellular carcinomas, mainly hepatic stellate cells and SM cells (Lepreux et al., 2013) are involved in the formation of the discrete tumoral stroma while, in cholangiocarcinoma, essentially portal fibroblasts are responsible for the large ECM deposition. Certainly, targeting cancer-associated myofibroblats could be the key for optimal treatment in future therapies and preventing or reversing the myofibroblast activation could inhibit or at least reduce tumor growth (Rizvi et al., 2014; Heindryckx and Gerwins, 2015).

Activation Spectrum of the Stromal Cells (From Unactivated Stromal Cells to Mature Myofibroblasts) and Reversibility of the Myofibroblastic Differentiation

The phenomenon of stromal cell activation is related to the transdifferentiation into myofibroblast. But, depending on the intensity and the chronicity of the stimulus, stromal cells can be activated at different degrees producing a spectrum from cells showing mixed features of quiescence and activation to cells presenting typical morphological and functional characteristics of myofibroblasts. It is particularly true for the hepatic stellate cells which can express overlapping features during the progression from the quiescent state with for example, the presence of vitamin A metabolism markers (intra-cytoplasmic lipid droplets, vitamin A autofluorescence, cellular retinol-binding protein-1 expression) to the fully activated state with for example, the overexpression of α-SM actin and the overproduction of ECM components (Ramadori, 1991; Gressner and Bachem, 1995; Hautekeete and Geerts, 1997; Lepreux

et al., 2001, 2004). From this point of view, hepatic stellate cells present a more malleable phenotype compared with portal fibroblasts. Indeed, differences have been reported between these two fibrogenic cell populations, concerning the mechanisms underlying myofibroblastic differentiation, activation, and deactivation (Guyot et al., 2006). After isolation from healthy rat liver and culturing under the same conditions, both hepatic stellate cells and portal fibroblasts acquire a myofibroblast phenotype. Hepatic stellate cell-derived myofibroblasts display rounded and spread morphological characteristics with an enlarged cytoplasm and, more important, a poor survival after two to three passages. In contrast, portal fibroblasts-derived myofibroblasts have more elongated morphological characteristics and proliferate over multiple passages. *In vivo*, during liver diseases, hepatic stellate cell- and portal fibroblast-derived myofibroblasts present different fates. By using a model of cultured precision-cut liver slices, the behavior of the myofibroblast subpopulations during remodeling differs depending on the experimental model, the pathological situation, and the disease cause (Guyot et al., 2007, 2010). Hepatic stellate cell-derived myofibroblasts can lose α-SM actin expression without undergoing cell death, whereas in similar conditions, portal fibroblast-derived myofibroblasts die by apoptosis. When liver myofibroblasts are cultured on a basement membrane-like substrate (Matrigel), they loss α-SM actin expression, reacquire cytoplasmic lipid droplets, and thus revert, at least partly, to quiescence (Sohara et al., 2002). In the mouse model of CCl_4 induced liver fibrosis, although some myofibroblasts die by apoptosis (Iredale et al., 1998), other myofibroblasts revert to an inactive phenotype during regression of fibrosis (Kisseleva et al., 2012). During the fetal liver portal tract maturation, a same phenomenon of replacement of the myofibroblastic cells by fibroblastic cells was observed (Villeneuve et al., 2009). These data are in accordance with a relative plasticity of the stromal cells; however, we suggest that this plasticity is well established in hepatic stellate cell-derived myofibroblasts, knowing in addition that hepatic stellate cells clearly present pericyte-like features (Costa et al., 2001), whereas portal fibroblast differentiation in myofibroblasts is more complete and less reversible. The question of the regulation of the stromal cell activation is important to consider therapeutic strategies. For example, the blockage of Hedgehog or Notch signaling pathways in rodent models of liver fibrosis leads to partial deactivation of activated hepatic stellate cells and inhibition of fibrotic process (Chen et al., 2012a,b; Xie et al., 2013).

Conclusion

In the past, many studies have been performed using cells derived from explants of human liver parenchyma (Win et al., 1993). Initially, it was suggested that mainly activation of hepatic stellate cells contributes to this population. It is now assumed that these cells are rather representative of many if not all the fibrogenic cell populations present in the liver. It is also accepted that these different fibrogenic cells present different features and that their mechanisms of activation and deactivation are definitively not identical. However, to our knowledge, no reliable markers have been identified that allow unambiguous discrimination between these different cell populations and particularly, between hepatic stellate cell- and portal fibroblast-derived myofibroblasts. However, clearly, depending on the cause of the lesion (e.g., virus, alcohol) and then the primary location of the injury, the fibrogenic cells involved are different. Knowing that the deactivation mechanisms of these different cells are not similar, the question of the reversibility of the liver fibrosis/cirrhosis remains a burning issue. Certainly, portal fibroblasts are involved in many pathological situations and must be considered as a major fibrogenic cell population beside the hepatic stellate cells. Finally, the pivotal role of the portal (myo)fibroblasts in the fetal liver development, as well as in wound healing, including tumoral stroma which could be assimilated to an overhealing wound (Schäfer and Werner, 2008), notably through their interactions with the proliferative bile structures, would require more investigations in the way of liver regeneration application.

References

Andrade, Z. A., Guerret, S., and Fernandes, A. L. (1999). Myofibroblasts in schistosomal portal fibrosis of man. *Mem. Inst. Oswaldo Cruz* 94, 87–93. doi: 10.1590/S0074-02761999000100018

Asahina, K., Zhou, B., Pu, W. T., and Tsukamoto, H. (2011). Septum transversum-derived mesothelium gives rise to hepatic stellate cells and perivascular mesenchymal cells in developing mouse liver. *Hepatology* 53, 983–995. doi: 10.1002/hep.24119

Bachem, M. G., Riess, U., and Gressner, A. M. (1989). Liver fat storing cell proliferation is stimulated by epidermal growth factor/transforming growth factor alpha and inhibited by transforming growth factor beta. *Biochem. Biophys. Res. Commun.* 162, 708–714. doi: 10.1016/0006-291X(89)92368-1

Beaussier, M., Wendum, D., Schiffer, E., Dumont, S., Rey, C., Lienhart, A., et al. (2007). Prominent contribution of portal mesenchymal cells to liver fibrosis in ischemic and obstructive cholestatic injuries. *Lab. Invest.* 87, 292–303. doi: 10.1038/labinvest.3700513

Bhunchet, E., and Wake, K. (1992). Role of mesenchymal cell populations in porcine serum-induced rat liver fibrosis. *Hepatology* 16, 1452–1473. doi: 10.1002/hep.1840160623

Blanc, J. F., Bioulac-Sage, P., Balabaud, C., and Desmoulière, A. (2005). Investigation of liver fibrosis in clinical practice. *Hepatol. Res.* 32, 1–8. doi: 10.1016/j.hepres.2005.03.001

Bosch, J. (2007). Vascular deterioration in cirrhosis: the big picture. *J. Clin. Gastroenterol.* 41, S247–S253. doi: 10.1097/mcg.0b013e3181572357

Ceballos, K. M., Nielsen, G. P., Selig, M. K., and O'Connell, J. X. (2000). Is anti-h-caldesmon useful for distinguishing smooth muscle and myofibroblastic tumors? An immunohistochemical study. *Am. J. Clin. Pathol.* 114, 746–753. doi: 10.1309/K5JP-A9EN-UWN7-B5GG

Chen, Y., Choi, S. S., Michelotti, G. A., Chan, I. S., Swiderska-Syn, M., Kraca, G. F., et al. (2012a). Hedgehog controls hepatic stellate cell fate by regulating metabolism. *Gastroenterology* 143, 1319–1329. doi: 10.1053/j.gastro.2012.07.115

Chen, Y., Zheng, S., Qi, D., Zheng, S., Guo, J., Zhang, S., et al. (2012b). Inhibition of Notch signaling by a γ-secretase inhibitor attenuates hepatic fibrosis in rats. *PLoS ONE* 7:e46512. doi: 10.1371/journal.pone.0046512

Choi, S. S., and Diehl, A. M. (2009). Epithelial-to-mesenchymal transitions in the liver. *Hepatology* 50, 2007–2013. doi: 10.1002/hep.23196

Crawford, A. R., Lin, X. Z., and Crawford, J. M. (1998). The normal adult human liver biopsy: a quantitative reference standard. *Hepatology* 28, 323–331. doi: 10.1002/hep.510280206

Costa, A. M. A., Tuchweber, B., Rubbia-Brandt, L., Peyrol, S., Chevallier, M., Adham, M., et al. (2001). Early activation of hepatic stellate cells and perisinusoidal extracellular matrix changes during *ex vivo* pig liver perfusion. *J. Submicrosc. Cytol. Pathol.* 33, 231–240. doi: 10.1002/jgm.1223

Darby, I. A., Vuillier-Devillers, K., Pinault, E., Sarrazy, V., Lepreux, S., Balabaud, C., et al. (2011). Proteomic analysis of differentially expressed proteins in peripheral cholangiocarcinoma. *Cancer Microenviron.* 4, 73–91. doi: 10.1007/s12307-010-0047-2

Desmoulière, A. (2007). Hepatic stellate cells: the only cells involved in liver fibrogenesis? A dogma challenged. *Gastroenterology* 132, 2059–2062. doi: 10.1053/j.gastro.2007.03.075

Desmoulière, A., Redard, M., Darby, I., and Gabbiani, G. (1995). Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar. *Am. J. Pathol.* 146, 56–66.

Enzan, H., Himeno, H., Hiroi, M., Kiyoku, H., Saibara, T., and Onishi, S. (1997). Development of hepatic sinusoidal structure with special reference to the Ito cells. *Microsc. Res. Tech.* 39, 336–349.

Fausther, M., Lavoie, E. G., and Dranoff, J. A. (2013). Contribution of myofibroblasts of different origins to liver fibrosis. *Curr. Pathobiol. Rep.* 1, 225–230. doi: 10.1007/s40139-013-0020-0

French, S. W., Miyamoto, K., Wong, K., Jui, L., and Briere, L. (1988). Role of the Ito cell in liver parenchymal fibrosis in rats fed alcohol and a high fat-low protein diet. *Am. J. Pathol.* 132, 73–85.

Frid, M. G., Shekhonin, B. V., Koteliansky, V. E., and Glukhova, M. A. (1992). Phenotypic changes of human smooth muscle cells during development: late expression of heavy caldesmon and calponin. *Dev. Biol.* 153, 185–193. doi: 10.1016/0012-1606(92)90104-O

Fujimiya, T., Liu, J., Kojima, H., Shirafuji, S., Kimura, H., and Fujimiya, M. (2009). Pathological roles of bone marrow-derived stellate cells in a mouse model of alcohol-induced fatty liver. *Am. J. Physiol. Gastrointest. Liver Physiol.* 297, G451–G460. doi: 10.1152/ajpgi.00055.2009

Gabbiani, G., Ryan, G. B., and Majno, G. (1971). Presence of modified fibroblasts in granulation tissue and their possible role in wound contraction. *Experientia* 27, 549–550. doi: 10.1007/BF02147594

Geerts, A. (2001). History, heterogeneity, developmental biology, and functions of quiescent hepatic stellate cells. *Semin. Liver Dis.* 21, 311–335. doi: 10.1055/s-2001-17550

Geerts, A. (2004). On the origin of stellate cells: mesodermal, endodermal or neuro-ectodermal? *J. Hepatol.* 40, 331–334. doi: 10.1016/j.jhep.2003.12.006

Grappone, C., Pinzani, M., Parola, M., Pellegrini, G., Caligiuri, A., DeFranco, R., et al. (1999). Expression of platelet-derived growth factor in newly formed cholangiocytes during experimental biliary fibrosis in rats. *J. Hepatol.* 31, 100–109. doi: 10.1016/S0168-8278(99)80169-X

Gressner, A. M., and Bachem, M. G. (1995). Molecular mechanisms of liver fibrogenesis–a homage to the role of activated fat-storing cells. *Digestion* 56, 335–346. doi: 10.1159/000201257

Guyot, C., Combe, C., Balabaud, C., Bioulac-Sage, P., and Desmoulière, A. (2007). Fibrogenic cell fate during fibrotic tissue remodelling observed in rat and human cultured liver slices. *J. Hepatol.* 46, 142–150. doi: 10.1016/j.jhep.2006.08.013

Guyot, C., Lepreux, S., Combe, C., Doudnikoff, E., Bioulac-Sage, P., Balabaud, C., et al. (2006). Hepatic fibrosis and cirrhosis: the (myo)fibroblastic cell subpopulations involved. *Int. J. Biochem. Cell Biol.* 38, 135–151. doi: 10.1016/j.biocel.2005.08.021

Guyot, C., Lepreux, S., Combe, C., Sarrazy, V., Billet, F., Balabaud, C., et al. (2010). Fibrogenic cell phenotype modifications during remodelling of normal and pathological human liver in cultured slices. *Liver Int.* 30, 1529–1540. doi: 10.1111/j.1478-3231.2010.02342.x

Hautekeete, M. L., and Geerts, A. (1997). The hepatic stellate (Ito) cell: its role in human liver disease. *Virchows Arch.* 430, 195–207. doi: 10.1007/BF01324802

He, Y., Wu, G. D., Sadahiro, T., Noh, S. I., Wang, H., Talavera, D., et al. (2008). Interaction of CD44 and hyaluronic acid enhances biliary epithelial proliferation in cholestatic livers. *Am. J. Physiol. Gastrointest. Liver Physiol.* 295, G305–G312. doi: 10.1152/ajpgi.90229.2008

Heindryckx, F., and Gerwins, P. (2015). Targeting the tumor stroma in hepatocellular carcinoma. *World J. Hepatol.* 7, 165–176. doi: 10.4254/wjh.v7.i2.165

Hinz, B., Phan, S. H., Thannickal, V. J., Prunotto, M., Desmoulière, A., Varga, J., et al. (2012). Recent developments in myofibroblast biology: paradigms for connective tissue remodeling. *Am. J. Pathol.* 180, 1340–1355. doi: 10.1016/j.ajpath.2012.02.004

Iredale, J. P., Benyon, R. C., Pickering, J., McCullen, M., Northrop, M., Pawley, S., et al. (1998). Mechanisms of spontaneous resolution of rat liver fibrosis. Hepatic stellate cell apoptosis and reduced hepatic expression of metalloproteinase inhibitors. *J. Clin. Invest.* 102, 538–549. doi: 10.1172/JCI1018

Jhandier, N. M., Kruglov, E. A., Lavoie, E. G., Sévigny, J., and Dranoff, J. A. (2005). Portal fibroblasts regulate the proliferation of bile duct epithelia via expression of NTPDase2. *J. Biol. Chem.* 280, 22986–22992. doi: 10.1074/jbc.M412371200

Kalluri, R., and Weinberg, R. A. (2009). The basics of epithelial-mesenchymal transition. *J. Clin. Invest.* 119, 1420–1428. doi: 10.1172/JCI39104

Kanta, J., Dooley, S., Delvoux, B., Breuer, S., D'Amico, T., and Gressner, A. M. (2002). Tropoelastin expression is up-regulated during activation of hepatic stellate cells and in the livers of CCl_4-cirrhotic rats. *Liver* 22, 220–227. doi: 10.1046/j.0106-9543.2002.01573.x

Kinnman, N., and Housset, C. (2002). Peribiliary myofibroblasts in biliary type liver fibrosis. *Front. Biosci.* 7, d496–d503. doi: 10.2741/kinnman

Kinnman, N., Francoz, C., Barbu, V., Wendum, D., Rey, C., Hultcrantz, R., et al. (2003). The myofibroblastic conversion of peribiliary fibrogenic cells distinct from hepatic stellate cells is stimulated by platelet-derived growth factor during liver fibrogenesis. *Lab. Invest.* 83, 163–173. doi: 10.1097/01.LAB.0000054178.01162.E4

Kisseleva, T., and Brenner, D. A. (2012). The phenotypic fate and functional role for bone marrow-derived stem cells in liver fibrosis. *J. Hepatol.* 56, 965–972. doi: 10.1016/j.jhep.2011.09.021

Kisseleva, T., Cong, M., Paik, Y., Scholten, D., Jiang, C., Benner, C., et al. (2012). Myofibroblasts revert to an inactive phenotype during regression of liver fibrosis. *Proc. Natl. Acad. Sci. U.S.A.* 109, 9448–9453. doi: 10.1073/pnas.1201840109

Kordes, C., Sawitza, I., Götze, S., Herebian, D., and Häussinger, D. (2014). Hepatic stellate cells contribute to progenitor cells and liver regeneration. *J. Clin. Invest.* 124, 5503–5515. doi: 10.1172/JCI74119

Lamireau, T., Le Bail, B., Boussarie, L., Fabre, M., Vergnes, P., Bernard, O., et al. (1999). Expression of collagens type I and IV, osteonectin and transforming growth factor beta-1 (TGFbeta1) in biliary atresia and paucity of intrahepatic bile ducts during infancy. *J. Hepatol.* 31, 248–255. doi: 10.1016/S0168-8278(99)80221-9

Lemoinne, S., Cadoret, A., El Mourabit, H., Thabut, D., and Housset, C. (2013). Origins and functions of liver myofibroblasts. *Biochim. Biophys. Acta.* 1832, 948–954. doi: 10.1016/j.bbadis.2013.02.019

Lepreux, S., Bioulac-Sage, P., Gabbiani, G., Sapin, V., Housset, C., Rosenbaum, J., et al. (2004). Cellular retinol-binding protein-1 expression in normal and fibrotic/cirrhotic human liver: different patterns of expression in hepatic stellate cells and (myo)fibroblast subpopulations. *J. Hepatol.* 40, 774–780. doi: 10.1016/j.jhep.2004.01.008

Lepreux, S., Dubuisson, L., Le Bail, B., Desmoulière, A., Balabaud, C., and Bioulac-Sage, P. (2001). Can hepatic stellate cells express alpha-smooth muscle actin in normal human liver? *Liver* 21, 293–294. doi: 10.1034/j.1600-0676.2001.021004293.x

Lepreux, S., Guyot, C., Billet, F., Combe, C., Balabaud, C., Bioulac-Sage, P., et al. (2013). Smoothelin, a new marker to determine the origin of liver fibrogenic cells. *World J. Gastroenterol.* 19, 9343–9350. doi: 10.3748/wjg.v19.i48.9343

Libbrecht, L., Cassiman, D., Desmet, V., and Roskams, T. (2002). The correlation between portal myofibroblasts and development of intrahepatic bile ducts and arterial branches in human liver. *Liver* 22, 252–258. doi: 10.1046/j.0106-9543.2002.01674.x

Lorena, D., Darby, I. A., Reinhardt, D. P., Sapin, V., Rosenbaum, J., and Desmoulière, A. (2004). Fibrillin-1 expression in normal and fibrotic rat liver and in cultured hepatic fibroblastic cells: modulation by mechanical stress and role in cell adhesion. *Lab. Invest.* 84, 203–212. doi: 10.1038/labinvest.3700023

Mak, K. M., and Lieber, C. S. (1988). Lipocytes and transitional cells in alcoholic liver disease: a morphometric study. *Hepatology* 8, 1027–1033. doi: 10.1002/hep.1840080508

Mederacke, I., Hsu, C. C., Troeger, J. S., Huebener, P., Mu, X., Dapito, D. H., et al. (2013). Fate tracing reveals hepatic stellate cells as dominant contributors to liver fibrosis independent of its aetiology. *Nat. Commun.* 4, 2823. doi: 10.1038/ncomms3823

Milani, S., Herbst, H., Shuppan, D., Stein, H., and Surrenti, C. (1991). Transforming growth factors beta 1 and beta 2 are differentially expressed in fibrotic liver disease. *Am. J. Pathol.* 139, 1221–1229.

Omenetti, A., Bass, L. M., Anders, R. A., Clemente, M. G., Francis, H., Guy, C. D., et al. (2011). Hedgehog activity, epithelial-mesenchymal transitions, and biliary dysmorphogenesis in biliary atresia. *Hepatology* 53, 1246–1258. doi: 10.1002/hep.24156

Ozaki, S., Sato, Y., Yasoshima, M., Harada, K., and Nakanuma, Y. (2005). Diffuse expression of heparin sulfate proteoglycan and connective tissue growth factor in fibrous septa with many mast cells relate to unresolving hepatic fibrosis of congenital hepatic fibrosis. *Liver Int.* 25, 817–828. doi: 10.1111/j.1478-3231.2005.01067.x

Perepelyuk, M., Terajima, M., Wang, A. Y., Georges, P. C., Janmey, P. A., Yamauchi, M., et al. (2013). Hepatic stellate cells and portal fibroblasts are the major cellular sources of collagens and lysyl oxidases in normal liver and early after injury. *Am. J. Physiol. Gastrointest. Liver Physiol.* 304, G605–G614. doi: 10.1152/ajpgi.00222.2012

Ramadori, G. (1991). The stellate cell (Ito-cell, fat-storing cell, lipocyte, perisinusoidal cell) of the liver. New insights into pathophysiology of an intriguing cell. *Virchows Arch. B Cell Pathol. Incl. Mol. Pathol.* 61, 147–158. doi: 10.1007/BF02890417

Reeves, H. L., and Friedman, S. L. (2002). Activation of hepatic stellate cells–a key issue in liver fibrosis. *Front. Biosci.* 7, d808–d826. doi: 10.2741/reeves

Rinn, J. L., Bondre, C., Gladstone, H. B., Brown, P. O., and Chang, H. Y. (2006). Anatomic demarcation by positional variation in fibroblast gene expression programs. *PLoS Genet.* 2:e119. doi: 10.1371/journal.pgen.0020119

Rinn, J. L., Wang, J. K., Allen, N., Brugmann, S. A., Mikels, A. J., Liu, H., et al. (2008). A dermal HOX transcriptional program regulates site-specific epidermal fate. *Genes Dev.* 22, 303–307. doi: 10.1101/gad.1610508

Rizvi, S., Borad, M. J., Patel, T., and Gores, G. J. (2014). Cholangiocarcinoma: molecular pathways and therapeutic opportunities. *Semin. Liver Dis.* 34, 456–464. doi: 10.1055/s-0034-1394144

Roskams, T., and Desmet, V. (2008). Embryology of extra- and intrahepatic bile ducts, the ductal plate. *Anat. Rec. (Hoboken)* 291, 628–635. doi: 10.1002/ar.20710

Sakaida, I. (2008). Autologous bone marrow cell infusion therapy for liver cirrhosis. *J. Gastroenterol. Hepatol.* 23, 1349–1353. doi: 10.1111/j.1440-1746.2008.05381.x

Schäfer, M., and Werner, S. (2008). Cancer as an overhealing wound: an old hypothesis revisited. *Nat. Rev. Mol. Cell Biol.* 9, 628–638. doi: 10.1038/nrm2455

Schuppan, D., Ruehl, M., Somasundaram, R., and Hahn, E. G. (2001). Matrix as a modulator of hepatic fibrogenesis. *Semin. Liver Dis.* 21, 351–372. doi: 10.1055/s-2001-17556

Sedlaczek, N., Jia, J. D., Bauer, M., Herbst, H., Ruehl, M., Hahn, E. G., et al. (2001). Proliferating bile duct epithelial cells are a major source of connective tissue growth factor in rat biliary fibrosis. *Am. J. Pathol.* 158, 1239–1244. doi: 10.1016/S0002-9440(10)64074-6

Sohara, N., Znoyko, I., Levy, M. T., Trojanowska, M., and Reuben, A. (2002). Reversal of activation of human myofibroblast-like cells by culture on a basement membrane-like substrate. *J. Hepatol.* 37, 214–221. doi: 10.1016/S0168-8278(02)00103-4

Swiderska-Syn, M., Syn, W. K., Xie, G., Krüger, L., Machado, M. V., Karaca, G., et al. (2014). Myofibroblastic cells function as progenitors to regenerate murine livers after partial hepatectomy. *Gut* 63, 1333–1344. doi: 10.1136/gutjnl-2013-305962

Tang, L., Tanaka, Y., Marumo, F., and Sato, C. (1994). Phenotypic change in portal fibroblasts in biliary fibrosis. *Liver* 14, 76–82. doi: 10.1111/j.1600-0676.1994.tb00051.x

Tanimizu, N., Kikkawa, Y., Mitaka, T., and Miyajima, A. (2012). α1- and α5-containing laminins regulate the development of bile ducts via β1 integrin signals. *J. Biol. Chem.* 287, 28586–28597. doi: 10.1074/jbc.M112.350488

Terada, T., and Nakanuma, Y. (1994). Expression of tenascin, type IV collagen and laminin during human intrahepatic bile duct development and in intrahepatic cholangiocarcinoma. *Histopathology* 25, 143–150. doi: 10.1111/j.1365-2559.1994.tb01570.x

Tschumperlin, D. J. (2013). Fibroblasts and the ground they walk on. *Physiology* 28, 380–390. doi: 10.1152/physiol.00024.2013

Tuchweber, B., Desmoulière, A., Bochaton-Piallat, M. L., Rubbia-Brandt, L., and Gabbiani, G. (1996). Proliferation and phenotypic modulation of portal fibroblasts in the early stages of cholestatic fibrosis in rat. *Lab. Invest.* 74, 265–278.

van der Loop, F. T., Gabbiani, G., Kohnen, G., Ramaekers, F. C., and van Eys, G. J. (1997). Differentiation of smooth muscle cells in human blood vessels as defined by smoothelin, a novel marker for the contractile phenotype. *Arterioscler. Thromb. Vasc. Biol.* 17, 665–671. doi: 10.1161/01.ATV.17.4.665

Villeneuve, J., Pelluard-Nehme, F., Combe, C., Carles, D., Chaponnier, C., Ripoche, J., et al. (2009). Immunohistochemical study of the phenotypic change of the mesenchymal cells during portal tract maturation in normal and fibrous (ductal plate malformation) fetal liver. *Comp. Hepatol.* 8:5. doi: 10.1186/1476-5926-8-5

Wake, K. (2006). Hepatic stellate cells: three-dimensional structure, localization, heterogeneity and development. *Proc. Jpn. Acad. Ser. B Phys. Biol. Sci.* 82, 155–164. doi: 10.2183/pjab.82.155

Wells, R. G. (2014). The portal fibroblast: not just a poor man's stellate cell. *Gastroenterology* 147, 41–47. doi: 10.1053/j.gastro.2014.05.001

Win, K. M., Charlotte, F., Mallat, A., Cherqui, D., Martin, N., Mavier, P., et al. (1993). Mitogenic effect of transforming growth factor-beta 1 on human Ito cells in culture: evidence for mediation by endogenous platelet-derived growth factor. *Hepatology* 18, 137–145. doi: 10.1002/hep.1840180121

Xie, G., and Diehl, A. M. (2013). Evidence for and against epithelial-to-mesenchymal transition in the liver. *Am. J. Physiol. Gastrointest. Liver Physiol.* 305, G881–G890. doi: 10.1152/ajpgi.00289.2013

Xie, G., Karaca, G., Swiderska-Syn, M., Michelotti, G. A., Krüger, L., Chen, Y., et al. (2013). Cross-talk between Notch and Hedgehog regulates hepatic stellate cell fate. *Hepatology* 58, 1801–1831. doi: 10.1002/hep.26511

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Culture Model of Rat Portal Myofibroblasts

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Myofibroblasts are matrix-producing cells with contractile properties, usually characterized by *de novo* expression of alpha-smooth muscle actin, that arise in fibrotic diseases. Hepatic stellate cells (HSCs), known as perisinusoidal cells containing auto-fluorescent vitamin A, are the major although not exclusive source of myofibroblasts in the injured liver. Portal myofibroblasts (PMFs) have been defined as liver myofibroblasts derived from cells that are distinct from HSCs and located in the portal tract. Here, we describe the protocol we have established to obtain rat PMFs in culture. In this method, the biliary tree is (i) separated from the liver parenchyma by *in situ* enzymatic perfusion of the liver, (ii) minced and further digested *in vitro,* until bile duct segments are isolated by sequential filtration. Bile duct isolates free of HSC contaminants, form small cell clusters, which initially comprise a large majority of epithelial cells. In culture conditions (fetal bovine serum) that provide a growth advantage to mesenchymal cells over epithelial cells, the epithelial cells die and detach from the substrate, while spindle-shaped cells outgrow from the periphery of the cell clusters, as shown by video-microscopy. These cells are highly proliferative and after 4–5 days, the culture is composed exclusively of fully differentiated myofibroblasts, which express alpha-smooth muscle actin and collagen 1, and secrete abundant collagen. We found no evidence for epithelial-mesenchymal transition, i.e., no co-expression of alpha-smooth muscle actin and cytokeratin at any stage, while cytokeratin becomes undetectable in the confluent cells. PMFs obtained by this method express the genes that were previously reported to be overexpressed in non-HSC or portal fibroblast-derived liver myofibroblasts as compared to HSC-derived myofibroblasts, including the most discriminant, collagen 15, fibulin 2, and Thy-1. After one passage, PMFs retain the same phenotypic features as in primary culture. In conclusion, this straightforward and reproducible method of PMF culture, can be used to identify new markers of PMFs at different stages of differentiation, to compare their phenotype with those of HSC-MFs and ultimately determine their progenitors and specific functions in liver wound-healing.

Keywords: alpha-smooth muscle actin, bile ducts, collagen-type XV-alpha 1, cytokeratin 19, fibulin 2, liver fibrosis, liver digestion, portal tract

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INTRODUCTION

Myofibroblasts are cells that arise in fibrotic diseases. They are matrix-producing cells with contractile properties, characterized by *de novo* expression of proteins shared with smooth muscle cells. Myofibroblasts form a heterogeneous population of cells with different possible origins. Alpha-smooth muscle actin (α-SMA) is their most commonly used marker (Hinz et al., 2012). Hepatic stellate cells (HSCs) have been identified as the major source of myofibroblasts in the injured liver (Friedman, 2008), in particular by studies of cell lineage tracing (Mederacke et al., 2013). HSCs are recognized in their quiescent state, as vitamin A-storing cells located in the perisinusoidal space of Disse. However, culture studies and *in situ* studies of fibrotic livers have provided evidence to indicate that liver myofibroblasts could also derive from cells that are distinct from HSCs and located in the portal area (Cassiman et al., 2002; Uchio et al., 2002; Kinnman et al., 2003; Beaussier et al., 2007; Li et al., 2007). Today, we refer to this sub-population of liver myofibroblasts as portal myofibroblasts (PMFs) (Lemoinne et al., 2013). Cells called portal fibroblasts have also been described. They are periductal α-SMA-negative fibroblastic cells found in the normal liver, that express ecto-nucleoside triphosphate diphosphohydrolase 2 (ENTPD2) (Tuchweber et al., 1996; Dranoff et al., 2002). There is evidence to indicate that portal fibroblasts can become activated, i.e., myofibroblastic, in biliary-type liver fibrosis. However, this does not mean that PMFs all derive from portal fibroblasts. In fact, the progenitor cells of PMFs have not been identified yet. To a large extent, our knowledge regarding PMFs is based on a rat culture model that we previously established (Kinnman et al., 2003; Bosselut et al., 2010). In this model, PMFs are obtained by outgrowth from bile duct preparations. We showed that PMFs obtained by this method expressed a number of genes at higher levels than HSC-derived myofibroblasts (HSC-MFs) (Lemoinne et al., 2015). This included fibulin 2 and other genes such as Thy-1, gremlin 1, and fibronectin 1 that were also found by others to be overexpressed in non-HSC derived liver myofibroblasts as compared to HSC-MFs (Knittel et al., 1999; Ogawa et al., 2007; Dudas et al., 2009). This also included the most discriminant marker of non-HSC derived myofibroblasts identified so far, with the highest expression ratio relative to HSC-MFs, i.e., collagen-type XV-alpha 1 (COL15A1).

Non-HSC derived liver myofibroblasts were previously obtained in culture, by different methods of cell isolation. The so-called rat liver myofibroblasts were isolated by enzymatic digestion of the liver, followed by separation of non-parenchymal cells by density gradient and purification of a fraction enriched in myofibroblast precursors by elutriation (Knittel et al., 1999). Vitamin A-free cells were also isolated from a stellate cell-enriched fraction of normal rat liver by fluorescence-activated cell sorting (FACS) (Ogawa et al., 2007). Cells that were negative for ultraviolet-autofluorescence of vitamin A were thus obtained by FACS and formed myofibroblasts in culture, with distinctive features compared to HSC-MFs. Another method has been described (Kruglov et al., 2002) and subsequently modified (Wen et al., 2012), to isolate cells assumed to be portal fibroblasts, from rat liver. First, the biliary tree is prepared by enzymatic digestion of the liver and isolated cells presumably enriched in portal fibroblasts, obtained by size-based filtration. Two markers of portal fibroblasts have been reported, ENTPD2, which is lost after myofibroblastic differentiation in culture, and elastin, which is maintained.

None of these methods, has yet allowed to clearly identify the progenitor cells distinct from HSCs that contribute to liver myofibroblasts, their fate and functions, compared to those of HSC-MFs in liver tissue repair. The advantage of our model is that (i) the outgrowth of myofibroblasts reproduces the pattern of fibrosis progression from the portal area toward the lobule observed *in vivo*; (ii) portal progenitors are not dissociated from their initial niche, avoiding cell selections; (iii) the protocol, phenotype and markers of myofibroblasts obtained by this method, are all very reproducible. The limitations are the abundance of contaminant bile duct epithelial cells in the initial preparation, and possibly, a diversity of myofibroblast progenitor cells. This protocol can be used to identify new markers of PMFs at different stages of differentiation, to compare the behavior and functions of these cells with those of HSC-MFs and determine their interactions with HSC-MFs.

Abbreviations: α-SMA, alpha-smooth muscle actin; CK, cytokeratin; COL, collagen; D, day; ENTPD2, ectonucleoside triphosphate diphosphohydrolase-2; H, hour; Hep, hepatocyte; HPRT, hypoxanthine guanine phosphoribosyl transferase; HSC, Hepatic stellate cell; NS, not significant; PMF, portal myofibroblast.

MATERIALS AND EQUIPMENT

N.B. European catalog numbers (Cat. No.) are provided.

Animals

Sprague Dawley rats weighing 150–200 g (Janvier Labs). Different strains (e.g., Wistar) can be used. The protocol is optimal when rats body weight is ≤250 g. Animals were housed under specific pathogen free conditions (PHEA, agreement No: B 75-12-01).

Enzymes/Chemicals

- Betadin®
- Bovine serum albumin (BSA, Sigma-Aldrich Cat. No. A7030)
- Collagenase P (Sigma-Aldrich Cat. No. 11 213 873 001)
- DNAse (Sigma-Aldrich Cat. No. DN25 > 400 units/mg)
- EDTA 0.5 mol/L pH 8.0 (Sigma-Aldrich Cat. No. 03690)
- Hyaluronidase (Sigma-Aldrich Cat. No. H3884 type IV-S750 3000 units/mg)
- Pronase (Roche Cat. No. 11 459 643 001)
- Sodium heparin (5000 IU/ml)
- Sodium pentobarbital (CEVA animal healthy 5.47 g/100 ml)

Culture Media

- DMEM (Sigma-Aldrich Cat. No. D6046)
- Fetal Bovine Serum (FBS, GIBCO Invitrogen Cat. No. 10270-098)
- HBSS with MgCl2 and CaCl2 (GIBCO Invitrogen Cat. No. 24020-022)

- HBSS without MgCl2 or CaCl2 (GIBCO Invitrogen Cat. No. 14170-088)
- Hepes (GIBCO Invitrogen Cat. No. 15630)
- L15 Leibovitz medium (Sigma-Aldrich Cat. No. L5520)
- MEM with Earl's without glutamin (GIBCO Invitrogen Cat. No. 21090-022)
- NaCl solution (9 g/L) (OTEC Cat. No. 600502)
- Penicillin/streptomycin (GIBCO Invitrogen Cat. No. 15140-122)

Equipment

- 12-well plates
- 16 gauge X 50 mm IV catheter (Jelco Cat. No. 4012)
- Bottle-top filter 0.22 μm (Thermo scientific Cat. No. 568-0020)
- Bubble trap (Medi-Globe Cat. No. 200927)
- Cell strainer 40 and 100 μm Nylon (Falcon Cat. Nos. 352340 and 352360)
- Cotton and linen threads
- Falcon tubes 50 mL
- Glass beakers 250 mL
- Heat lamp
- Orbital water bath
- Perfusion line (Masterflex Cat. No. 96410-16)
- Perfusion pump (Masterflex Cat. No. 751800)
- Plate with magnetic stirrer
- Refrigerated (+4°C) bench-top centrifuge
- Schott Pyrex bottles 150, 250, and 500 mL
- Sterile filter for syringe (0.22 μm)
- Sterile Petri dishes
- Sterile pipettes 5, 10, 25 mL
- Sterilized fork and scissors
- Syringes 3 and 50 mL
- Water bath

Reagent Set-Up

- Dilute pentobarbital at 1/10 in NaCl solution.
- EDTA solution (19 mg/L): Prepare the solution by dissolving 6.5 mL EDTA in 43.5 mL sterile water and filter through a 0.22-μm syringe filter. This solution can be prepared up to 4 weeks in advance and stored at +4°C.
- L15 1% Hepes solution: Under sterile condition, dilute 5 mL Hepes (1 mol/L) in 500 mL L15 medium.
- Wash solution (HBSS without MgCl2 or CaCl2, 1% EDTA): Dilute 2 mL of 19-mg/L EDTA solution in 200 mL HBSS without CaCl2 or MgCl2. Adjust the pH to 7.35–7.40 and filter it through 0.22-μm bottle-top filter.
- Enzyme solution 1: Add 15 mg collagenase P in 200 mL HBSS with MgCl2 and CaCl2. Place the mixture on a magnetic shaker until dissolution and adjust the pH to 7.35–7.40 and filter it through 0.22-μm bottle-top filter.
- Enzyme solution 2. In 200 mL MEM, dissolve 15 mg collagenase P, 140 mg pronase, 13 mg DNAse and 200 mg BSA. Supplement with 1% penicillin/streptomycin, 1% Hepes and 3% FBS. Place the mixture on a magnetic shaker until dissolution, adjust the pH to 7.35–7.40 and filter it through 0.22-μm bottle-top filter.
- Enzyme solution 3. In 100 mL MEM, dissolve 6 mg collagenase P, 20 mg hyaluronidase, 6 mg DNAse and 100 mg BSA. Supplement with 1% penicillin/streptomycin, 1% Hepes and 3% FBS. Place the mixture on a magnetic shaker until dissolution, adjust the pH to 7.35–7.40 and filter it through 0.22-μm bottle-top filter.
- Complete DMEM: Supplement DMEM with 1% penicillin/streptomycin, 1% Hepes and 10% FBS.

▲ CRITICAL Wash and enzyme solutions should be prepared no later than 18 h before starting the isolation procedure.

Equipment Set-Up

Fill perfusion line and bubble trap with wash solution and adjust the flow rate of perfusion pump at 10 mL/min.

PROCEDURE

Procedure Set-Up (60 min)

Described in Section Reagent Set-Up and Equipment Set-Up.

▲ CRITICAL Wash solution and enzyme solution 1 should be preheated in the water bath (38°C).

In situ Digestion of Rat Liver (90 min)

1- Anesthetize the rat according to the institutional approved animal protocol. We perform pentobarbital intra-peritoneal injection (1 mL/100 g body weight).
2- Shave the abdomen and clean with Betadin®.
3- Immobilize the rat by the upper and lower extremities on a dissecting board.
4- Perform a laparatomy to expose the liver, and move the visceral organs to the right side to expose the inferior vena cava and the portal vein.
5- Ligate the gastro-duodenal vein with a cotton thread. Then, prepare two linen threads around the portal vein under the gastro-duodenal vein, not to ligate at this stage. Prepare also a linen thread around the infrahepatic vena cava above the renal vein, not to ligate at this stage.
6- Inject 500 IU heparin in the femoral vein.
7- Place a 16-gauge catheter into the portal vein, entering under the gastro-duodenal vein. Check the position of the catheter that should not point upward in the vein, and ligate the two linen threads around the catheter placed in the portal vein. **!** Troubleshooting (**Table 1**).
8- Start the pump, connect the catheter to the perfusion line and stabilize the set-up to avoid exit of the catheter. ▲ CRITICAL STEP Before connecting the perfusion line, make certain that no bubbles are introduced in the catheter, which would result in improper digestion.
9- Section the infrahepatic vena cava beneath the unligated linen thread that was prepared in step 5.
10- Place a 16-gauge catheter through the right auricle into the suprahepatic vena cava and fix it with a linen thread.
11- Ligate the infrahepatic vena cava by using the linen thread that was prepared in step 5. Thereafter, perfused solutions will outflow through the auricle catheter (**Figure 1A**).

TABLE 1 | Troubleshooting.

Step	Problem	Possible reason	Solution
7	Catheter exit from the portal vein	Abrupt manipulation	Inverse perfusion direction by placing the catheter in the right auricle and use it for perfusion; linen threads around the portal vein are removed to allow outflow
14	Catheter exit from the explanted liver	Abrupt manipulation	Inject L15 medium through the liver capsule using a syringe
23	Tan color of hepatocytes visible in preparations remaining on the 40-μm cell strainer	Contamination during filtration steps	Increase the number of filtration through 40-μm cell strainers
25	Low purity (contaminating hepatocytes)	Incomplete digestion	Make sure that the enzyme concentrations are correct Make sure that all liver lobules are perfused

12- Perfuse the liver with wash solution for a total of 20 min. Switch on the heat lamp directed toward the rat liver to maintain a temperature of 37°C and hydrate the liver with NaCl solution during perfusion. ▲ CRITICAL STEP Make sure that all liver lobules are perfused. If it was not the case, you can gently move the catheter.

13- Switch from wash solution to enzyme solution 1 and perfuse the liver for 15–20 min, until digestion becomes visible.

14- Disconnect the perfusion line from the catheter but do not remove the intra-portal catheter from the liver. Explant the liver and place it in a sterile 100-mm Petri dish. Inject 20 mL of cold L15 medium (+4°C) with a syringe through catheter to dislocate hepatocytes, and repeat this injection twice. ! Troubleshooting (**Table 1**).

15- Under cell culture hood, transfer the liver to a new sterile 100-mm Petri dish with L15 medium at room temperature. Peel off the liver capsule and detach the liver parenchyma by scrubbing with a fork, until the biliary tree is isolated (**Figure 1B**).

In vitro Biliary Tree Digestion (150 min)

▲ CRITICAL Enzyme solutions 2 and 3 should be preheated in the water bath (38°C).

16- Transfer the biliary tree to a 100-mm sterile Petri dish containing pre-warmed enzyme solution 2. Cut the biliary tree into small pieces with scissors and transfer the minced biliary tree into a 250-mL Schott Pyrex bottle. Add enzyme solution 2 to a final volume of 100 mL and place the bottle in an orbital water bath at 37°C. Shake the bottle during 30 min. ▲ CRITICAL STEP The result of mincing should be a homogeneous preparation of very small fragments.

17- Filter this preparation through a 100-μm cell strainer and then through a 40-μm cell strainer. Discard the filtrate from the 40-μm cell strainer.

18- Suspend the preparations remaining on the 100-μm and 40-μm cell strainers in 100 mL of enzyme solution 2. Place the mixture under shaking at 37°C, for 30 min. ▲ CRITICAL STEP Increase the digestion time if chunks are visible.

19- Repeat step 17.

20- Suspend the preparations remaining on the 100 and 40-μm cell strainers in 100 mL of enzyme solution 3. Place the mixture under shaking at 37°C for 30 min. ▲ CRITICAL STEP Exceeding 40 min of incubation may result in a poor yield of bile duct segments.

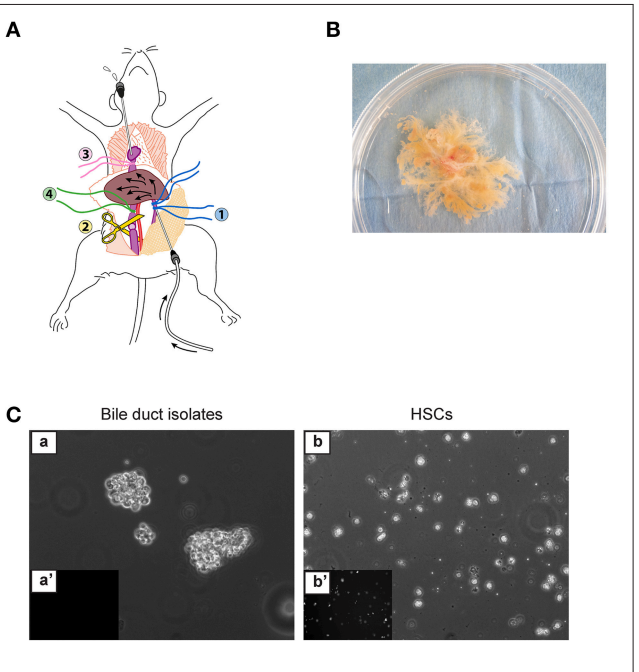

FIGURE 1 | Enzymatic digestion of the liver. (A) Set-up for *in situ* liver perfusion. A catheter is inserted into the portal vein (blue threads) and connected to the perfusion tubing. After starting the perfusion, the vena cava is sectioned under the liver to prevent hyper-pressure in the liver. Then, a catheter is inserted through the right auricle into the inferior vena cava (pink thread) to drain the outflow from the liver, and the thread (green) around the vena cava, just above section, is ligated. **(B)** Biliary tree isolated by *in situ* digestion, placed in a 100-mm culture dish. **(C)** Bile duct isolates obtained immediately after *in vitro* digestion of the biliary tree (a,a'), in comparison with freshly isolated HSCs (b,b'), under phase-contrast microscopy (a,b) or epifluorescence microscopy at 328-nm UV excitation (a',b'), form small cell clusters free of autofluorescent HSCs.

21- Filter the mixture through a 100-μm cell strainer and collect the filtrate for two additional filtrations, each time with a new 100-μm cell strainer.

22- Subject the filtrate obtained in step 21 to filtration through a 40-μm cell strainer. Discard the filtrate.

23- Suspend the preparation remaining on the 40-μm cell strainer in L15 medium. Proceed to a last filtration

through a 40-μm cell strainer and discard the filtrate. **!** Troubleshooting (**Table 1**).

24- Suspend the preparation remaining on the 40-μm cell strainer in 50 mL complete DMEM and transfer it into a 50-mL Falcon tube.

Cell Culture

25- Centrifuge at 1900 rpm during 8 min at room temperature. Discard the supernatant. Suspend the cell pellet in complete DMEM and plate the cells in 12-well culture dishes (1 mL/well). The yield of cell clusters is 7000–10,000 per rat liver. ▲ CRITICAL STEP The volume of complete DMEM should be adapted to the size of the cell pellet so as to obtain four to six cell clusters per microscope field at magnification x10. Perform microscope observation under 328-nm UV excitation to exclude contamination by HSCs (**Figure 1C**). **!** Troubleshooting (**Table 1**).

26- Maintain cell culture at 37°C in a 5% CO_2 incubator. Replace the culture medium 24 h after seeding and every 48 h, thereafter. Generally, cells are confluent after 4–5 days in primary culture, a stage we refer to as passage 0 (P0).

27- Confluent cells can undergo several passages. They are fully differentiated in myofibroblasts, in primary culture (P0) and after one passage (P1).

RESULTS

Methods Used to Characterize PMF Culture

RT-PCR

Total RNA was used to prepare cDNA. Quantitative real-time PCR was performed using Sybr Green Master Mix on a Lightcycler 96 (Roche). Target gene mRNA levels were reported relative to a calibrator according to the $2^{-\Delta\Delta Ct}$ method with hypoxanthine guanine phosphoribosyl transferase (*Hprt*) used as the reference gene. Primer sequences are provided in Supplementary Table 1.

Fluorescence

To monitor cell viability in culture, NucGreen® Dead 488 reagent (ThermoFisher) was added to the culture medium, following the manufacturer's instructions. For immunofluorescence, cell preparations were fixed in 4% paraformaldehyde for 15 min, then blocked and permeabilized, by incubation in 2% albumin (Roche) supplemented with 0.1% Triton X100 (Sigma-Aldrich) for 1 h. Fixed cells were incubated with the primary antibodies against: COL15A1 (HPA017913, 1/30, Sigma-Aldrich), α-SMA (1A4, 1/100, Dako), or pan-cytokeratin (sc-8018, 1/10, Santa Cruz Biotechnology) overnight at 4°C. Primary antibody was revealed by Alexa Fluor 488 or 568-conjugated antibodies (1/200, Life Technologies) and DAPI was used for nuclear staining. For dual α-SMA and pan-cytokeratin immunofluorescence, Alexa Fluor 488-conjugated α-SMA (1/100, Abcam) and Alexa Fluor 647-conjugated pan-CK (1/10, Cell Signaling) antibodies were used. Image acquisition was performed, using a SP8 confocal microscope (Leica). For proliferation assay, fixed cells were incubated with the Alexa Fluor 488-conjugated Ki67 antibody (1/50, Cell Signaling) overnight at +4°C and DAPI was used for nuclear staining. Ki67-positive cells was counted in 5 random fields at magnification x20 using ImageJ software and reported to the total cell numbers (%).

Collagen Assay

Conditioned medium prepared from cells incubated with or without 10% FBS for the last 24 h, was analyzed for soluble collagen using the Sircol collagen assay, according to the manufacturer (Sigma-Aldrich). Briefly, Sircol reagent was added to the conditioned medium to form collagen-dye complex.

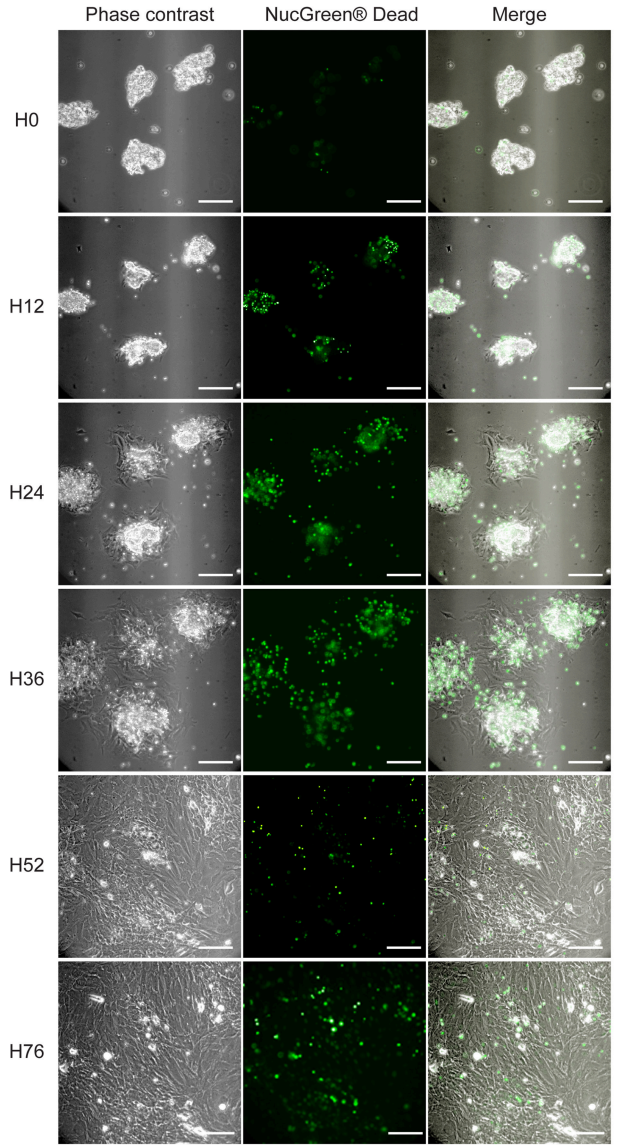

FIGURE 2 | PMF outgrowth in culture. PMF culture in complete medium (i.e., supplemented with 10% FBS), containing NucGreen® Dead reagent to monitor cell death, was observed by **Video-Microscopy** (Supplementary Video). Phase-contrast, fluorescent and merge images at different time points are shown. H, hours; Scale bars, 50 μm.

The precipitates were collected by centrifugation, dissolved in 0.5 mol/L NaOH and dye concentration was estimated by spectrophotometry at 540 nm.

PMF Culture Characterization

In the culture model of PMFs presented here, myofibroblasts are obtained by outgrowth from bile duct preparations. These preparations form small cell clusters that are entirely free of HSC contamination, as ascertained by the absence of vitamin A autofluorescence (**Figure 1C**). They initially contain a majority of bile duct epithelial cells as shown by cytokeratin staining (**Figure 3Ba**), and trace amounts of vascular structures as shown by α-SMA staining detectable in approximately one bile duct segment out of five (data not shown). They are placed in culture conditions with FBS, which contains factors such as TGF-β, that provide a growth advantage to mesenchymal cells over epithelial cells, in primary culture. In these conditions, bile duct epithelial cells die and detach from the substrate, while cells with a spindle-shape morphology, outgrow from the cell clusters. The time course of this evolution in culture can be observed by **Video-Microscopy** (Supplementary Video). Morphology of the cells observed under video-microscopy at different time points of culture, is shown in **Figure 2**. The time course of gene expression assessed by RT-PCR in the cultured cells (**Figure 3A**),

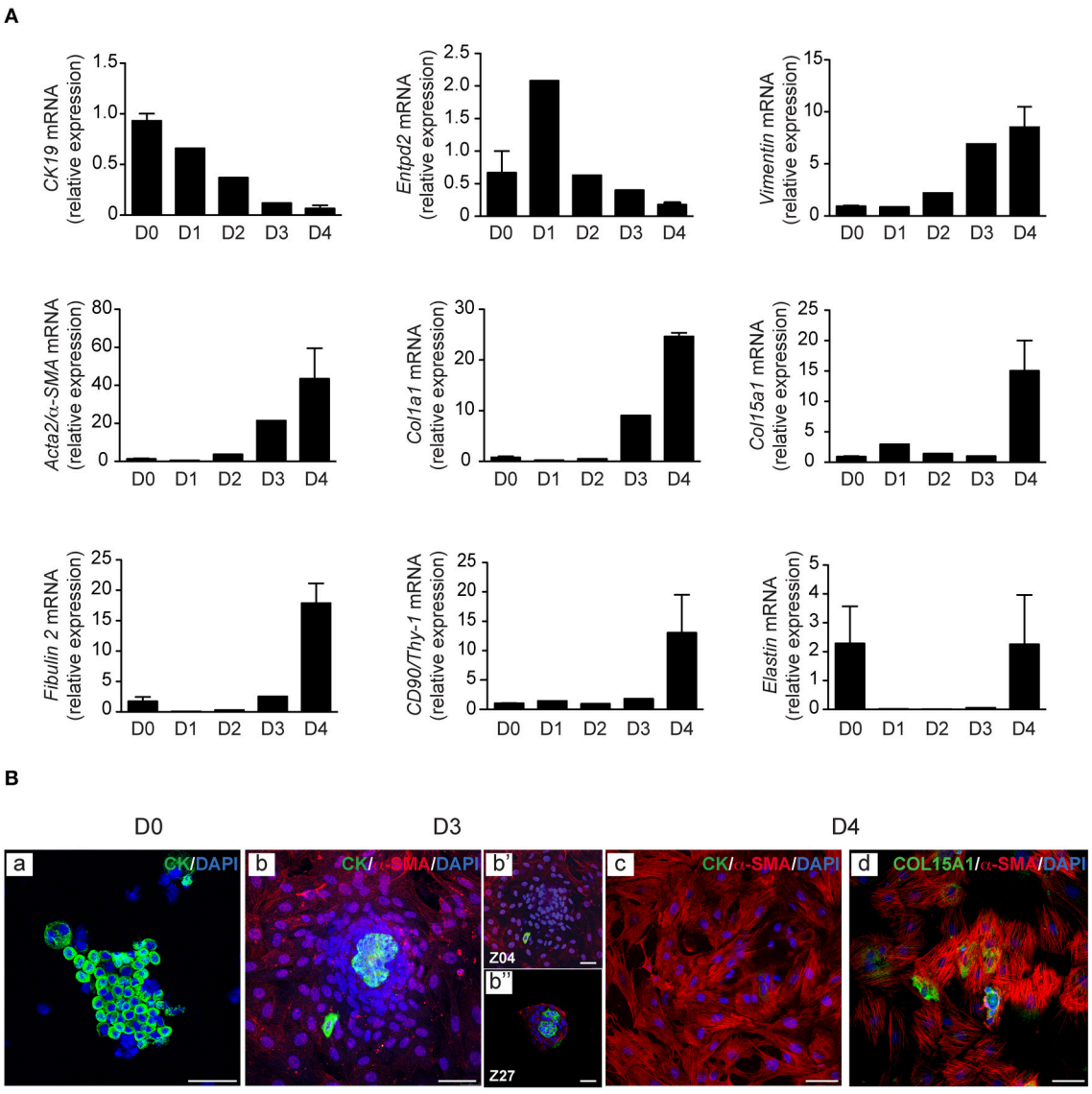

FIGURE 3 | Time course of cell markers in PMF culture. PMF culture was subjected at different time points, to **(A)** RT-PCR analysis of cytokeratin 19 (*CK19*), ecto-nucleoside triphosphate diphosphohydrolase 2 (*Entpd2*), *Vimentin*, *Acta2/α-SMA*, *Col1a1*, *Col15a1*, *Fibulin 2, and CD90/Thy-1*; mRNA levels are normalized for *Hprt* and reported relative to those measured immediately after bile duct isolation (D0). They represent the means ± standard error of 2–3 preparations. **(B)** Immunofluorescence of cytokeratin (CK), and of α-SMA co-labeled with CK or COL15A1; nuclei were stained with DAPI; z images at 5.7 (Z04) and 13.6 (Z27) μm from the substrate are shown. D, days; Scale bars, 50 μm.

shows that the expression of cytokeratin 19 (*CK19*) used as a marker of bile duct epithelial cells, progressively decreases over time, to become virtually absent at the stage of confluence, after 4 days in culture. The expression of *Entpd2*, previously shown to be a marker of portal fibroblasts and to undergo down-regulation during the myofibroblastic differentiation of these cells (Dranoff et al., 2002; Li et al., 2007; Wen et al., 2012), increases at day 1, when the fibroblastic cells emerge and decline thereafter. The expressions of *Vimentin*, a general marker of mesenchymal cells, and of *Acta2/α-SMA*, a marker of myofibroblasts, progressively increase after day 2. The expression of collagen, type I, alpha 1 (*Col1a1*) increases after day 3, whereas expressions of the PMF markers *Col15a1*, *Fibulin 2,* and *CD90/Thy-1*, are induced at the stage of confluence, after 4 days. We also found that the expression of elastin, a marker of portal fibroblasts, could be detected in bile duct isolates

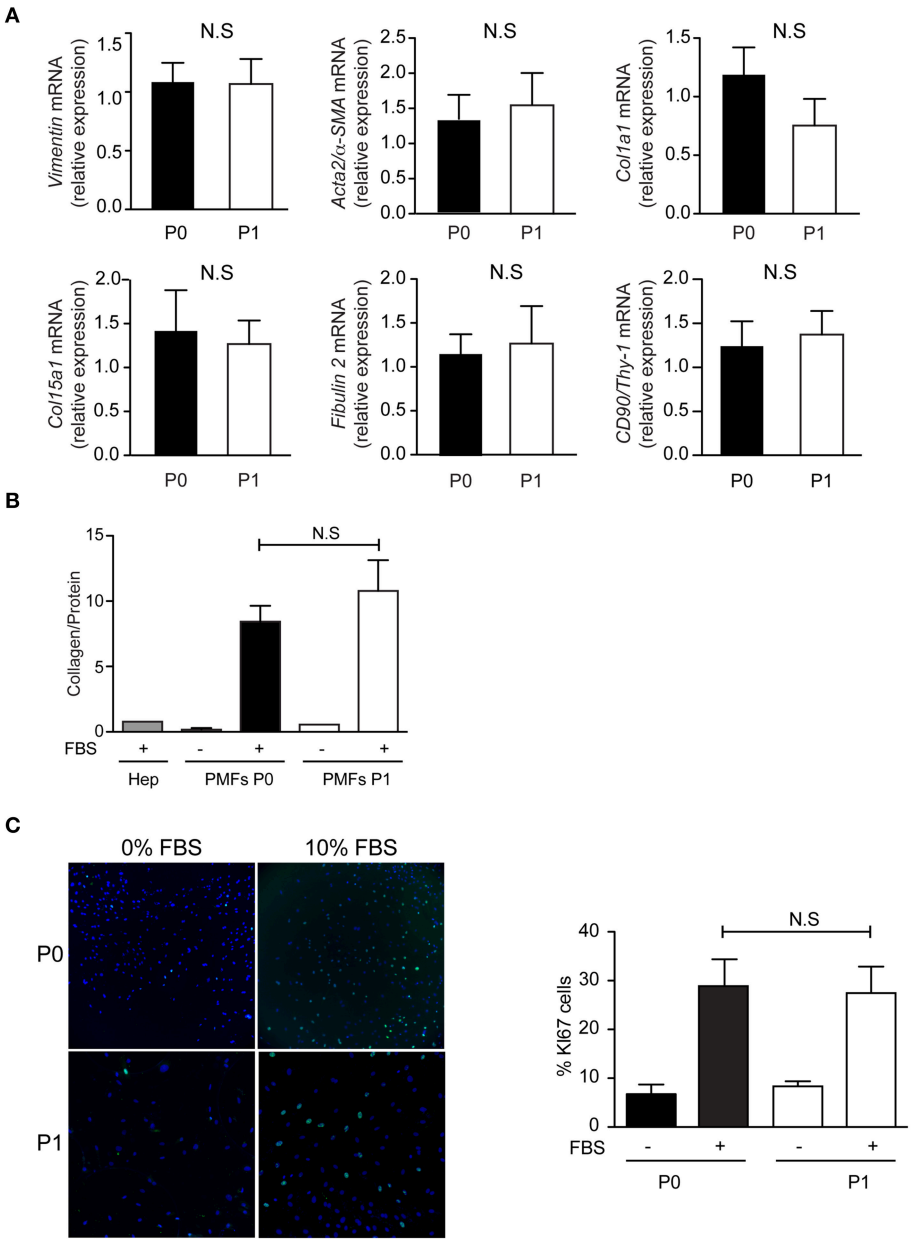

FIGURE 4 | Phenotype of PMFs in culture. Characterization of PMFs at confluence in primary culture (P0) and after one passage (P1), by **(A)** RT-PCR analysis of *Vimentin*, *Acta2/α-SMA*, *Col1a1*, *Col15a1*, *Fibulin 2, and CD90/Thy-1*; mRNA levels are normalized for *Hprt* and reported relative to those measured at P0. They represent the means ± standard error of 7 preparations. **(B)** Collagen secretion, measured in the conditioned media of PMFs ($n = 3$–9) or hepatocytes (Hep, $n = 1$), used as controls, incubated with or without 10% FBS for the last 24 h; Results are normalized for total protein amount and represent the means ± standard error. **(C)** Proliferation assay by Ki67 immunofluorescence, in sub-confluent PMFs incubated with or without 10% FBS for the last 24 h; results are expressed as a percentage of Ki67-positive cells and represent the means ± standard error of 5–6 preparations. NS, not significant.

and in confluent PMFs, but not during the period of cell expansion in culture. In two independent studies of Collagen-α1(I)-green fluorescent protein (*Col1a1*^{GFP}) mice with induced liver fibrosis, Fibulin 2, Thy-1 and elastin were overexpressed in cells considered as activated portal fibroblasts (Iwaisako et al., 2014; Lua et al., 2016). Consistent with RT-PCR analyses, dual immunolabeling shows that after 3 days, most of the cells are cytokeratin-negative (**Figure 3Bb**). Only a small number of cytokeratin-positive cells localized in the apex of the cell clusters are observed under confocal microscopy, whereas α-SMA is expressed in cells that adhere to the substrate (**Figure 3Bbb'b"**). Occasionally, a cytokeratin-positive cell adhering and migrating on the substrate, can be seen at the periphery of the cluster. However, we never detected co-expression of α-SMA and cytokeratin in the same cell, providing evidence against epithelial-mesenchymal transition in the emergence of PMF, although this mechanism could not be formally excluded. After 4 days, virtually all the cells are α-SMA-positive, whereas cytokeratin immunofluorescence is undetectable (**Figure 3Bc**). Co-expression of α-SMA and COL15A1 can be detected, although the intensity of COL15A1 immunofluorescence varies between individual cells (**Figure 3Bd**).

The comparison of confluent PMFs after 4–5 days, in primary culture (P0) and after one passage (P1), shows similar characteristics. The mRNA levels of *Vimentin*, *Acta2*/α-*SMA*, *Col1a1*, *Col15a1*, *Fibulin 2,* and *CD90/Thy-1,* are not significantly different between PMFs at P0 and P1 (**Figure 4A**). The mRNA levels of *Elastin* and *Entpd2* are not significantly different between P0 and P1 either (Data not shown), but too low to qualify them as PMF markers. Compared to serum-free conditions or primary rat hepatocytes, PMFs grown to confluence secrete collagen abundantly, without significant difference between P0 and P1 (**Figure 4B**). PMFs are also highly proliferative in the presence of serum, without significant difference between P0 and P1 (**Figure 4C**).

In conclusion, the protocol that we herein describe, provides a straightforward and reproducible method to obtain PMFs in culture. There is strong evidence to indicate that the progenitor cells giving rise to PMFs *in vivo*, are present in the initial bile duct isolates. These cells proliferate intensely, they acquire a fully differentiated myofibroblastic phenotype in primary culture and maintain this phenotype after one passage.

AUTHOR CONTRIBUTIONS

HE: acquisition, analysis and interpretation of data, drafting and revision of the manuscript; EL: acquisition, analysis and interpretation of data, drafting and revision of the manuscript; SL: acquisition, analysis and interpretation of data, revision of the manuscript; AC: design and supervision of the work, acquisition, analysis and interpretation of data, drafting and revision of the manuscript; CH: conception and design of the work, interpretation of data, writing.

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SUPPLEMENTARY MATERIAL

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Video-Microscopy | PMF outgrowth in culture. Bile duct isolates were plated in complete DMEM on u-Slide 2 Well Ph+ ibiTreat (Clinisciences), and placed at 37°C in a 5% CO_2 incubator. A time-lapse video was acquired using phase contrast and 488 nm channels with a camera associated to an Olympus IX83 microscope at x20 magnification. This movie stands for a 76-h experiment (assembly of images of the same PMF culture, acquired from H0 to H36 and from H52 to H76).

REFERENCES

Beaussier, M., Wendum, D., Schiffer, E., Dumont, S., Rey, C., Lienhart, A., et al. (2007). Prominent contribution of portal mesenchymal cells to liver fibrosis in ischemic and obstructive cholestatic injuries. *Lab. Invest.* 87, 292–303. doi: 10.1038/labinvest.3700513

Bosselut, N., Housset, C., Marcelo, P., Rey, C., Burmester, T., Vinh, J., et al. (2010). Distinct proteomic features of two fibrogenic liver cell populations: hepatic stellate cells and portal myofibroblasts. *Proteomics* 10, 1017–1028. doi: 10.1002/pmic.200900257

Cassiman, D., Libbrecht, L., Desmet, V., Denef, C., and Roskams, T. (2002). Hepatic stellate cell/myofibroblast subpopulations in fibrotic human and rat livers. *J. Hepatol.* 36, 200–209. doi: 10.1016/S0168-8278(01)00260-4

Dranoff, J. A., Kruglov, E. A., Robson, S. C., Braun, N., Zimmermann, H., and Sévigny, J. (2002). The ecto-nucleoside triphosphate diphosphohydrolase NTPDase2/CD39L1 is expressed in a novel functional compartment within the liver. *Hepatology* 36, 1135–1144. doi: 10.1053/jhep.2002.36823

Dudas, J., Mansuroglu, T., Batusic, D., and Ramadori, G. (2009). Thy-1 is expressed in myofibroblasts but not found in hepatic stellate cells following liver injury. *Histochem. Cell Biol.* 131, 115–127. doi: 10.1007/s00418-008-0503-y

Friedman, S. L. (2008). Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. *Physiol. Rev.* 88, 125–172. doi: 10.1152/physrev.00013.2007

Hinz, B., Phan, S. H., Thannickal, V. J., Prunotto, M., Desmouliere, A., Varga, J., et al. (2012). Recent developments in myofibroblast biology: paradigms for connective tissue remodeling. *Am. J. Pathol.* 180, 1340–1355. doi: 10.1016/j.ajpath.2012.02.004

Iwaisako, K., Jiang, C., Zhang, M., Cong, M., Moore-Morris, T. J., Park, T. J., et al. (2014). Origin of myofibroblasts in the fibrotic liver in mice. *Proc. Natl. Acad. Sci. U.S.A.* 111, E3297–E3305. doi: 10.1073/pnas.1400062111

Kinnman, N., Francoz, C., Barbu, V., Wendum, D., Rey, C., Hultcrantz, R., et al. (2003). The myofibroblastic conversion of peribiliary fibrogenic cells distinct from hepatic stellate cells is stimulated by platelet-derived growth factor during liver fibrogenesis. *Lab. Invest.* 83, 163–173. doi: 10.1097/01.LAB.0000054178.01162.E4

Knittel, T., Kobold, D., Saile, B., Grundmann, A., Neubauer, K., Piscaglia, F., et al. (1999). Rat liver myofibroblasts and hepatic stellate cells: different cell populations of the fibroblast lineage with fibrogenic potential. *Gastroenterology* 117, 1205–1221. doi: 10.1016/S0016-5085(99)70407-5

Kruglov, E. A., Jain, D., and Dranoff, J. A. (2002). Isolation of primary rat liver fibroblasts. *J. Investig. Med.* 50, 179–184. doi: 10.2310/6650.2002.33431

Lemoinne, S., Cadoret, A., El Mourabit, H., Thabut, D., and Housset, C. (2013). Origins and functions of liver myofibroblasts. *Biochim. Biophys. Acta* 1832, 948–954. doi: 10.1016/j.bbadis.2013.02.019

Lemoinne, S., Cadoret, A., Rautou, P. E., El Mourabit, H., Ratziu, V., Corpechot, C., et al. (2015). Portal myofibroblasts promote vascular remodeling underlying cirrhosis formation through the release of microparticles. *Hepatology* 61, 1041–1055. doi: 10.1002/hep.27318

Li, Z., Dranoff, J. A., Chan, E. P., Uemura, M., Sévigny, J., and Wells, R. G. (2007). Transforming growth factor-beta and substrate stiffness regulate portal fibroblast activation in culture. *Hepatology* 46, 1246–1256. doi: 10.1002/hep.21792

Lua, I., Li, Y., Zagory, J. A., Wang, K. S., French, S. W., Sévigny, J., et al. (2016). Characterization of hepatic stellate cells, portal fibroblasts, and mesothelial cells in normal and fibrotic livers. *J. Hepatol.* doi: 10.1016/j.jhep.2016.01.010. [Epub ahead of print].

Mederacke, I., Hsu, C. C., Troeger, J. S., Huebener, P., Mu, X., Dapito, D. H., et al. (2013). Fate tracing reveals hepatic stellate cells as dominant contributors to liver fibrosis independent of its aetiology. *Nat. Commun.* 4, 2823. doi: 10.1038/ncomms3823

Ogawa, T., Tateno, C., Asahina, K., Fujii, H., Kawada, N., Obara, M., et al. (2007). Identification of vitamin A-free cells in a stellate cell-enriched fraction of normal rat liver as myofibroblasts. *Histochem. Cell Biol.* 127, 161–174. doi: 10.1007/s00418-006-0237-7

Tuchweber, B., Desmoulière, A., Bochaton-Piallat, M. L., Rubbia-Brandt, L., and Gabbiani, G. (1996). Proliferation and phenotypic modulation of portal fibroblasts in the early stages of cholestatic fibrosis in the rat. *Lab. Invest.* 74, 265–278.

Uchio, K., Tuchweber, B., Manabe, N., Gabbiani, G., Rosenbaum, J., and Desmoulière, A. (2002). Cellular retinol-binding protein-1 expression and modulation during *in vivo* and *in vitro* myofibroblastic differentiation of rat hepatic stellate cells and portal fibroblasts. *Lab. Invest.* 82, 619–628. doi: 10.1038/labinvest.3780456

Wen, J. W., Olsen, A. L., Perepelyuk, M., and Wells, R. G. (2012). Isolation of rat portal fibroblasts by *in situ* liver perfusion. *J. Vis. Exp.* 3669. doi: 10.3791/3669

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REVIEW
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Cytoglobin as a Marker of Hepatic Stellate Cell-derived Myofibroblasts

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Myofibroblasts play important roles in inflammation, fibrosis and tumorigenesis in chronically inflamed liver. Liver myofibroblasts originate from hepatic stellate cells, portal fibroblasts or mesothelial cells, and they are localized in and around fibrotic septum and portal tracts. Liver myofibroblasts are the source of extracellular matrix materials, including type I collagen and multiple fibrogenic growth factors, such as transforming growth factor-β and vascular endothelial growth factor. Although a detailed characterization of the function of individual myofibroblasts has not been conducted, owing to the lack of appropriate cell markers, recent lineage-tracing technology has revealed the limited contribution of myofibroblasts that are derived from portal fibroblasts to various types of liver fibrosis, as compared with the contribution of hepatic stellate cells. In addition, cytoglobin, which is the fourth globin in mammals and function as a local gas sensor, provides a new perspective on the involvement of stellate cells in fibrosis and carcinogenesis, possibly through its anti-oxidative properties and is a promising new marker that discriminates between myofibroblasts derived from stellate cells and those from portal fibroblasts.

Keywords: stellate cell, cytoglobin, myofibroblast, liver fibrosis, hypoxia

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INTRODUCTION

The myofibroblast was originally described by Gabbiani in the granulation tissue of healing wounds as a cell type that had characteristics of fibroblasts and smooth muscle cells (Gabbiani et al., 1971).

In the liver, myofibroblasts were first identified in hepatic schistosomal fibrosis (Grimaud and Borojevic, 1977); this was followed by the identification of contractile fibroblasts (myofibroblasts) in chronic alcoholic cirrhosis (Rudolph et al., 1979).

Liver myofibroblasts are featured by the expression of α-smooth muscle actin (α-SMA), which is encoded by *ACTA2* gene located in 10q22-q24 in the human genome (Schmitt-Gräff et al., 1991). The origin of liver myofibroblasts is complex: at least three cellular sources—hepatic stellate cells (HSCs), portal fibroblasts (PFs), and mesothelial cells (MCs)—are considered to transdifferentiate to liver myofibroblasts (Yin et al., 2013). The contribution of each type of myofibroblast to the development or resolution of liver fibrosis varies depending on the etiology of liver disease.

Abbreviations: ABCRYS, α-B-crystallin; α-SMA, α-smooth muscle actin; BDNF, brain-derived nerve growth factor; Cygb, cytoglobin; ET-1, endothelin-1; ECM, extracellular matrix material; GFAP, glial fibrillar acidic protein; HSC, hepatic stellate cell; HIF, hypoxia inducible factor; LOX, lysyl oxidase; MMP, matrix metalloproteinase; MC mesothelial cell; NGF, neuronal growth factor; NT, neurotrophin; NO, nitric oxide; PDGF, platelet-derived growth factor; PF, portal fibroblast; TIMP, tissue inhibitor of matrix metalloproteinases; TGF-β, transforming growth factor β; TNF, tumor-necrosis factor; VEGF, vascular endothelial growth factor.

Conversely, cytoglobin (Cygb) belongs to the mammalian globin family, which consists of myoglobin, hemoglobin, neuroglobin, and androglobin (Burmester and Hankeln, 2014). Cygb was accidentally discovered in rat HSCs during the proteomics analysis of proteins that are expressed in HSCs. The amino acid sequence and gas-binding ability of Cygb are similar to those of myoglobin, thus suggesting that these two globins have the same primary function in pathophysiology (Kawada et al., 2001; Burmester et al., 2002; Trent and Hargrove, 2002). However, recent studies have suggested that Cygb is involved in the carcinogenesis of various organs, which is different from the function of myoglobin, a gas-binding sensor that affects muscle cell contraction (Shivapurkar et al., 2008; Shaw et al., 2009; Latina et al., 2015).

In this review, we will discuss the role of Cygb, which is uniquely expressed in HSCs in the liver, in the myofibroblastic transformation of HSCs and carcinogenesis in the liver.

LIVER MYOFIBROBLASTS

In 2002, Cassiman et al. proposed the presence of at least four different subpopulations of myofibroblasts in human liver. Portal myofibroblasts and septal myofibroblasts are located in the extended collagenous area around portal tracts and in the internal region of fibrotic septa, respectively. These myofibroblasts are strongly positive for α-SMA, and they exhibit a variable positivity to glial fibrillar acidic protein (GFAP), brain-derived nerve growth factor (BDNF) and α-B-crystallin (ABCRYS). The third type of myofibroblasts is interface myofibroblasts, which present at the edge between fibrotic septa and the surrounding hepatic parenchyma, which are positive for α-SMA, GFAP, and ABCRYS as well as BDNF, neuronal cell adhesion molecule (N-CAM), neuronal growth factor (NGF) and neurotrophin 4 (NT-4). The fourth type is activated HSCs that are located in or around sinusoidal capillary and within pseudo-lobules and express intensively α-SMA and N-CAM and are positive for GFAP, NGF, BDNF, NT-3, and synaptophysin as well as NT-4, ABCRYS, p75 and the NT receptors tyrosine kinase A and B. Cassiman et al. proposed that portal myofibroblasts, in addition to activated HSCs, may originate from PFs (Cassiman et al., 2002). Recent cell-tracing technology has clarified the origin of myofibroblasts in further detail, as discussed below.

Hepatic Stellate Cells

HSCs reside in Disse's space in the hepatic sinusoid, a space between sinusoidal endothelial cells and hepatocytes (Wake, 1980). HSCs constitute approximately 10% of total liver cells in the adult healthy liver. In healthy situation, quiescent HSCs principally store vitamin A in droplets in their cytoplasm. It is calculated that HSCs in the liver store 45–72% of vitamin A in the body. Quiescent HSCs are positive for neural crest markers like GFAP and NTs, nerve growth factor receptor (p75), and desmin. Quiescent HSCs are the source of extracellular matrix materials (ECMs) and the component of basement membrane-like structure, such as laminin, proteoglycan, and type IV collagen (Friedman, 2008; Yin et al., 2013; Pellicoro et al., 2014). HSCs also function as liver-specific pericytes surrounding sinusoidal endothelial cells, and thus their contractility is regulated by the exposure to endothelin-1 (ET-1), angiotensin-II, and their relaxation by nitric oxide (NO) control the diameter of sinusoids and regulates the hepatic microcirculation (Kawada et al., 1993).

Liver trauma caused by hepatitis virus B or C infection, alcohol abuse, drug toxicity, autoimmunity, or steatohepatitis, triggers the activation of HSCs and their transdifferentiation from quiescent phenotype to myofibroblast-like one (Novo et al., 2014). The myofibroblast-like HSCs contain less amount of vitamin A droplets, express increased α–SMA and growth factor receptors, possess augmented contractility, and generate multiple ECMs, including type I and III collagens (Friedman, 2008; Yin et al., 2013; Pellicoro et al., 2014). This activation process of HSCs is initiated by paracrine stimulation by neighboring and activated sinusoidal endothelial cells, Kupffer cells (liver macrophages), hepatocytes and cholangiocytes, as well as platelets. When activated, HSCs synthesize transforming growth factor β (TGF-β), which stimulates HSCs themselves as an autocrine loop. Hepatic sinusoidal endothelial cells take part in the activation of TGF-β from its latency-associated peptide-binding form by its proteolytic cleavage (Schuppan and Kim, 2013). Cholangiocytes and platelets are also an important origin of growth factors, including platelet-derived growth factor (PDGF), TGF-β, and epidermal growth factor. Activation of HSCs is additionally initiated by damaged hepatocytes; they secrete fibrogenic lipid peroxides, and their apoptosis mediated by Fas and tumor-necrosis factor (TNF)-related apoptosis-inducing ligand initiates the activation of HSCs via damage-associated molecular pattern molecules including high-mobility group box 1, RNA and DNA, S100 molecules, and purine metabolites. In culture system, it was also demonstrated that apoptotic fragments derived from damaged hepatocytes stimulate HSC activation, and that fibrogenic activity of myofibroblasts are augmented by the phagocytosis of apoptotic hepatocytes due to NADPH oxidase 2 and the janus kinase/signal transducer and activator of transcription and phosphoinositide 3-kinase/Akt pathways (Wree et al., 2014). Activated HSCs also produce an increased amount of matrix metalloproteinases (MMPs), especially MMP13, and their inhibitors, tissue inhibitor of matrix metalloproteinases (TIMPs).

Transcription factors including activated protein-1, Jun D, Sp1, Kruppel-like factor 6, and nuclear factor kappa B, whose functions are strictly regulated by intracellular signaling molecules, such as Smad, Ras, Raf-1, and mitogen-activated protein kinase, control the activation of HSC, leading to the transcriptional upregulation of latent TGF-β (Mann and Smart, 2002; Lopez-Sanchez et al., 2014). Epigenetic regulation, specifically DNA methylation and histone modification, is also important in the regulation of the activation of HSCs. For example, the abnormal DNA methylation of phosphatase and tension homolog (PTEN) and MeCP2 has been identified in activated HSCs (Lee et al., 2015). Among epigenetic signals, microRNAs also participate in the activation of HSCs. The microRNAs that have been implicated in HSC activation

include miR-199a,b, miR-221, miR-27, miR-21, miR-125, miR-195, miR-214, and miR-221/222 as profibrotic miRNAs, and miR-29, miR-15b, miR-200, miR-16, miR-133b, and miR-122 as anti-fibrotic miRNAs. Among these miRNAs, miR-29 has been the most intensively analyzed for its ability to suppress collagen 1A1 production in HSCs (Sekiya et al., 2011; Ogawa et al., 2012). In addittion, the augmented production of TIMPs impedes ECM degradation and triggers ECM accumulation in the damaged liver (Perugorria et al., 2013). It should be noted that leptin and other adipocytokines are involved in the process of HSC activation (Choi et al., 2010) and that activated HSCs are additionally characterized by augmented expression of receptors for PDGF, TGF-β, vascular endothelial growth factor (VEGF), angiotensin-II, and ET-1 (Novo et al., 2012). Through study of reporter Collagen α1(I)-GFP (Col-GFP) mice, GFP^+Vitamin-$A^+$$Desmin^+$ activated HSCs have been shown to comprise more than 92% of myofibroblasts in the fibrotic liver induced by CCl_4 intoxication (Scholten et al., 2010).

Hepatocellular carcinoma (HCC) is a consequence of chronic liver disease with many etiologies, and it develops after repetitive hepatocyte death and the development of fibrosis. In fact, it is thought that 80% of HCC develop in fibrotic and cirrhotic liver. Thus, fibrosis consisting of HSC-derived myofibroblasts may be involved in cancer development. Because activated HSCs are the source for growth factors and ECMs as mentioned above, they may contribute to the development of a microenvironment that is suitable for cancer growth. In fact, Coulouarn et al. demonstrated the importance of HSC-hepatocyte interaction due to the production of VEGFA and MMP-9 (Coulouarn et al., 2012). Very recently, Yoshimoto et al. elucidated that senescent HSCs activated by a bile acid derived from intestinal microbiota shows a senescent-associated secretary phenotype, in which a panel of cytokines, such as IL-1, IL-6, and GROα, are synthesized. This phenomenon is suggested in the central scenario for the development of liver cancer in obesity (Yoshimoto et al., 2013).

Portal Fibroblasts

PFs are the resident fibroblasts around the portal tract existing in the mesenchyme surrounding the bile ducts. PFs were first described more than 50 years ago in rat liver. It is considered that PFs in addition to HSC-derived myofibroblasts produce ECMs particularly in case of biliary fibrosis (Wells, 2014).Although it is considered that the septum tranversum-derived mesothelial cells are origin of PFs and HSCs, the definition of the roles of PFs in normal and injured liver has been unsatisfactory confirmed due to the lack of reliable markers to distinguish PFs from HSCs. PFs are generally positive for gremlin, Thy-1, fibulin 2, elastin, interleukin 6 (IL-6), cofilin, and the ectonucleotidase NTPDase 2. Lemoinne et al. demonstrated that COL15A1-positive portal and periportal cells give rise to portal myofibroblasts and release VEGFA-laden microparticles, thus promoting endothelial tubulogenesis (Lemoinne et al., 2015). Unlike HSCs, PFs lack desmin, cytoglobin, β2-macroglobulin, Hand2, GFAP, $p75^{NGFr}$, and Vitamin A. Using a mouse model carrying a bacterial artificial chromosome with a Cre reporter derived from the lecithin-retinol acyltransferase, Mederacke et al. recently showed that HSCs are the source of 82–96% of myofibroblasts in seven models of fibrosis in mice, although they could not eliminate the possibility that PFs are required for biliary fibrosis. Particularly, bridging fibrosis occurs from the portal vein area to the central vein area where PFs and desmin–negative cells are localized (Mederacke et al., 2013). Iwaisako et al. showed the important role of myofibroblasts derived from PFs during the initial stages of bile-duct ligation-induced liver fibrosis by contributing more than 70% of myofibroblasts, although HSC-derived myofibroblasts become dominant at the last phase (Iwaisako et al., 2014). In their study, Iwaisako et al. also identified mesothelin as a useful marker to distinguish PF-myofibroblasts from HSC-myofibroblasts.

PFs can be differentiated from HSCs-myofibroblasts by their production of elastin. As mentioned before, PFs were originally defined by their fibulin 2 expression (Knittel et al., 1999). Fibulin 2 is a linker protein that occupies the interface between microfibrils and their elastin core. Fibulin 2 mediates the progression of fibrosis by sequestering latent TGF-β binding proteins from TGF-β. In addition, PFs produce lysyl oxidase (LOX) and the related protein LOX-like 1, which cross-links elastin. Taken together, the function of PFs should be estimated regarding to the metabolism of elastin since elastin fibers are able to provide the bile ducts and the portal vasculature with mechanical stability (Perepelyuk et al., 2013).

The interaction of PFs with cholangiocytes is another important issue. Tanimizu et al. described that neurotrophin receptor p75-positive progenitors of PFs and cholangiocytes express laminin α1 and α5 subunits, respectively, which interact with the β1 integrin subunit to maintain the polarity and lumen of the bile ducts (Tanimizu et al., 2012). On the other hand, Jhandier et al. reported that PF-derived myofibroblasts do not express ectonucleotidase NTPDase2, which is equivalent to ecto-ATPase or CD39L1 and metabolizes extracellular nucleotides. This fact may explain how cholangiocyte proliferation is initiated by P2Y receptor activation in chronic cholangiopathies (Jhandier et al., 2005).

Mesodermal Mesenchymal Cells

Although myofibroblasts are able to be derived from epithelial cells like hepatocytes and cholangiocytes, during the epithelial-to-mesenchymal transition, recent genetic cell lineage tracing has clearly revealed that mesenchymal cells including HSCs, PFs, and vascular smooth muscle cells are derived from MCs in the mouse embryonic liver. In addition, in adult $Wt1^{CreERT2}$; $R26T/G^f$ mice after the induction of fibrosis by CCl_4 or bile duct ligation, MCs that are positive for glycoprotein M6a, podoplanin and CD200 acquire mesenchymal characteristics—expression of collagen 1α1 and vimentin—but the epithelial cell markers are decreased. This phenomenon of the conversion of liver MCs to mesenchymal cells is called the mesothelial-mesenchymal transition. The canonical TGF-β pathway plays a central role in MMT via SMAD3 (Li et al., 2013; Yin et al., 2013; Lua et al., 2014).

CYTOGLOBIN

Cygb belongs to the mammalian globin family including myoglobin in muscle cells, hemoglobin in red blood cells,

neuroglobin in the nervous system, and androglobin in testis. Cygb, originally named stellate cell activation-associated protein by our group and histoglobin by Trent and Hagrove, was discovered in 2001 from cultured rat HSCs by proteomics analysis, namely the separation of proteins expressed in HSCs by 2D SDS-PAGE, followed by the identification of individual proteins by time of flight mass spectrometry. The molecular weight of Cygb is 21,496 Da, and it is composed of 190 amino acids (Kawada et al., 2001; Trent and Hargrove, 2002; Burmester and Hankeln, 2014).

Cygb is uniquely expressed in HSCs, but not in hepatocytes, endothelial cells, or Kupffer cells, in the liver (**Figure 1**). Cygb is also expressed in extrahepatic organs, including the pancreas, spleen, kidney, digestive tract, and heart. Cygb-positive cells usually localize proximally to capillaries, and they are considered to be pericytes that store vitamin A, produce collagen and closely correlate with vascular endothelium (Nakatani et al., 2004). In addition, Cygb is expressed in chondrocytes, osteoblasts and osteocytes (Schmidt et al., 2004). Furthermore, a recent report has indicated that Cygb is expressed in melanocytes and several types of melanoma, and it is involved in the melanocyte-to-melanoma transition through hyper-methylation in the promoter region of the CYGB gene (Fujita et al., 2014).

The 3D structure of Cygb is almost identical to that of myoglobin, and it has a three-over-three α-helical sandwich structure consisting of eight helices, whereas Cygb is a bis-histidyl hexa-coordinate globin similar to neuroglobin and different from myoglobin and hemoglobin, which are penta-coordinate globins. However, Cygb can bind gas molecules including oxygen, nitric oxide, and carbon monoxide with kinetic properties similar to those of myoglobin. Thus, Cygb is thought to function as a local gas-sensor (Makino et al., 2006, 2011). A recent report has indicated the role of Cygb as a nitric oxide dioxygenase [$Cygb(Fe^{2+}) \rightarrow O_2 \rightarrow Cygb(Fe^{2+}-O_2) \rightarrow NO \rightarrow Cygb(Fe^{3+}) + NO_3^-$] resulting in the regulation of the local concentration of nitric oxide and hence vascular tonus (Liu et al., 2012).

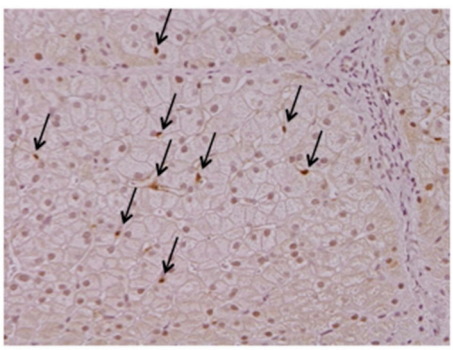

FIGURE 1 | Cytoglobin expression in intact human liver. Cytoglobin (arrows) is localized along hepatic sinusoid, namely these cells are identical to hepatic stellate cells, but not hepatocytes. Intact human liver tissue was obtained at Osaka City University Hospital under the patient's consent (Motoyama et al., 2014). Written informed consent was obtained from the patient. NK contributed to the evaluation of diagnosis of human liver histopathology.

Cygb gene expression is regulated by hypoxia inducible factor-1 (HIF-1), which binds to the hypoxia response element of the Cygb gene promoter. In fact, exposure of HeLa cells to 1% O_2 upregulates Cygb mRNA expression 1.7-fold (3 h) and 1.6-fold (6 h) in culture. HIF-1 and erythropoietin have 2 (-141 and -448) and 1 (-144) binding site, respectively, within the 5′-UTR lesion of the Cygb gene; these sites are important for Cygb gene transcription under hypoxia (Guo et al., 2007). The other transcription factors involved in Cygb gene expression include c-Ets-1, Sp1, nuclear factor of activated T cell and activator protein (Guo et al., 2006). In case of the liver, Cygb is induced during hypoxia (24 and 48 h at 7% O2) and reoxygenetion in mice (Fordel et al., 2007). Man et al. demonstrated that more than 3.5-fold upregulation of Cygb mRNA expression and the increase in the number of Cygb-expressing cells after the administration of CCl_4 in mice (Man et al., 2008). Gninsky et al. reported the induction of Cygb in thioacetamide-intoxicated liver fibrosis model in rats, which was clearly suppressed by the treatment with halofuginone, an inhibitor of TGF-β-dependent Smad3 phosphorylation (Gnainsky et al., 2007). In culture studies, Cui et al. demonstrated the induction of Cygb in primary-cultured rat HSCs by arundic acid, an inhibitor of S100b synthesis in astrocytes (Cui et al., 2012). Stone et al. recently clarified the regulation of Cygb expression in HTC-T6 cells in culture; Cygb expression is upregulated by laminin via integrin α1β4 signaling while it is downregulated by type I collagen by phosphorylation of focal-adhesion kinase via integrin α2β1 signaling (Stone et al., 2015).

Although Cygb's function as a fas-binding molecule is well-established, its pathophysiological role has not been fully elucidated. Cygb protects SH-SY5Y neuroblastoma cells from apoptosis under exposure to 300 μM H_2O_2 (Fordel et al., 2006). Li et al. demonstrated that siCygb treatment facilitates N2a neuronal cell death under exposure to 500 μM H_2O_2 for 4 h (Li et al., 2007). These and other studies have revealed that Cygb is a possible cytoprotector against oxidative stress.

Recently, the role of Cygb in human carcinogenesis in various organs has been paid special attention. The downregulation of Cygb gene expression has been demonstrated in human esophageal cancer, head and neck cancer, lung cancer, ovarian cancer and prostatic cancer via the hyper-methylation of the promoter lesion of the Cygb gene (Shivapurkar et al., 2008; Shaw et al., 2009; Latina et al., 2015). In detail, Oleksiewicz et al. showed (1) marked down-regulation of Cygb protein in human lung cancer cell line compared to normal human epithelial cell lines and possible involvement of promoter methylation and (2) significantly reduced Cygb mRNA expression in non-small cell lung cancer tissues compared with normal lung tissue (Mann-Whitney test, $P = 2.3 \times 10^{-7}$), suggesting the possible tumor suppressor function of Cygb (Oleksiewicz et al., 2013). Based on these reports, Hubers et al. studied Cygb gene methylation in spontaneous sputum in order to utilize it for lung cancer diagnosis. As a result, they reported the sensitivity of Cygb for

the diagnosis of lung cancer was 22% (Hubers et al., 2014). Except for the lung cancer, Wojnarowicz et al. demonstrated that Cygb gene expression was lowered in the majority of tumors with low malignant potential and cancer compared to benign tumors and normal ovarian surface epithelial cell samples (Wojnarowicz et al., 2012). Chen et al. also clarified the involvement of Cygb in ovarian cancer development at human tissue level and in cell culture models (Chen et al., 2014). Similar observations were additionally reported in human atrophy and adenocarcinoma of the prostate, suggesting that prostatic malignancy is accompanied with low level of Cygb, which play a role in protecting from oxidative damage (Mogal et al., 2012).

Using Cygb-knockout mice that were generated in our laboratory, we studied the role of Cygb in liver cancer development. First, mice were treated with 0.05 ppm diethylnitrosamine, an established carcinogen to the liver, for 36 weeks, thus resulting in the occurrence of liver tumors in 57.1% of the knockout mice compared with 0% of the wild-type mice. In this model, background liver tissues showed a marked development of liver fibrosis, augmented inflammatory reactions and overproduction of peroxynitrite ($ONOO^-$) in knockout mice (Thuy le et al., 2011). Second, mice were given a choline-deficient L-amino acid-defined diet for 32 weeks to induce steatohepatitis. Unexpectedly, 100% of Cygb knockout mice developed multiple liver tumors compared with 0% of the wild type mice. Again, background liver tissues showed marked development of liver fibrosis and augmented inflammatory reactions, which were accompanied by DNA double strand breaks (γH2AX expression) in hepatocytes. These results show the protective role of Cygb against oxidative stress and liver fibrosis development under chronic inflammation, suggesting Cygb's role in tissue carcinogenesis (Thuy le et al., 2015). In accordance with these observations, previous two reports demonstrated the anti-fibrotic function of Cygb. Xu et al. reported that Cygb overexpression in the liver by recombinant adeno-associated virus-2 encoding full-length rat Cygb suppressed liver injury and fibrosis induced by CCl_4 adminstration or bile-duct ligation in rats (Xu et al., 2006) and, furthermore, He at al. showed that administration of recombinant Cygb (10 mg/Kg) attenuates liver fibrosis in thioacetamide-induced liver fibrosis in rats (He et al., 2011), suggesting the hepato-protective role of Cygb.

CYTOGLOBIN AS A MARKER OF HEPATIC STELLATE CELL-DERIVED MYOFIBROBLASTS

As mentioned above, Cygb was initially discovered from rat HSCs in primary culture (Kawada et al., 2001). Cygb is expressed in mouse and human HSCs (**Figure 1**). Thus, whether Cygb can be utilized as a specific marker to distinguish myofibroblasts derived from HSCs from those derived from PFs is an interesting issue. Schmidt et al. first provided a detailed description of Cygb-expressing cells *in vivo* in mice, and they demonstrated that, in the liver, Cygb is expressed in HSCs and fibroblasts that surround the portal vein, but not in hepatocytes (Schmidt et al., 2004). However, in this study, fibroblasts around the portal vein were insufficiently characterized. Using fibulin 2 as a PF marker, Tateaki et al. demonstrated that there are several types of fibroblastic cells in the liver, namely fibulin 2^+/$Cygb^+$, fibulin 2^+/$Cygb^-$, fibulin 2^-/$Cygb^+$, fibulin 2^-/$Cygb^-$, and they concluded that fibulin 2^+/$Cygb^+$ and fibulin 2^-/$Cygb^+$ are identical to portal myofibroblasts and activated HSCs, respectively. A limitation of this study is that the identification of individual cell types was performed only by immunofluorescence staining (Tateaki et al., 2004). Ogawa et al. isolated the mouse liver non-parenchymal cell fraction by the Nycodenz density gradient method and further separated the cells in the fraction on the basis of vitamin A autofluorescence with FACS. UV^+ cells had minimum growth potential, expressed desmin and Cygb, but not αSMA and fibulin 2 at 1 day in culture, and later became positive for desmin, Cygb, and αSMA at 7 day in culture. Hence, UV^+ cells (vitamin A-storing cells) are identical to HSCs. Conversely, UV^- cells showed high proliferation activity and expressed αSMA, fibulin 2, and Gremlin, but not desmin and Cygb, thus indicating that UV^- cells are derived from portal fibroblasts (Ogawa et al., 2007). Bosselut at al. demonstrated that isolated rat portal myofibroblasts do not express Cygb, and thus are different from rat HSCs, as determined by 2-D MS/MS (Bosselut et al., 2010). Recent study by Fausther et al. described that rat portal fibroblasts and portal myofibroblast cell lines, RGF and RGF-N2, express Cygb at least in mRNA level in culture model (Fausther et al., 2015). In contrast, Motoyama et al. demonstrated that in human liver damaged by chronic

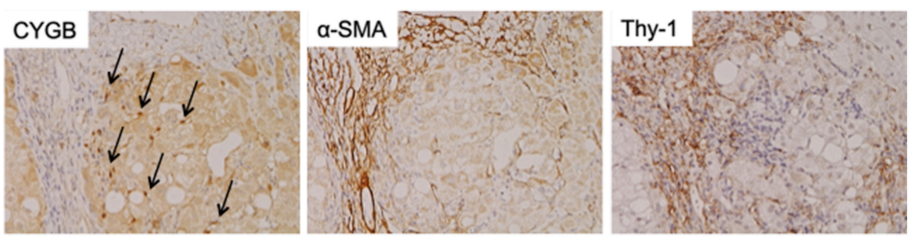

FIGURE 2 | Cytoglobin expression in fibrotic human liver. Cytoglobin (arrows) is localized along hepatic sinusoid, while α-SMA and Thy-1, myofibroblast markers, were strongly positive at fibrotic septum. Human liver tissue with fibrosis caused by chronic hepatitis C was obtained at Osaka City University Hospital under the patient's consent (Motoyama et al., 2014). Written informed consent was obtained from the patients. NK contributed to the evaluation of diagnosis of human liver histopathology.

hepatitis C, Cygb- and cellular retinol-binding protein-positive cells localize along hepatic sinusoids in liver parenchyma and at the margin of fibrotic septum, and they are not identical to αSMA, fibulin 2, and Thy-1-positive myofibroblasts (Motoyama et al., 2014; **Figure 2**). In summary, in mouse, rat, and human liver, Cygb is expressed in quiescent HSCs, and its expression level increases in activated HSCs that also express αSMA. In contrast, myofibroblasts derived from PFs are positive for fibulin 2 and αSMA, and they are negative for Cygb in human, while in rodent Cygb expression in PF-derived myofibroblasts needs to be characterized further by, for example, using genetic cell-labeling technology.

AUTHOR CONTRIBUTIONS

NK contributed to the evaluation of diagnosis of human liver histopathology.

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REFERENCES

Bosselut, N., Housset, C., Marcelo, P., Rey, C., Burmester, T., Vinh, J., et al. (2010). Distinct proteomic features of two fibrogenic liver cell populations: hepatic stellate cells and portal myofibroblasts. *Proteomics* 10, 1017–1028. doi: 10.1002/pmic.200900257

Burmester, T., Ebner, B., Weich, B., and Hankeln, T. (2002). Cytoglobin: a novel globin type ubiquitously expressed in vertebrate tissues. *Mol. Biol. Evol.* 19, 416–421. doi: 10.1093/oxfordjournals.molbev.a004096

Burmester, T., and Hankeln, T. (2014). Function and evolution of vertebrate globins. *Acta Physiol.* 211, 501–514. doi: 10.1111/apha.12312

Cassiman, D., Libbrecht, L., Desmet, V., Denef, C., and Roskams, T. (2002). Hepatic stellate cell/myofibroblast subpopulations in fibrotic human and rat livers. *J. Hepatol.* 36, 200–209. doi: 10.1016/S0168-8278(01)00260-4

Chen, H., Zhao, X., and Meng, T. (2014). Expression and biological role of cytoglobin in human ovarian cancer. *Tumour Biol.* 35, 6933–6939. doi: 10.1007/s13277-014-1941-x

Choi, S. S., Syn, W. K., Karaca, G. F., Omenetti, A., Moylan, C. A., Witek, R. P., et al. (2010). Leptin promotes the myofibroblastic phenotype in hepatic stellate cells by activating the hedgehog pathway. *J. Biol. Chem.* 285, 36551–35560. doi: 10.1074/jbc.M110.168542

Coulouarn, C., Corlu, A., Glaise, D., Guénon, I., Thorgeirsson, S. S., and Clément, B. (2012). Hepatocyte-stellate cell cross-talk in the liver engenders a permissive inflammatory microenvironment that drives progression in hepatocellular carcinoma. *Cancer Res.* 72, 2533–2542. doi: 10.1158/0008-5472.CAN-11-3317

Cui, W., Wang, M., Maegawa, H., Teranishi, Y., and Kawada, N. (2012). Inhibition of the activation of hepatic stellate cells by arundic acid via the induction of cytoglobin. *Biochem. Biophys. Res. Commun.* 425, 642–648. doi: 10.1016/j.bbrc.2012.07.126

Fausther, M., Goree, J. R., Lavoie, É. G., Graham, A. L., Sévigny, J., and Dranoff, J. A. (2015). Establishment and characterization of rat portal myofibroblast cell lines. *PLoS ONE* 10:e0121161. doi: 10.1371/journal.pone.0121161

Fordel, E., Thijs, L., Martinet, W., Lenjou, M., Laufs, T., Van Bockstaele, D., et al. (2006). Neuroglobin and cytoglobin overexpression protects human SH-SY5Y neuroblastoma cells against oxidative stress-induced cell death. *Neurosci. Lett.* 410, 146–151. doi: 10.1016/j.neulet.2006.09.027

Fordel, E., Thijs, L., Moens, L., and Dewilde, S. (2007). Neuroglobin and cytoglobin expression in mice. Evidence for a correlation with reactive oxygen species scavenging. *FEBS J.* 274, 1312–1317. doi: 10.1111/j.1742-4658.2007.05679.x

Friedman, S. L. (2008). Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. *Physiol. Rev.* 88, 125–172. doi: 10.1152/physrev.00013.2007

Fujita, Y., Koinuma, S., De Velasco, M. A., Bolz, J., Togashi, Y., Terashima, M., et al. (2014). Melanoma transition is frequently accompanied by a loss of cytoglobin expression in melanocytes: a novel expression site of cytoglobin. *PLoS ONE* 9:e94772. doi: 10.1371/journal.pone.0094772

Gabbiani, G., Ryan, G. B., and Majno, G. (1971). Presence of modified fibroblasts in granulation tissue and their possible role in wound contraction. *Experientia* 27, 549–550. doi: 10.1007/BF02147594

Gnainsky, Y., Kushnirsky, Z., Bilu, G., Hagai, Y., Genina, O., Volpin, H., et al. (2007). Gene expression during chemically induced liver fibrosis: effect of halofuginone on TGF-beta signaling. *Cell Tissue Res.* 328, 153–166. doi: 10.1007/s00441-006-0330-1

Grimaud, J. A., and Borojevic, R. (1977). Myofibroblasts in hepatic schistosomal fibrosis. *Experientia* 33, 890–892. doi: 10.1007/BF01951264

Guo, X., Philipsen, S., and Tan-Un, K. C. (2007). Study of the hypoxia-dependent regulation of human CYGB gene. *Biochem. Biophys. Res. Commun.* 364, 145–150. doi: 10.1016/j.bbrc.2007.09.108

Guo, X., Philipsen, S., and Tan-Un, K. C. (2006). Characterization of human cytoglobin gene promoter region. *Biochim. Biophys. Acta* 1759, 208–215. doi: 10.1016/j.bbaexp.2006.04.002

He, X., Lv, R., Wang, K., Huang, X., Wu, W., Yin, L., et al. (2011). Cytoglobin exhibits anti-fibrosis activity on liver *in vivo* and *in vitro*. *Protein J.* 30, 437–446. doi: 10.1007/s10930-011-9340-2

Hubers, A. J., van der Drift, M. A., Prinsen, C. F., Witte, B. I., Wang, Y., Shivapurkar, N., et al. (2014). Methylation analysis in spontaneous sputum for lung cancer diagnosis. *Lung Cancer* 84, 127–133. doi: 10.1016/j.lungcan.2014.01.019

Iwaisako, K., Jiang, C., Zhang, M., Cong, M., Moore-Morris, T. J., Park, T. J., et al. (2014). Origin of myofibroblasts in the fibrotic liver in mice. *Proc. Natl. Acad. Sci. U.S.A.* 111, E3297–E3305. doi: 10.1073/pnas.1400062111

Jhandier, M. N., Kruglov, E. A., Lavoie, E. G., Sévigny, J., and Dranoff, J. A. (2005). Portal fibroblasts regulate the proliferation of bile duct epithelia via expression of NTPDase2. *J. Biol. Chem.* 280, 22986–22992. doi: 10.1074/jbc.M412371200

Kawada, N., Kristensen, D. B., Asahina, K., Nakatani, K., Minamiyama, Y., Seki, S., et al. (2001). Characterization of a stellate cell activation-associated protein (STAP) with peroxidase activity found in rat hepatic stellate cells. *J. Biol. Chem.* 276, 25318–25323. doi: 10.1074/jbc.M102630200

Kawada, N., Tran-Thi, T. A., Klein, H., and Decker, K. (1993). The contraction of hepatic stellate (Ito) cells stimulated with vasoactive substances. Possible involvement of endothelin 1 and nitric oxide in the regulation of the sinusoidal tonus. *Eur. J. Biochem.* 213, 815–823.

Knittel, T., Kobold, D., Saile, B., Grundmann, A., Neubauer, K., Piscaglia, F., et al. (1999). Rat liver myofibroblasts and hepatic stellate cells: different cell populations of the fibroblast lineage with fibrogenic potential. *Gastroenterology* 117, 1205–1221. doi: 10.1016/S0016-5085(99)70407-5

Latina, A., Viticchiè, G., Lena, A. M., Piro, M. C., Annicchiarico-Petruzzelli, M., Melino, G., et al. (2015). ΔNp63 targets cytoglobin to inhibit oxidative stress-induced apoptosis in keratinocytes and lung cancer. *Oncogene*. doi: 10.1038/onc.2015.222. [Epub ahead of print].

Lee, Y. A., Wallace, M. C., and Friedman, S. L. (2015). Pathobiology of liver fibrosis: a translational success story. *Gut* 64, 830–841. doi: 10.1136/gutjnl-2014-306842

Lemoinne, S., Cadoret, A., Rautou, P. E., El Mourabit, H., Ratziu, V., Corpechot, C., et al. (2015). Portal myofibroblasts promote vascular remodeling underlying cirrhosis formation through the release of microparticles. *Hepatology* 61, 1041–1055. doi: 10.1002/hep.27318

Li, D., Chen, X. Q., Li, W. J., Yang, Y. H., Wang, J. Z., and Yu, A. C. (2007). Cytoglobin up-regulated by hydrogen peroxide plays a protective role in

oxidative stress. *Neurochem. Res.* 32, 1375–1380. doi: 10.1007/s11064-007-9317-x

Li, Y., Wang, J., and Asahina, K. (2013). Mesothelial cells give rise to hepatic stellate cells and myofibroblasts via mesothelial-mesenchymal transition in liver injury. *Proc. Natl. Acad. Sci. U.S.A.* 110, 2324–2329. doi: 10.1073/pnas.1214136110

Liu, X., Follmer, D., Zweier, J. R., Huang, X., Hemann, C., Liu, K., et al. (2012). Characterization of the function of cytoglobin as an oxygen-dependent regulator of nitric oxide concentration. *Biochemistry* 51, 5072–5082. doi: 10.1021/bi300291h

Lopez-Sanchez, I., Dunkel, Y., Roh, Y. S., Mittal, Y., De Minicis, S., Muranyi, A., et al. (2014). GIV/Girdin is a central hub for profibrogenic signalling networks during liver fibrosis. *Nat. Commun.* 5, 4451. doi: 10.1038/ncomms5451

Lua, I., James, D., Wang, J., Wang, K. S., and Asahina, K. (2014). Mesodermal mesenchymal cells give rise to myofibroblasts, but not epithelial cells, in mouse liver injury. *Hepatology* 60, 311–322. doi: 10.1002/hep.27035

Makino, M., Sawai, H., Shiro, Y., and Sugimoto, H. (2011). Crystal structure of the carbon monoxide complex of human cytoglobin. *Proteins* 79, 1143–1153. doi: 10.1002/prot.22950

Makino, M., Sugimoto, H., Sawai, H., Kawada, N., Yoshizato, K., and Shiro, Y. (2006). High-resolution structure of human cytoglobin: identification of extra N- and C-termini and a new dimerization mode. *Acta Crystallogr. D Biol. Crystallogr.* 62, 671–677. doi: 10.1107/S0907444906013813

Man, K. N., Philipsen, S., and Tan-Un, K. C. (2008). Localization and expression pattern of cytoglobin in carbon tetrachloride-induced liver fibrosis. *Toxicol. Lett.* 183, 36–44. doi: 10.1016/j.toxlet.2008.09.015

Mann, D. A., and Smart, D. E. (2002). Transcriptional regulation of hepatic stellate cell activation. *Gut* 50, 891–896. doi: 10.1136/gut.50.6.891

Mederacke, I., Hsu, C. C., Troeger, J. S., Huebener, P., Mu, X., Dapito, D. H., et al. (2013). Fate tracing reveals hepatic stellate cells as dominant contributors to liver fibrosis independent of its aetiology. *Nat. Commun.* 4, 2823. doi: 10.1038/ncomms3823

Mogal, A. P., Watson, M. A., Ozsolak, F., Salavaggione, L., and Humphrey, P. A. (2012). Gene expression profiles and differential cytoglobin expression in atrophy and adenocarcinoma of the prostate. *Prostate* 72, 931–937. doi: 10.1002/pros.21494

Motoyama, H., Komiya, T., Thuy le, T. T., Tamori, A., Enomoto, M., Morikawa, H., et al. (2014). Cytoglobin is expressed in hepatic stellate cells, but not in myofibroblasts, in normal and fibrotic human liver. *Lab. Invest.* 94, 192–207. doi: 10.1038/labinvest.2013.135

Nakatani, K., Okuyama, H., Shimahara, Y., Saeki, S., Kim, D. H., Nakajima, Y., et al. (2004). Cytoglobin/STAP, its unique localization in splanchnic fibroblast-like cells and function in organ fibrogenesis. *Lab. Invest.* 84, 91–101. doi: 10.1038/labinvest.3700013

Novo, E., Cannito, S., Paternostro, C., Bocca, C., Miglietta, A., and Parola, M. (2014). Cellular and molecular mechanisms in liver fibrogenesis. *Arch. Biochem. Biophys.* 548, 20–37. doi: 10.1016/j.abb.2014.02.015

Novo, E., Povero, D., Busletta, C., Paternostro, C., di Bonzo, L. V., Cannito, S., et al. (2012). The biphasic nature of hypoxia-induced directional migration of activated human hepatic stellate cells. *J. Pathol.* 226, 588–597. doi: 10.1002/path.3005

Ogawa, T., Enomoto, M., Fujii, H., Sekiya, Y., Yoshizato, K., Ikeda, K., et al. (2012). MicroRNA-221/222 upregulation indicates the activation of stellate cells and the progression of liver fibrosis. *Gut* 61, 1600–1609. doi: 10.1136/gutjnl-2011-300717

Ogawa, T., Tateno, C., Asahina, K., Fujii, H., Kawada, N., Obara, M., et al. (2007). Identification of vitamin A-free cells in a stellate cell-enriched fraction of normal rat liver as myofibroblasts. *Histochem. Cell Biol.* 127, 161–174. doi: 10.1007/s00418-006-0237-7

Oleksiewicz, U., Liloglou, T., Tasopoulou, K. M., Daskoulidou, N., Bryan, J., Gosney, J. R., et al. (2013). Cytoglobin has bimodal: tumour suppressor and oncogene functions in lung cancer cell lines. *Hum. Mol. Genet.* 22, 3207–3217. doi: 10.1093/hmg/ddt174

Pellicoro, A., Ramachandran, P., Iredale, J. P., and Fallowfield, J. A. (2014). Liver fibrosis and repair: immune regulation of wound healing in a solid organ. *Nat. Rev. Immunol.* 14, 181–194. doi: 10.1038/nri3623

Perepelyuk, M., Terajima, M., Wang, A. Y., Georges, P. C., Janmey, P. A., Yamauchi, M., et al. (2013). Hepatic stellate cells and portal fibroblasts are the major cellular sources of collagens and lysyl oxidases in normal liver and early after injury. *Am. J. Physiol. Gastrointest. Liver Physiol.* 304, G605–G614. doi: 10.1152/ajpgi.00222.2012

Perugorria, M. J., Murphy, L. B., Fullard, N., Chakraborty, J. B., Vyrla, D., Wilson, C. L., et al. (2013). Tumor progression locus 2/Cot is required for activation of extracellular regulated kinase in liver injury and toll-like receptor-induced TIMP-1 gene transcription in hepatic stellate cells in mice. *Hepatology* 57, 1238–1249. doi: 10.1002/hep.26108

Rudolph, R., McClure, W. J., and Woodward, M. (1979). Contractile fibroblasts in chronic alcoholic cirrhosis. *Gastroenterology* 76, 704–709.

Schmidt, M., Gerlach, F., Avivi, A., Laufs, T., Wystub, S., Simpson, J. C., et al. (2004). Cytoglobin is a respiratory protein in connective tissue and neurons, which is up-regulated by hypoxia. *J. Biol. Chem.* 279, 8063–8069. doi: 10.1074/jbc.M310540200

Schmitt-Gräff, A., Krüger, S., Bochard, F., Gabbiani, G., and Denk, H. (1991). Modulation of alpha smooth muscle actin and desmin expression in perisinusoidal cells of normal and diseased human livers. *Am. J. Pathol.* 138, 1233–1242.

Scholten, D., Osterreicher, C. H., Scholten, A., Iwaisako, K., Gu, G., Brenner, D. A., et al. (2010). Genetic labeling does not detect epithelial-to-mesenchymal transition of cholangiocytes in liver fibrosis in mice. *Gastroenterology* 139, 987–998. doi: 10.1053/j.gastro.2010.05.005

Schuppan, D., and Kim, Y. O. (2013). Evolving therapies for liver fibrosis. *J. Clin. Invest.* 123, 1887–1901. doi: 10.1172/JCI66028

Sekiya, Y., Ogawa, T., Yoshizato, K., Ikeda, K., and Kawada, N. (2011). Suppression of hepatic stellate cell activation by microRNA-29b. *Biochem. Biophys. Res. Commun.* 412, 74–79. doi: 10.1016/j.bbrc.2011.07.041

Shaw, R. J., Omar, M. M., Rokadiya, S., Kogera, F. A., Lowe, D., Hall, G. L., et al. (2009). Cytoglobin is upregulated by tumour hypoxia and silenced by promoter hypermethylation in head and neck cancer. *Br. J. Cancer* 101, 139–144. doi: 10.1038/sj.bjc.6605121

Shivapurkar, N., Stastny, V., Okumura, N., Girard, L., Xie, Y., Prinsen, C., et al. (2008). Cytoglobin, the newest member of the globin family, functions as a tumor suppressor gene. *Cancer Res.* 68, 7448–7456. doi: 10.1158/0008-5472.CAN-08-0565

Stone, L. C., Thorne, L. S., Weston, C. J., Graham, M., and Hodges, N. J. (2015). Cytoglobin expression in the hepatic stellate cell line HSC-T6 is regulated by extracellular matrix proteins dependent on FAK-signalling. *Fibrogenesis Tissue Repair* 8, 15. doi: 10.1186/s13069-015-0032-y

Tanimizu, N., Kikkawa, Y., Mitaka, T., and Miyajima, A. (2012). α1- and α5-containing laminins regulate the development of bile ducts via β1 integrin signals. *J. Biol. Chem.* 287, 28586–28597. doi: 10.1074/jbc.M112.350488

Tateaki, Y., Ogawa, T., Kawada, N., Kohashi, T., Arihiro, K., Tateno, C., et al. (2004). Typing of hepatic nonparenchymal cells using fibulin-2 and cytoglobin/STAP as liver fibrogenesis-related markers. *Histochem. Cell Biol.* 122, 41–49. doi: 10.1007/s00418-004-0666-0

Thuy le, T. T., Matsumoto, Y., Thuy, T. T., Hai, H., Suoh, M., Urahara, Y., et al. (2015). Cytoglobin deficiency promotes liver cancer development from hepatosteatosis through activation of the oxidative stress pathway. *Am. J. Pathol.* 185, 1045–1060. doi: 10.1016/j.ajpath.2014.12.017

Thuy le, T. T., Morita, T., Yoshida, K., Wakasa, K., Iizuka, M., Ogawa, T., et al. (2011). Promotion of liver and lung tumorigenesis in DEN-treated cytoglobin-deficient mice. *Am. J. Pathol.* 179, 1050–1060. doi: 10.1016/j.ajpath.2011.05.006

Trent, J. T. III, and Hargrove, M. S. (2002). A ubiquitously expressed human hexacoordinate hemoglobin. *J. Biol. Chem.* 277, 19538–19545. doi: 10.1074/jbc.M201934200

Wake, K. (1980). Perisinusoidal stellate cells (fat-storing cells, interstitial cells, lipocytes), their related structure in and around the liver sinusoids, and vitamin A-storing cells in extrahepatic organs. *Int. Rev. Cytol.* 66, 303–353. doi: 10.1016/S0074-7696(08)61977-4

Wells, R. G. (2014). The portal fibroblast: not just a poor man's stellate cell. *Gastroenterology* 147, 41–47. doi: 10.1053/j.gastro.2014.05.001

Wojnarowicz, P. M., Provencher, D. M., Mes-Masson, A. M., and Tonin, P. N. (2012). Chromosome 17q25 genes, RHBDF2 and CYGB, in ovarian cancer. *Int. J. Oncol.* 40, 1865–1880. doi: 10.3892/ijo.2012.1371

Wree, A., Eguchi, A., McGeough, M. D., Pena, C. A., Johnson, C. D., Canbay, A., et al. (2014). NLRP3 inflammasome activation results in hepatocyte pyroptosis, liver inflammation, and fibrosis in mice. *Hepatology* 59, 898–910. doi: 10.1002/hep.26592

Xu, R., Harrison, P. M., Chen, M., Li, L., Tsui, T. Y., Fung, P. C., et al. (2006). Cytoglobin overexpression protects against damage-induced fibrosis. *Mol. Ther.* 13, 1093–1100. doi: 10.1016/j.ymthe.2005.11.027

Yin, C., Evason, K. J., Asahina, K., and Stainier, D. Y. (2013). Hepatic stellate cells in liver development, regeneration, and cancer. *J. Clin. Invest.* 123, 1902–1910. doi: 10.1172/JCI66369

Yoshimoto, S., Loo, T. M., Atarashi, K., Kanda, H., Sato, S., Oyadomari, S., et al. (2013). Obesity-induced gut microbial metabolite promotes liver cancer through senescence secretome. *Nature* 499, 97–101. doi: 10.1038/nature 12347

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REVIEW
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Evolving Insights on Metabolism, Autophagy, and Epigenetics in Liver Myofibroblasts

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Liver myofibroblasts (MFB) are crucial mediators of extracellular matrix (ECM) deposition in liver fibrosis. They arise mainly from hepatic stellate cells (HSCs) upon a process termed "activation." To a lesser extent, and depending on the cause of liver damage, portal fibroblasts, mesothelial cells, and fibrocytes may also contribute to the MFB population. Targeting MFB to reduce liver fibrosis is currently an area of intense research. Unfortunately, a clog in the wheel of antifibrotic therapies is the fact that although MFB are known to mediate scar formation, and participate in liver inflammatory response, many of their molecular portraits are currently unknown. In this review, we discuss recent understanding of MFB in health and diseases, focusing specifically on three evolving research fields: metabolism, autophagy, and epigenetics. We have emphasized on therapeutic prospects where applicable and mentioned techniques for use in MFB studies. Subsequently, we highlighted uncharted territories in MFB research to help direct future efforts aimed at bridging gaps in current knowledge.

Keywords: liver myofibroblasts, metabolism, autophagy, epigenetics, fibrosis, hepatic stellate cells

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INTRODUCTION

Long-term exposure of the liver to injurious xenobiotic insults is a major cause of liver fibrosis and its sequelae, notably cirrhosis, acute liver failure, and liver cancer. Fibrosis is characterized by the net accumulation of extracellular matrix (ECM) and scar formation. This process is driven by a heterogeneous population of liver myofibroblasts (MFB) that are recruited to and accumulate at the site of injury. Hepatic stellate cells (HSCs) are widely accepted as the major source of liver MFB. Studies have consistently shown that upon activation to MFB, HSCs play a crucial role in the development of liver fibrosis. The activation process is induced by various stimulatory factors,

Abbreviations: 3PO, Cell-permeable inhibitor of PFKFB3; ACC, Acyl CoA carboxylase; ACLY, ATP citrate lyase; ALD, Alcoholic liver disease; ADRP, Adipose differentiation-related protein; BDL, Bile duct ligation; CCl4, Carbon tetrachloride; CTGF, Connective tissue growth factor; DHA, Docosahexaenoic acid; ECM, Extracellular matrix; FA, Fatty acid; FASN, Fatty acid synthase complex; FDFT1, Farnesyl-diphosphate farnesyltransferase 1; GLUT1, Glucose transporter 1; GSK3β, Glycogen synthase kinase 3 beta; H3K9me2, Histone H3 dimethyl Lys9; HCC, Hepatocellular carcinoma; HDAC, Histone deacetylase; HK2, Hexokinase 2 (muscle isoform); HMGCR, 3-hydroxy-3-methylglutaryl-Coenzyme A reductase; HMGCS, 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1; HSCs, Hepatic stellate cells; IL, Interleukin; LDs, Lipid droplets; LX-2, immortalized human hepatic stellate cells; MCT4, Monocarboxylate transporter 4; MFB, Myofibroblasts; MMP, Matrix metalloproteinase; NASH, Non-alcoholic steatohepatitis; OA, Oleic acid; PA, Palmitic acid; PPARγ, Peroxisome proliferator-activated receptor gamma; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3; PKM2, Pyruvate kinase M2 (muscle isoform); PUFAs, Polyunsaturated fatty acids; ROS, Reactive oxygen species; TCA, Tricarboxylic acid; TGF-β, Transforming growth factor beta; VPA, Valproic acid; α-SMA, alpha smooth muscle actin.

including transforming growth factor beta (TGF-β) and inflammatory cytokines (Dooley et al., 2003; Gabbiani, 2003; Mederacke et al., 2013; Seki and Schwabe, 2015). Besides HSCs, other cell types such as portal fibroblasts, bone marrow-derived fibrocytes and mesothelial cells may also contribute to liver MFB in response to chronic injury (Iwaisako et al., 2014; Xu et al., 2015).

Regardless of their origin, MFB are highly contractile, proliferative, and produce ECM components such as collagen types I, III, and fibronectin (Bataller and Brenner, 2005). MFB mediate reconstruction of connective tissues upon injury (Gabbiani, 2003; Swiderska-Syn et al., 2014). For example, following partial hepatectomy, MFB not only accumulate at the site of injury to initiate liver regeneration, but also activate liver progenitor cells, and subsequently induce proliferation of hepatocytes and cholangiocytes via hedgehog signaling pathway (Swiderska-Syn et al., 2014). It is therefore conceivable that extremely sophisticated mechanisms are responsible for the timely activation, recruitment, homing, and perpetuation of MFB functions at injured sites. There is also evidence that activated HSCs may undergo a coordinated reversion to quiescence once "their job" is done (Pellicoro et al., 2014).

In the last decade, new understanding of cellular metabolism arose especially with regards to cancer cells. This followed consistent *in vitro* and *in vivo* experimental proofs that tumor cells reprogram their metabolism to ensure continual survival (Vander Heiden et al., 2009; Hanahan and Weinberg, 2011). However, quite contrary to prevailing views, metabolic alterations or reprogramming are not exclusive to cancer cells. In fact, many other cell types, including dendritic cells, macrophages, T-cells, myeloid derived suppressor cells, cortical astrocytes, microglia, and skeletal muscle cells may also undergo metabolic changes under a variety of initiating factors (Bentaib et al., 2014; Gimeno-Bayón et al., 2014; Kelly and O'Neill, 2015; Maekawa et al., 2015; Pallett et al., 2015; Ryall et al., 2015; Shi et al., 2015; Xu et al., 2015). Hence, after years of focus on cell signaling, it is time to refocus efforts on how metabolic perturbations might influence the activity of MFB, including any therapeutic prospects it holds.

Closely linked to metabolism is autophagy (Galluzzi et al., 2014; Filomeni et al., 2015), and in many contexts, both processes have the same goal—energy generation. In autophagy, cells "eat up" their cellular components to produce sufficient energy to meet other immediate needs; however, autophagy could also be a cell death process (Elmore, 2007; Green and Levine, 2014). Such a dynamic system could be pivotal in MFB homeostasis. Metabolic alterations and autophagic responses may have epigenetic twists, e.g., via the transcriptional switch of critical gene networks (Hanley et al., 2010). Thus, epigenetic processes could enhance or suppress gene functions as the need arises during HSC-MFB transdifferentiation.

In this review, we have highlighted current knowledge on metabolism, autophagy and epigenetics in liver MFB. We also briefly mention recent technical advances that could help unravel new insights on the three topics in discourse. Finally, we offer perspectives to stimulate further questions on the role of metabolism, autophagy, and epigenetics in liver MFB.

METABOLIC ALTERATIONS IN LIVER MYOFIBROBLASTS

There is a growing knowledge of metabolic alterations in various types of cells. Despite paucity of experimental evidences, it is plausible that metabolic alterations are critical in the transdifferentiation of HSCs to MFB. Key intermediary metabolic pathways previously implicated in malignant transformation and cell survival, may be intricately involved in the maintenance of membrane integrity, morphology, energy production, signaling among other functions in MFB. Thus, metabolism could control the balance between MFB and the reversal to quiescent HSCs (**Figure 1**).

Glycolysis

The role of glycolysis in MFB origin or function is currently understudied. HSCs gain a glycolytic phenotype upon activation (Chen et al., 2012). Specifically, several glycolytic targets including GLUT1, HK2, PKM2, and lactate transporter MCT4 were simultaneously upregulated with alpha smooth muscle actin (α-SMA) during culture activation of HSCs and in animal liver fibrosis models (Chen et al., 2012). The glycolytic feature was mediated via Hedgehog (Hh) signaling and strongly correlated with expression of hypoxia inducible factor 1α (HIF1α), a known transcriptional regulator of glycolytic genes (Chen et al., 2012). Mechanistically, damaged hepatocytes release Hedgehog ligands, which activate HSCs via Hh signaling mediator Smoothened (SMO), and HIF1α induction. Deletion of SMO in quiescent HSCs suppressed basal mRNA expression of *Glut1, Hk2, Pkm2*, and HIF1α, while the opposite effect was observed with SMO agonist (SAG). Hence, the authors confirmed a direct link between MFB glycolytic activity and progression of liver fibrosis since inhibition of Hh signaling, HIF1α, glycolysis, or lactate accumulation all converted MFB to quiescent HSCs. In addition, the number of glycolytic stromal cells, as determined by *PKM2* expression, also correlated with the severity of fibrosis in diseased livers of animals and patients (Chen et al., 2012). Consistent with the above findings, Hh signaling inhibitors with potent antifibrotic effects (i.e., cyclopamine and curcumin) were recently shown to decrease intracellular levels of adenosine triphosphate (ATP), lactate, and the expression of glycolytic targets HK, PFK2, and Glut4 in HSCs (Lian et al., 2015). These evidences support the role of glycolysis in HSC activation and highlight the possibilities of targeting this metabolic pathway toward ameliorating fibrosis. Obviously, more studies are required to investigate the direct effect of modulating glycolytic targets in HSCs. Specifically, findings from MFB of other cellular origin could be tested in liver MFB. For example, glycolytic alterations are observed during MFB differentiation in the lung and prostate. Lung MFB at their early activation stage have increased and sustained expression of glycolytic enzymes PFK1, HK2, and notably PFKFB3. Inhibition of PFKFB3 with 3PO suppressed fibroblast differentiation to MFB (Xie et al., 2015). In the prostate, however, TGF-β1-induced fibroblast-to-MFB transdifferentiation led to suppression of pyruvate kinase, PKM2 (Untergasser et al., 2005). In cancer-associated fibroblasts, the glycolytic product lactate is associated with increased

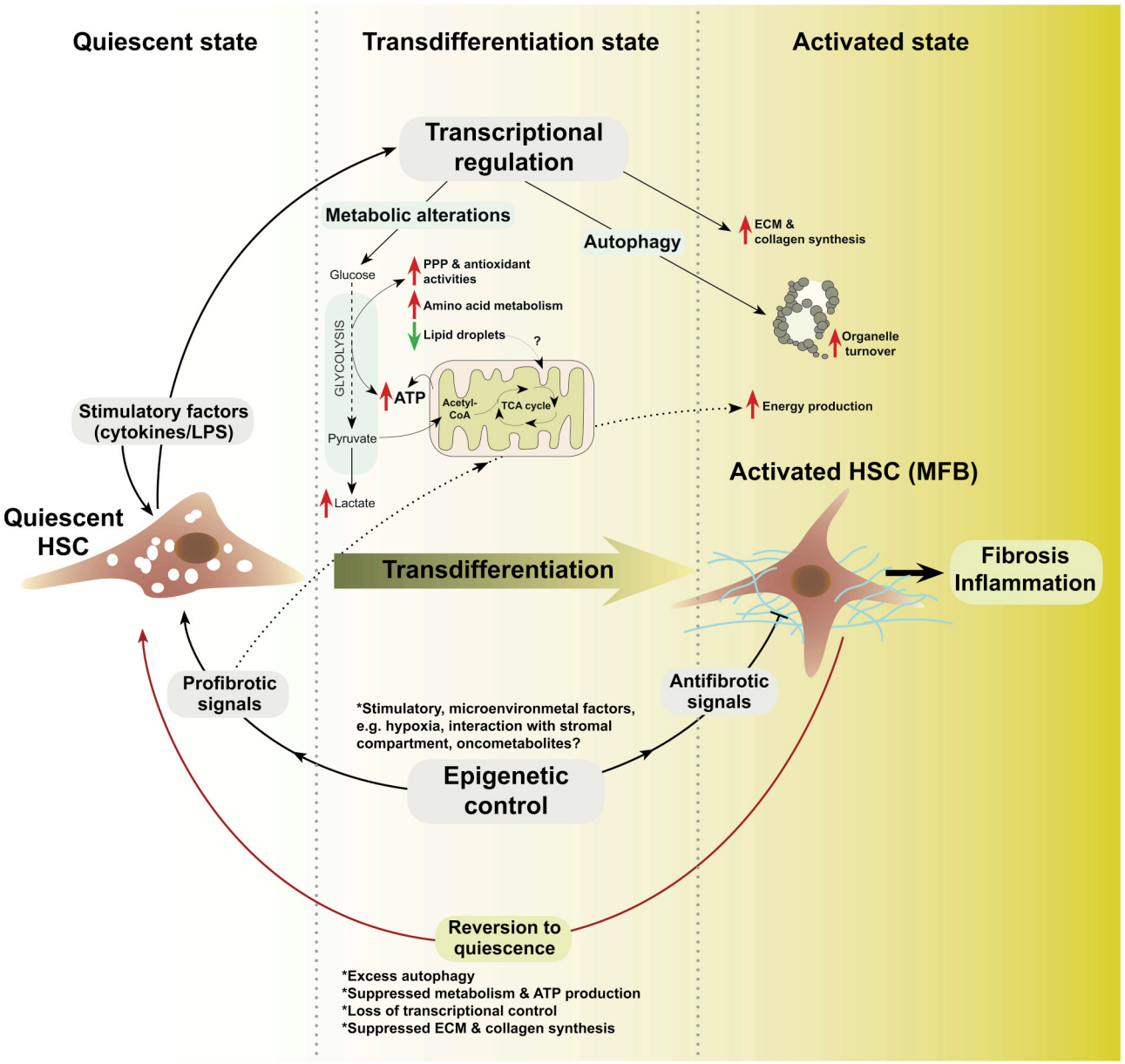

FIGURE 1 | A schematic model of metabolism, autophagy, and epigenetics in HSC-to-MFB transdifferentiation based on evolving research insights. In this model, quiescent HSC exposed to various stimuli transdifferentiate to activated HSC (MFB) and loose lipid droplets (LDs). Prior to or during transdifferentiation, transcription-level alterations modulate the expression of relevant metabolic, autophagy, and epigenetic mediators (?). Epigenetic modifiers (e.g., HDACs, DNMTs, MECP2, etc.) may determine, which transcriptional networks are switched on or off. In the transdifferentiated state, increased glycolysis, pentose phosphate pathway (PPP), and antioxidant system as well as loss of LDs could synergize to sustain energy production (Hernández-Gea et al., 2012) and provide the metabolite pool for extracellular matrix (ECM) and collagen synthesis (?). In the activated state, MFB may rely on nutrients from accelerated *de novo* metabolism, microenvironment, or autophagic breakdown of organelles to sustain their function in fibrosis and inflammation, e.g., cytokine production. Microenvironmental factors may signal the end of healing by (a) activating antifibrotic epigenetic modifiers, (b) attenuating transcriptional activators of metabolism and autophagy, or (c) by inducing self-destructive autophagy in MFB (?). LPS, lipopolysaccharides; ?, unknown mechanisms.

stemness, and constitutive TGF-β receptor activation led to metabolic reprogramming with increased lactate production (Martinez-Outschoorn et al., 2011; Guido et al., 2012). This suggests that lactate may be crucial in TGF-β-mediated HSC activation. In line, baboon liver MFB treated with lactate had an increased intracellular proline pool and upregulated collagen synthesis (Savolainen et al., 1984). Since MFB produce high amount of collagen (Bataller and Brenner, 2005), it will

be interesting to test whether glucose-derived lactate could modulate human or murine liver MFB, including analysis on the role of lactate dehydrogenases in this context. Together, the currently available information suggests that glycolysis is critical in MFB physiology and offers hints for further investigations.

Tricarboxylic Acid (TCA) Cycle

The role of TCA in HSC activation or MFB function is still unclear and the relevance of most TCA enzymes is yet to be delineated. It is also unclear if loss of lipid droplets (LDs), which occurs during HSC activation (Blaner et al., 2009; Kluwe et al., 2011), is aimed at supplying acetyl-CoA for TCA via β-oxidation. The sole evidence of TCA involvement in HSC activation stems from a recent study showing that succinate induces G protein-coupled receptor 91 (GPR91) to increase the production of TGF-β, collagen type I, and α-SMA (Li et al., 2015). This link between succinate and HSC activation suggests a likely relevance of TCA intermediates in metabolism and signaling regulation during HSC-MFB transdifferentiation. Noteworthy, succinate level is significantly increased in lung MFB and fibrotic lungs. Succinate accumulation enhanced TGF-β1-induced HIF-1α stabilization and MFB differentiation (Xie et al., 2015). Thus, it will be interesting to further investigate the effect of other TCA intermediates, including α-ketoglutarate that was recently shown to maintain pluripotency in embryonic stem cells via epigenetic control (Carey et al., 2015). Other interesting intermediates are fumarate and 2-hydroxyglutarate, which are called "oncometabolites" due to their oncogenic effect on rapidly proliferating cells (Xu et al., 2011; Sullivan et al., 2013; Nowicki and Gottlieb, 2015). Whether these "oncometabolites" exert profibrotic effects on MFB is yet to be explored. On a broader perspective, any abnormal accumulation of TCA intermediates may alter transcriptomic or signaling networks to initiate or sustain MFB phenotype.

Glutamine Metabolism

Glutamine is a very abundant amino acid and a highly energy-rich metabolite in humans. Research on mechanisms of cellular glutamine flux have rapidly evolved in recent years to elucidate its relevance in cell metabolism—e.g., in sustaining nucleotide biosynthesis, TCA, and lipogenesis (DeBerardinis et al., 2007; Metallo et al., 2012; Mullen et al., 2012; Son et al., 2013). In HSCs, the mechanism of glutamine utilization or its relevance to activation is currently unknown. Nevertheless, activated HSCs are long known to express high glutamine synthetase (GS; Bode et al., 1998). GS expression and glutamine metabolism have been severally linked to the Wnt/β-catenin signaling pathway (Cadoret et al., 2002; Austinat et al., 2008; Schmidt et al., 2011; Karner et al., 2015). Thus, the evidence that activation of the Wnt pathway elevates GS expression, while suppressing HSC activation marker α-SMA (Kordes et al., 2008) suggests that Wnt may control HSC fate by modulating glutamine metabolism. However, since the authors measured neither the intracellular nor extracellular glutamine level in their experiment, it is hard to discuss the effect of the elevated GS on glutamine metabolism. Therefore, further studies are needed to clarify the role of glutamine in activation and MFB bioenergetics, including any prospects of targeting glutamine utilization in MFB.

Fatty Acid Metabolism

Fatty acids (FA) are important in liver physiology, notably in the maintenance of membrane integrity, signaling, energy production, and regulation of inflammation in various cellular and tissue compartments (Freigang et al., 2013; Bazinet and Layé, 2014; Serhan, 2014). When deregulated, FA metabolism accounts for several liver diseases such as hepatic steatosis, steatohepatitis, and cirrhosis (Rinella, 2015). Specifically, polyunsaturated fatty acids (PUFAs) as substrates for the cyclooxygenase pathway vitally regulate initiation and resolution of inflammation (Alhouayek and Muccioli, 2014; Buckley et al., 2014), and have been linked to HSC activation. Rat HSCs at early activation stage replace retinyl esters with PUFAs in LDs (Testerink et al., 2012). However, the mechanism by which the incorporated PUFAs later contribute to HSC activation is not yet known, especially given that HSCs loose LDs during activation (Blaner et al., 2009).

Saturated FA such as oleic acid (OA) and palmitic acids (PA) also participate in MFB activity (Lee et al., 2010, 2014). Further, palmitate and retinol supplementation suppress activation of human immortalized LX-2 (Xu et al., 2005) and primary human HSCs (Lee et al., 2010). Lee et al. found that palmitate and retinol induced adipose differentiation-related protein (ADRP), which regulates the formation of LDs. Mechanistically, ADRP induction led to LDs formation, and the suppression of activation and fibrogenic targets including α-SMA, collagen, and MMP1 (Lee et al., 2010). The effect of OA and PA on increasing lipid storage was also corroborated by a recent study, in which these FA were reported to further synergize with natural compounds, such as rutin and curcumin, to increase LDs and suppress proliferation of HSCs (Lee et al., 2014). On the contrary, OA treatment induced TGF-β, which ostensibly promoted the MFB phenotype in mesangial cells by inducing the expression of collagen I, fibronectin,and α-SMA (Mishra and Simonson, 2008); whether OA similarly induces TGF-β in HSCs is yet unknown. Besides activating MFB, lipids may participate in MFB-mediated inflammatory functions. For instance, human liver MFB were found to trigger activation of monocytes by secreting prostaglandin-E2 (PGE2) *in vitro*. Accordingly, blocking PGE2 production with cyclooxygenase 2 inhibitor (NS-398) reduced the expression of the monocyte marker CD163 (Zhang et al., 2014).

Evidences also suggest that FA regulates MFB activation and function in other tissues. For instance, arachidonic acid and docosahexaenoic acid (DHA) reversed the MFB phenotype of valvular interstitial cells from porcine aortic valves by decreasing contractility and expression of α-SMA via a mechanism involving suppression of RhoA/G-actin/MRTF signaling (Witt et al., 2014). In the prostate, DHA also suppressed fibroblast to MFB differentiation. Specifically DHA prevented TGF-β-induced differentiation, α-SMA expression,

and migration of prostate associated fibroblasts (Bianchini et al., 2012). Furthermore, DHA suppressed matrix metalloproteinase 2 (MMP2) release and reversed the myofibroblast phenotype of prostate adenocarcinoma-associated fibroblasts (Bianchini et al., 2012). In other studies, dietary supplementation with fish oil blocked cardiac fibroblast activation and prevented cardiac fibrosis. Accordingly, eicosapentaenoic acid and DHA increased cyclic GMP levels, attenuated cardiac fibroblast transformation, proliferation, and collagen synthesis, and also blunted TGF-β1-induced phospho-Smad2/3 nuclear translocation through activation of cyclic GMP/protein kinase G pathway (Chen et al., 2011). Nitrated fatty acids (NFAs), formed when nitric oxide (NO) and NO-derived species react with unsaturated FA, are critical mediators of signaling and inflammation-related functions (reviewed by Trostchansky and Rubbo, 2008). NFAs upregulated PPARγ and blocked TGF-β signaling/activity in human lung fibroblasts (Reddy et al., 2014). *In vivo*, NFA treatment led to reduction of disease severity and reversal of existing MFB numbers and collagen deposition in a mouse model of pulmonary fibrosis (Reddy et al., 2014). Furthermore, resolvins, a family of lipid mediators derived from omega-3 PUFA, and known to have anti-inflammatory potency (Xu et al., 2010), inhibited interstitial fibrosis by blocking proliferation of resident fibroblasts (Qu et al., 2012). Despite these evidences that fatty acids influence the MFB phenotype, no study has directly interrogated the role of key enzymes in fatty acid metabolism, including FASN, ACLY, ACC in HSC activation. Hence, further studies will help to resolve the exact molecular regulation and relevance of fatty acid metabolism in HSC biology.

Cholesterol Metabolism

Recently, it was shown that cholesterol accumulation drives liver fibrosis (Tomita et al., 2014). According to the authors, increased cholesterol intake in a model of non-alcoholic steatohepatitis (NASH) led to free cholesterol accumulation in HSCs. Cholesterol accumulation consequently sensitized HSCs to TGF-β-induced activation by upregulating toll-like receptor 4 protein (TLR4), which suppressed TGF-β pseudoreceptor Bambi (Tomita et al., 2014). Similarly, in hypercholesterolemic mice with aortic valve disease, rapid normalization of cholesterol levels by genetic switching led to normalized superoxide levels, decreased myofibroblast activation, and a suppressed disease progression (Miller et al., 2009). The molecular mechanism by which cholesterol mediates activation and fibrosis is not fully understood. However, blocking cholesterol metabolism has offered prospects in ameliorating MFB-mediated fibrogenesis (**Table 1**).

Statins are known blockers of cholesterol metabolism and act by inhibiting 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR). HMGCR catalyzes the rate-limiting step in cholesterol metabolism (i.e., HMG-CoA → mevalonate), which commits acetyl-CoA to cholesterol production (Sirtori, 2014). Compelling evidences suggest that commonly used statins, such as simvastatin, pravastatin, fluvastatins, and atorvastatin are beneficial in targeting fibrogenesis. For instance, simvastatin suppressed HSC activation and liver fibrosis by increasing endothelial nitric oxide synthase expression, while suppressing the expression of inducible nitric oxide synthase (Wang et al., 2013)—a proinflammatory mediator (Brenner et al., 2013). Simvastatin also suppressed rat HSC proliferation and collagen I production, and reversed the morphology of activated HSCs toward quiescence (Rombouts et al., 2003). Further, pravastatin in combination with protein kinase c (PKC) inhibitor (enzastaurin) yielded a synergistic antifibrotic effect in *in vitro* and *in vivo* liver fibrosis models, notably by inducing cell apoptosis (Yang et al., 2010). Fluvastatin was recently shown to suppress palmitate-induced HSC activation *in vitro*. In addition, fluvastatin suppressed inflammation and oxidative stress to ameliorate steatosis-induced HSC activation and hepatic fibrogenesis in *in vivo* NASH model (Chong et al., 2015). Recently, Trebicka et al. (2010) investigated the antifibrotic effect of atorvastatin in rats after bile duct ligation (BDL). In their study, early (prophylactic) therapy with atorvastatin significantly reduced fibrosis and HSC activation. On the contrary, late atorvastatin therapy (against severe fibrosis) transduced HSCs into a more quiescent state, and led to suppression of MMP2 and profibrotic targets (e.g., TGF-β1, CTGF, and PDGFβ-R), but without affecting inflammation and fibrosis (Trebicka et al., 2010). In addition to suppressing activation, atorvastatin also induced senescence in MFB, both *in vitro* and *in vivo* as determined by p21 expression and β-galactosidase staining (Klein et al., 2012). Together, these evidences show that cholesterol-lowering agents have antifibrotic potency. It is noteworthy that the regulatory mechanisms linking statins to MFB deactivation are still vaguely defined. Studies suggest that besides inhibiting cholesterol metabolism, statins may suppress activation by attenuating membrane Ras and cytosolic RhoA levels (Rombouts et al., 2003; Porter et al., 2004). Statins may also mediate their antifibrotic effects by activating the transcription factor Kruppel-like factor 2 (KLF2; Marrone et al., 2013, 2015). In addition, the findings that atorvastatin exerted divergent effects depending on timing of therapy (Trebicka et al., 2010) suggest that the stage of fibrosis may determine the mechanism of action or effects of statins on MFB.

Besides inhibition of HMGCR, reduction of cholesterol levels by other mechanisms may also be of therapeutic benefits in limiting fibrogenesis. For instance, ezetimibe, which inhibits cholesterol absorption, was found to improve hepatic fibrosis in a controlled trial of 80 non-alcoholic fatty liver disease patients (Takeshita et al., 2014). Noteworthy, many targets in carnithine metabolism/transport and steroid biosynthesis, including *CPT1A, CPT1B, SQLE, SREBF, SC5DL,* and *HMGCS1* were deregulated in this patient cohort. However, despite genomic evidence of suppressed HSC to MFB transition, patients treated with ezetimibe had adverse effects, including increased long-chain fatty acid and glycated hemoglobin (HbA1c), which led to premature termination of the study (Takeshita et al., 2014). It remains to be elucidated how the cholesterol biosynthetic pathway modulates MFB features, and no study has yet reported the role of cholesterogenic targets like *HMGCS, HMGCR,* and *FDFT1* in HSC activation and MFB function.

TABLE 1 | Summary of selected research findings on liver myofibroblast metabolism, autophagy. and epigenetics with notes on evidence of therapeutic prospects.

Molecular process	Findings/Evidence	Models	[1]Evidence of therapeutic prospects with inhibitor (s)?	References
Glycolysis	• ↑Glycolytic phenotype and targets (GLUT1, HK2, PKM2) during HSC differentiation ↑Number of glycolytic stromal cells	1° mouse HSCs (*in vitro*) MCD (*in vivo*) BDL (*in vivo*)	Yes—with 2-deoxy glucose	Chen et al., 2012
	• Hedgehog inhibitors suppress activation and also lactate output and glycolytic targets (e.g., HK, PFK2, and Glut4)	1° rat HSCs (*in vitro)* CCl4-induced rat fibrosis liver (*in vivo)*	No	Lian et al., 2015
TCA/Glutamine metabolism	• Succinate treatment increases α-SMA via GPR91 activation	LX-2 and 1° mouse HSCs cultured in MCD (*in vitro*)	No	Li et al., 2015
	• Stimulation of β-catenin-dependent Wnt signaling prevents HSC activation	1° rat HSCs (*in vitro*)	Yes—with GSK3β inhibitor TWS119	Kordes et al., 2008
	• ↑Glutamine synthetase (GS) in activated HSCs GS as potential marker of HSC activation	1° rat HSCs (*in vitro*)	No	Bode et al., 1998
Fatty acid/Cholesterol metabolism	• HSCs replace retinyl esters with PUFAs in lipid droplets during activation process ↑Incorporation of exogenous arachidonic acid	1° rat HSCs (*in vitro*)	No	Testerink et al., 2012
	• Palmitate suppress activation by ↑ADRP	LX-2 and human 1° HSCs (*in vitro*)	No	Lee et al., 2010
	• ↑Accumulation of oleic and palmitic acids increases autophagy in activated HSCs	LX-2 (*in vitro*)	No	Lee et al., 2014
	• Simvastatin—↓HSC proliferation, ↓collagen I, revert HSCs to quiescence	1° rat HSCs (*in vitro*)	Yes—with Simvastatin	Rombouts et al., 2003
	• Fluvastatin—↓palmitate-induced HSC activation; alleviated steatosis-induced HSC activation; ↓hepatic fibrogenesis	Rat immortalized HSCs (T6 cells; *in vitro*) NASH (*in vivo*)	Yes—with Fluvastatin	Chong et al., 2015
	• Atorvastatin attenuates HSC activation and fibrosis	BDL mice (*in vivo*)	Yes—with Atorvastatin	Trebicka et al., 2010
	• Inflammatory and profibrotic function and effect of leptin was blocked by inhibition of NADPH oxidase	1° human and mouse HSCs (*in vitro*)	Yes—with NADPH oxidase inhibitor diphenylene-iodonium (DPI)	De Minicis et al., 2008
Autophagy	• Autophagy promotes fibrogenesis Blocking autophagy via Atg7 suppress liver fibrosis	1° mouse and human HSCs Mouse immortalized HSCs (JS1; *in vitro*) *Atg7 transgenic* mice (*in vivo*) CCl4 and TAA-treated mice (*in vivo*)	Yes—with 3-Methyladenine (3-MA)	Hernández-Gea et al., 2012
	• Inhibition of autophagy suppress HSC activation	1° mouse HSCs (*in vitro*)	Yes—with Bafilomycine A1	Thoen et al., 2011
Epigenetics	• JMJD1A—novel epigenetic regulator in HSCs ↓JMJD1A correlates with reinforced H3K9me2 in the PPARγ gene promoter, ↑α-SMA and collagen	CCl4-treated mice (*in vivo*)	No	Jiang et al., 2015
	• Epigenetic silencing of Smad7 enables TGF-β1-induced fibrosis via Smad2/3	Rat HSCs (*in vitro*)	Yes—with 5-aza-2'-deoxycytidine (5-azadC)	Bian et al., 2014
	• Ethanol induce multiple epigenetic regulators, including a histone 3 lysine 4 (H3K4) methyltransferase (MLL1) during HSC activation	1° rat HSCs (*in vitro*)	No	Page et al., 2015
	• VDR ligands inhibit TGF-β1-induced HSC activation by blocking recruitment of histone modifiers (CBP and p300) and histone H3 hyperacetylation in profibrotic genes	*LX-2 cells 1° rat and mouse HSCs $Vdr^{-/-}$ mice (*in vivo*) CCl4-treated mice (*in vivo*)	Yes—with Vit-D agonist Calcipotriol	Ding et al., 2013

(Continued)

TABLE 1 | Continued

Molecular process	Findings/Evidence	Models	[1]Evidence of therapeutic prospects with inhibitor (s)?	References
	• MRTF-A mediates fibrosis by recruiting histone methyltransferase complex to the promoters of fibrogenic genes to activate transcription	1° mouse HSCs (*in vitro*) HSC-T6 cells (*in vitro*) MRTF-A KO mice treated with CCl4 (*in vivo*)	No	Tian et al., 2015
	• HDAC inhibition blocks activation markers α-SMA, lysyl oxidase, collagens.	1° mouse HSCs (*in vitro*) **CCl4-treated mice (*in vivo*)	Yes—with HDAC class II inhibitor, MC1568	Mannaerts et al., 2013
	• HDAC inhibition blocks HSC proliferation, activation, and suppress liver fibrosis	1° mouse and human HSCs (*in vitro*) BDL rat (*in vivo*)	Yes—with HNHA	Park et al., 2014
	• HDAC inhibition blocks HSC activation and fibrosis	1° mouse HSCs (*in vitro*) CCl4-treated mice (*in vivo*)	Yes—with Valproate	Mannaerts et al., 2010

[1]*Refers only to part of the study showing inhibitors that directly target a gene or pathway in the respective molecular process; 1°, Primary; BDL, Bile duct ligation; CCl4, Carbon tetrachloride; MCD, Methionine choline-deficient; MRTF, Myocardin-related transcription factor; NASH, Non-alcoholic steatohepatitis; TAA, Thioacetamide ↑, increase or upregulation; KO, knockout; ↓, decrease or downregulation; HNHA, N-hydroxy-7-(2-naphthylthio)heptanomide; *The main model used to demonstrate the antagonistic role of VDR ligand on TGFβ1-induced activation; **In vivo data was inconclusive due to variabilities within samples (Mannaerts et al., 2013).*

Oxidative Stress and Anti-oxidant Defense System

Oxidative stress and the cellular anti-oxidant defense system are regulated in a coordinated fashion during inflammation. It is known that reactive oxygen species (ROS) such as hydrogen peroxide and superoxides are released and scavenged during hepatic wound healing (Prosser et al., 2006). This process must be coordinated by a plethora of tightly regulated mechanisms to ensure homeostatic balance. Some well-studied anti-oxidant mediators, such as NADPH Oxidase, galectin-3, glutathione, and superoxide dismutases are involved in MFB physiology and could be prospective targets in fibrotic therapy.

NADPH Oxidase 4 (NOX4) is a key mediator of the cellular antioxidant system, which is known to be upregulated in fibrosis and linked to TGF-β fibrotic action. Targeted inhibition of NOX4 suppresses HSC activation and also the initiation or progression of fibrogenesis in other organs including lung, breast, kidney, and heart (Cucoranu et al., 2005; Aoyama et al., 2012; Sancho et al., 2012; Chan et al., 2013; Hecker et al., 2014; Manickam et al., 2014; Sampson et al., 2014; Tobar et al., 2014; Lan et al., 2015). In line, pharmacological and genetic inhibition of NADPH oxidase in HSCs blocked inflammatory and profibrotic functions of leptin, e.g., enhanced HSC proliferation; up-regulation of fibrogenic markers, inflammatory mediators, and chemokine expression, thus supporting a role of NOX in mediating fibrogenesis via signaling control (De Minicis et al., 2008).

Galectin-3 is a pleiotropic β-galactoside-binding lectin. Galectin-3 associates with cell adhesion molecules to mediate its downstream functions such as cell apoptosis, adhesion, migration, angiogenesis, fibrosis, and inflammatory responses (Li et al., 2014). Galectin-3 is overexpressed upon injury and regulates hepatic progenitor cell expansion and HSC activation (Henderson et al., 2006; Hsieh et al., 2015). In addition, several studies show that galectin-3 expression has direct correlation with HSC phagocytosis, matrix production, and hepatic fibrosis (Maeda et al., 2003; Henderson et al., 2006; Jiang et al., 2012; Martínez-Martínez et al., 2014). Recently, galectin-3 was found to function as a scavenging receptor for advanced lipoxidation endproducts (ALEs) in the liver. Consequently, galectin-3 deficient mice fed with atherogenic diet present with less steatosis and reduced tissue uptake of ALEs (Iacobini et al., 2011).

Glutathione is another prominent player in the cellular antioxidant function (Lu, 2013; Espinosa-Diez et al., 2015). Increased glutathione suppresses HSC growth and activation (Fu et al., 2008). Accordingly, stimulation with TGF-β suppressed expression of glutamate-cysteine ligase (GCL), the rate-limiting enzyme in glutathione biosynthesis, leading to lower glutathione levels in cultured HSCs (Fu et al., 2008). This suggests that suppression of glutathione levels is a mechanism of TGF-β-induced fibrosis. Similarly, increased intracellular glutathione levels in lung fibroblast inhibit Smad3 phosphorylation to suppress TGF-β1-induced profibrotic effects, such as expression of CTGF, collagen I, fibronectin, and transformation to MFB (Ono et al., 2009).

Superoxide dismutase (SOD) is also suggested to have antifibrotic activity. A study with a skin fibrosis model indicates that exogenous Cu/Zn SOD exerts antifibrotic activity by suppressing MFB features, such as expression of α-SMA, TGF-β1, and ECM (Vozenin-Brotons et al., 2001). Manganese superoxide dismutase (MnSOD) is a downstream target of the AKT-dependent forkhead transcription factor FOXO1 (Adachi et al., 2007). MnSOD induction via active FOXO1 partly inhibits HSC proliferation and transdifferentiation by suppressing ROS production (Adachi et al., 2007), further showing that anti-oxidant mediators are crucial in HSC activation and MFB function.

In human alcoholic hepatitis, disturbance of the antioxidant system occurs in the background of advanced fibrosis (Colmenero et al., 2007). Those patients present with significant accumulation of fibrogenic MFB and overexpression of genes involved in oxidative stress, including NOX4, as well as dual oxidases 1 and 2. Oxidative stress is also associated with corneal

and alveolar MFB functions (Yang et al., 2013b; Vyas-Read et al., 2014). Furthermore, production of mitochondrial complex III ROS is essential for TGF-β-driven MFB differentiation and profibrotic gene expression in human lung fibroblasts (Jain et al., 2013). However, ROS scavengers trigger TGF-β1-mediated differentiation of human subcutaneous fibroblasts into MFB (Cat et al., 2006; Popova et al., 2010). Taken together, oxidative stress and anti-oxidant mediators are pivotal in activation and MFB function. Therefore, metabolic processes that generate or remove ROS, e.g., oxidative phosphorylation, pentose pathways, and glutathione metabolism, may critically participate in liver MFB activities and so represent yet untapped areas in the search for antifibrotic therapies.

AUTOPHAGY—A PROSPECTIVE FACET IN LIVER MYOFIBROBLASTS PATHOPHYSIOLOGY

Autophagy, literarily meaning "self-eating," is a rapidly emerging facet in cellular bioenergetics. It defines a process whereby cells eat up their cytoplasmic components in order to generate metabolites for energy sustainability (Green and Levine, 2014; Hurley and Schulman, 2014). In normal and disease states, autophagy has critical survival, protective, and immune modulatory functions—the latter including suppression of proinflammatory cytokines (Levine et al., 2011; Choi et al., 2013). There are several known markers of autophagy, including the most studied ATG8/LC3, and SQSTM1/p62, ATG1/ULK1, ATG9, and BECN1/ATG6 (Klionsky et al., 2012). However, little and conflicting information currently exist on the role of autophagy in HSCs, MFB, and fibrosis as discussed below.

Autophagy is Profibrotic

Recent findings implicate autophagy as promoter of liver fibrosis (Mallat et al., 2014; Lee et al., 2015). Increased expression of autophagy markers positively correlates with ductular reaction (Hung et al., 2015), a process that goes hand in hand with HSC activation in a subset of liver diseases, and thus may directly participate in tissue repair and hepatic fibrogenesis (Williams et al., 2014). Further, autophagy markers, notably microtubule-associated protein 1 light chain 3B (LC3B), ATG12-5, and ATG7 were significantly upregulated in the livers of cirrhotic patients and in 2-acetylaminofluorene (AAF)/CCl_4-induced liver fibrosis in rat (Hung et al., 2015). With LC3B as marker, the authors showed that autophagy correlated with severity of fibrosis and was consistently increased in cirrhosis regardless of varying etiologies. However, while the autophagy markers correlated with protein expression of α-SMA and bile duct proliferation marker CK19, a direct overlap between MFB and autophagy was not apparent in this study, as immunofluorescent staining showed no co-localization of LC3B with α-SMA in α-SMA+ MFB (Hung et al., 2015). Considering that lipids are among its metabolic triggers (Galluzzi et al., 2014), autophagy may induce HSC activation by crosstalk with lipid molecules. Indeed, autophagy facilitates loss of LDs and concomitantly promotes the supply of free fatty acids as energy-building substrates during HSC activation (Hernández-Gea et al., 2012). Consequently, inhibition of autophagy with 3-Methyladenine (3-MA) decreased ATP levels in HSCs. Furthermore, blocking autophagy by interfering with its Atg7 attenuated CCl_4 or thioacetamide-induced liver fibrosis and matrix accumulation (Hernández-Gea et al., 2012). The reduction of ECM accumulation and fibrosis upon loss of autophagic function in mouse HSCs led to the suggestion to target autophagy in fibrotic diseases (Hernández-Gea et al., 2012). Consistent with this, earlier studies had shown that inhibition of autophagy in cultured hepatocytes and in mouse liver led to increased triglyceride storage and LDs (Singh et al., 2009). Furthermore, autophagy was significantly increased upon HSC activation, while treatment with autophagy inhibitor Bafilomycine A1 blunted HSC activation (Thoen et al., 2011). Treatment of rat AAF/CCl_4 fibrotic model with chloroquine, which blocks autophagic degradation in the lysosome, also ameliorated liver injury, decreased the expression of CK19 and pro-fibrogenic targets (COL1A1, α-SMA, TGF-β), and blunted liver fibrosis (Hung et al., 2015). These findings suggest that autophagy is relevant in MFB generation and is potentially druggable toward inhibition of excessive MFB function.

Autophagy is Antifibrotic

Contrary to a direct correlation between autophagy and HSC activation, autophagy has also been found to be protective in fibrosis (Mallat et al., 2014). For instance, mutations in the autophagy gene, *Atg5*, apparently interfered with HSC-to-MFB transdifferentiation to protect mice against chronic CCl4-induced liver fibrosis (Lodder et al., 2015). $Atg5^{-/-}$ mice treated with CCl4 had higher hepatic levels of interleukins (IL-1A, IL-1B), enhanced inflammatory cell recruitment, and were more susceptible to liver fibrosis (Lodder et al., 2015). Indeed, in studying the potential therapeutic benefits of natural compounds in alleviating fibrosis, it was observed that activated HSCs have increased light chain I/II (LC3 I/II) protein expression when pre-treated with fatty acids (OA and PA) and then post-treated with various natural compounds, including rutin and curcumin (Lee et al., 2014). While the study confirms that FA induces autophagy as mentioned earlier, the subsequent conclusion that the natural compounds are potential antifibrotic agents (Lee et al., 2014) seems to suggest that autophagy induction (as caused by the compounds) is antifibrotic in activated HSCs. Similarly, tonsil-derived mesenchymal stem cells could ameliorate CCl4-induced liver fibrosis in mice via autophagy activation, notably by reducing TGF-β and type I collagen expression (Park et al., 2015). In pulmonary fibrosis, reduced autophagy in aged animals also worsened the fibrotic phenotype (Sosulski et al., 2015). In addition, TGF-β promotes lung fibrosis by suppressing autophagy (Sosulski et al., 2015). Taken together, autophagy may represent a highly context-dependent facet in MFB pathophysiology. Whether autophagy is protective or induces cell death may largely depend on the initiating factor. Supporting this view, Rautou and colleagues argued that in most liver diseases, autophagy is mainly protective, e.g., by allowing the degradation of LDs in fatty acid disease and protein aggregates in alcohol liver disease. Contrarily, Hepatitis B/C virus can subvert autophagy for their replicative advantage (Rautou et al., 2010).

Here, it is worthy to highlight that the loss of LDs attributed to "protective" autophagy (Rautou et al., 2010) is also a mechanism through which autophagy provides energy substrates to promote HSC activation and fibrosis (Hernández-Gea et al., 2012). Therefore, it will be of interest to further interrogate protective and detrimental autophagy in HSC activation, MFB functions, and in the switch between activation and dedifferentiation to quiescence (**Figure 1**).

EPIGENETIC ALTERATIONS IN LIVER MYOFIBROBLASTS

Epigenetics refer to heritable traits resulting from chromosomal alterations that do not alter DNA sequence. Epigenetic alterations maintain cell identity and include DNA methylation, histone modifications, chromatin remodeling, transcriptional control, and post-translational modification of non-coding RNA (Berger et al., 2009; Cedar and Bergman, 2009; Portela and Esteller, 2010; Mann, 2014). Interestingly, several epigenetic targets, including DNA methyltransferases (DNMTs), histone deacetylases (HDACs), histone methyltransferases (*DOT1L, EZH2, G9A*), histone demethylases (JmjC-domain proteins, *LSD1*), and binding domains (*BET, BAZ2B, L3MBTL1*) are druggable in human diseases (Helin and Dhanak, 2013). Epigenetic alterations occur in liver fibrosis and chronic liver diseases (Mann, 2014; Atta, 2015; Lleo et al., 2015) and are relevant in HSC activation (Kang et al., 2015).

DNA Methylation

During activation, HSCs accumulate methylation changes that significantly modulate the expression of genes involved in cell activation and inflammation (Götze et al., 2015). Specifically, expression of DNA methyltransferases, DNMT3A and DNMT3B, increased with HSC activation (Götze et al., 2015). One consequence of hypermethylation is gene silencing. For instance, transcriptional silencing of PPARγ, which occurs during HSC activation (Hazra et al., 2004), has been attributed to methylation based epigenetic control (Mann et al., 2007; Yang et al., 2012). Recently, the Jumonji Domain-Containing Protein 1A (JMJD1A)—a histone H3K9 demethylase—was found to regulate HSC activation and liver fibrosis by targeting PPARγ gene expression (Jiang et al., 2015). Knockdown of JMJD1A in HSCs correlated with reinforced H3K9me2 in the PPARγ gene promoter; increased α-SMA and collagen expression, and enhanced necrosis in the CCl_4 mouse fibrosis model (Jiang et al., 2015). Consistent with this finding, blocking CpG methylation with the nucleotide analog 5-aza-2′-deoxycytidine (5-azadC) prevented loss of PPARγ expression (Mann et al., 2007). Methylation-based control in HSCs is also evident from methyl-CpG-binding protein (MECP2), known to repress chromatin structures. MECP2 is induced during HSC activation, correlates with α-SMA expression and contributes to MFB transdifferentiation by regulating fibrogenic targets (Mann et al., 2007; Yang et al., 2013a). Mechanistically, MECP2 repressed Patched (PTCH1), whose loss upon hypermethylation is necessary for sustained fibroblast activation and liver fibrosis (Yang et al., 2013a). Furthermore, DNA methylation is responsible for epigenetic silencing of Smad7, which enables fibrogenic TGF-β effects via Smad2 and Smad3 phosphorylation (Bian et al., 2014). Hence, RNA interference and 5-azadC-mediated inhibition of the methylation gene *DNMT1* prevented TGF-β-induced proliferation and upregulation of activation markers in HSCs (Bian et al., 2014). More studies are required to further validate methylation switches at various MFB differentiation stages under normal and perturbed microenvironments.

Histone Modification

Histone modification is another active epigenetic alteration during HSC activation. For instance, HSCs in a mouse model of acute liver failure secrete IL-1, which induces high MMP9 levels, leading to collagen IV degradation (Yan et al., 2008). If uncontrolled, MMP9 expression could oppose MFB-mediated accumulation of ECM. Hence maintenance of appropriate balance is necessary during HSC activation or MFB function. Interestingly, epigenetic repression of MMPs has been suggested as a mechanism that controls HSC transdifferentiation (Qin and Han, 2010). Consequently, MMP9 and MMP13 promoters in MFB display impaired histone acetylation and assembly of transcription machinery. These alterations blocked docking of transcription factor c-Jun on the MMP promoters (Qin and Han, 2010). Similarly, ectopic expression of *HDAC4* in quiescent HSCs suppressed intrinsic and IL-1-induced MMP promoter activity and repressed MMP9 expression. These findings implicate accumulation of HDACs at MMP promoters, specifically *HDAC4*, as an epigenetic mechanism to repress MMP expression during HSC activation (Qin and Han, 2010). Similar regulation is provided via *HDAC7*, which represses hepatocyte growth factor (HGF) and thus increases susceptibility to hepatocellular damage, inflammation, and fibrosis in liver injury (Pannem et al., 2014). *HDAC7*-mediated repression of HGF in HSCs is antagonized by the tumor suppressor gene cylindromatosis (*CYLD*). Accordingly, *CYLD* interacts with and removes HDAC7 from the HGF promoter, hence enabling HGF induction, which subsequently is secreted and protects against hepatocellular injury and fibrosis (Pannem et al., 2014). In cultured human skin fibroblasts, *HDAC6, HDAC8*, but most potently *HDAC4* were identified as crucial epigenetic regulators of TGF-β-induced MFB differentiation, ostensibly by blocking the expression of TGF-β signaling repressors 5′-TG-3′-Interacting Factor (TGIF) and TGIF2 (Glenisson et al., 2007).

Recently, the myocardin-related transcription factor (MRTF), ethanol and vitamin D receptor (VDR) have been identified as epigenomic modifiers during HSC activation. MRTF promotes MFB differentiation, fibrosis, and TGF-β-induced HSC activation (Crider et al., 2011; O'Connor and Gomez, 2013; Velasquez et al., 2013; O'Connor et al., 2015; Sisson et al., 2015). Mechanistically, MRTF-A mediates fibrosis via recruitment of the histone methyltransferase complex to the promoters of fibrogenic genes and subsequent transcriptional activation (Tian et al., 2015). Ethanol exposure was found to promote rat HSC transdifferentiation by inducing global changes in histone

modifying enzymes that upregulate ECM components elastin (*ELN*) and collagens (Page et al., 2015). The authors found that ethanol induced the expression of histone 3 lysine 4 (H3K4) methyltransferases, mainly *MLL1*. *MLL1* binding was enriched on *ELN* gene promoter and consequently induced *ELN* expression in transitioning HSCs. In addition, *MLL1* expression also correlated with *ELN* and collagens in ALD liver explants further confirming that ethanol induced pro-fibrogenic processes via epigenetic regulators (Page et al., 2015). VDR ligands also induce chromatin remodeling as a mechanism to counteract TGF-β-driven HSC activation (Ding et al., 2013). TGF-β induced activation by promoting the recruitment of histone-modifying cofactors, p300 and CBP, and by promoting histone H3 hyperacetylation at a VDR/SMAD co-occupied regulatory region of *COL1A1*. Treatment with VDR ligands antagonized activation by disrupting TGF-β-mediated SMAD/VDR interaction. Consequently, synthetic VDR agonist Calcipotriol reduced collagen deposition and fibrotic gene expression *in vitro* and *in vivo* (Ding et al., 2013). These studies underscore the critical role of histone modification in HSC transdifferentiation (**Figure 1**).

Indeed, evolving links between epigenetics and MFB function justifies targeting histone modifiers in antifibrotic therapies. In line, various HDAC inhibitors are effective against TGF-β-induced MFB generation (Glenisson et al., 2007; Guo et al., 2009; Liu et al., 2013), including MC1568, valproic acid (VPA), trichostatin A (TSA), and butyrate. MC1568 inhibited HSC activation markers, such as type I/III collagen, SMA, and lysyl oxidase. In addition, MC1568 induced antifibrotic microRNA-29 and also suppressed the proliferation of freshly isolated mouse HSCs (Mannaerts et al., 2013). Unfortunately, the authors were unable to reproduce the result in CCl_4 fibrosis model owing to technical issues, probably due to inefficient delivery or fast metabolization of the drug (Mannaerts et al., 2013). VPA also suppresses liver fibrosis and HSC activation *in vitro* and *in vivo* (Mannaerts et al., 2010; Aher et al., 2015). However, given the pivotal role of TGF-β in HSC activation, it is important to mention that VPA did not interfere with early TGF-β targets, SMAD 6 and 7 (Mannaerts et al., 2010), thus raising further questions on the exact mechanism, by which epigenetics influences HSC activation by TGF-β. Furthermore, TSA and RNA interference against *HDAC4* prevented MFB differentiation as measured by α-SMA expression (Glenisson et al., 2007). Nilotinib, a tyrosine kinase inhibitor, selectively induces apoptotic and autophagic cell death in HSCs by blocking HDAC 1, 2, and 4 (Shaker et al., 2013). Other HDAC inhibitors that suppress HSC activation include a chalcone derivative 2',4',6'-tris(methoxymethoxy) chalcone (TMMC; (Lee et al., 2011)), and N-hydroxy-7-(2-naphthylthio)heptanomide (HNHA; (Park et al., 2014)). HNHA not only suppressed HSC proliferation, activation and liver fibrosis, but also restored liver function and prolonged survival in the BDL rat model (Park et al., 2014). Further evidence for targeting HDACs in fibrotic diseases have been shown in other settings, e.g., cardiac and lung fibroblasts (Zhang et al., 2013; Sanders et al., 2014; Schuetze et al., 2014). Together, these data highlight the relevance of epigenetics in HSC activation and encourage the exploitation of epigenetic targets in the control of MFB-mediated fibrogenesis.

TECHNICAL ADVANCES THAT COULD HELP DELINEATE METABOLISM, AUTOPHAGY, AND EPIGENETICS IN LIVER MYOFIBROBLASTS

Within the last few decades, several cutting edge techniques have emerged for the study of complex biological processes.

In metabolism, mass spectrometry based metabolomics techniques have been developed for measuring metabolic flux. Consequently, it is now possible to precisely determine utility of metabolites with a very high degree of precision (Zamboni et al., 2009; Hiller and Metallo, 2013). Thus, metabolic flux analyses could enable (a) holistic and simultaneous quantification of labeled and unlabeled metabolites derived from parent carbon sources (e.g., glucose or glutamine), (b) help to delineate the metabolic properties of MFB at different differentiation stages, and (c) offer hints on prospective metabolic pathways of therapeutic relevance in HSC activation or MFB function. In addition, cellular respiration can be easily measured by fiber optic oxygen sensors, clark electrode, and extracellular flux analyzers—the latter offering the advantage of assessing oxygen consumption and extracellular acidification rates in living cells (Zhang et al., 2012; Perry et al., 2013). Furthermore, *in vivo* measurement of metabolism using hyperpolarized, (13)C-labeled cells has been successfully applied (Rodrigues et al., 2014; Brindle, 2015). The latter *in vivo* approach could enhance accuracy given the challenges of potential artifacts from cell cultures.

For autophagy studies, techniques such as time-lapse microscopy (Muzzey and van Oudenaarden, 2009) and transmission electron microscopy could be adopted with molecular biology methods (Klionsky et al., 2012) to dissect how autophagy affects the evolution of fibrosis. This could help identify novel potential mediators of MFB autophagic mechanisms for therapeutic purposes. Also, research on MFB autophagy will benefit from other advances in microscopy, e.g., super-resolution microscopy that allow high-resolution imaging and protein tracking in living cells (Bergner et al., 2013; Barden et al., 2015; Chéreau et al., 2015). Tools for measuring autophagy based on biochemical features, e.g., quenching of GFP fluorescent signals in the lysosome at low pH (Bampton et al., 2005), could enable a more improved understanding of autophagy during HSC transdifferention (**Figure 1**).

Delineating epigenetic alterations in HSCs will be greatly enhanced by bisulfite conversion, chromatin immunoprecipitation and high throughput DNA methylome analyses (Shull et al., 2015; Tang et al., 2015). Other techniques, including single cell analysis, sequencing techniques, and multi-color fluorescence activated cell sorting could be applied to uncover yet unknown epigenetic alterations specific to HSC activation and MFB functions (**Figure 1**).

To accelerate understanding of MFB function, it is important to consider and possibly tackle the fact that *in vivo* changes in gene expression during HSC activation may differ markedly from

those that occur in *in vitro* culture (De Minicis et al., 2007). Such differences may be due to a plethora of factors, including but not limited to cell culture conditions, contamination by other liver cell populations, and sample handling. Where possible, a strategy to overcome this challenge would be to interface the assays mentioned above with lineage tracing. Lineage tracing has been successfully applied as a powerful innovative tool for tracking MFB origin (Mederacke et al., 2013; Lua et al., 2014; Swiderska-Syn et al., 2014). Hence, lineage tracing opens up the feasibility of *in vivo* studies of alterations in HSCs at quiescent, transitory, and activated states, and will highly complement genomic and functional assays.

Finally, optimal application of novel gene editing techniques, such as CRISPR/Cas9, TALENs, etc. (Cho et al., 2014; Jamal et al., 2015; LaFountaine et al., 2015; Laufer and Singh, 2015) will hugely accelerates the identification and understanding of metabolic, autophagic, and epigenetic targets in MFB, especially when complemented with proteomic and transcriptomic profilings. Ultimately, perhaps the most important technical step in understanding MFB physiology, especially in the context of metabolism, autophagy, and epigenetics, is to explore all possible strategies to eliminate analytical variables that distort results.

UNCHARTED TERRITORIES IN THE STUDY OF METABOLISM, AUTOPHAGY AND EPIGENETICS IN LIVER MYOFIBROBLASTS

Currently, few studies have focused on how MFB feed, regulate survival via autophagy or via epigenetic alterations that activate or silence key genes in spatio-temporal cell fate decisions. Consequently, knowledge of metabolism, autophagy, and epigenetics in MFB is still at a very nascent stage with many convoluted parts worth further clarifications (**Figure 1**). The studies that so far focused on the above subjects have offered exciting platforms for further questions. However, more efforts should be dedicated to delineate their molecular relevance to MFB origin and function in health and disease. Lessons can also be learned from other settings, e.g., cancer. For example, metabolism has evolved as a potentially druggable process in cancer entities and many metabolic targets are in preclinical and clinical trials (Galluzzi et al., 2013). Whether those therapeutic strategies would find application in MFB origin/liver fibrosis remains an open question. It is also unknown which metabolic priorities are exploited by MFB, e.g., particular substrates that are indispensable for their survival. The timing of metabolic alterations is also critical to any studies, as changes in mRNA transcripts occur within hours in cultured HSCs (Chen et al., 2012). Unresolved questions also abound in the area of nutrient exchange between MFB and other liver cell populations in the microenvironment, including the extent of their capacity to sustain *de novo* anabolism. Regarding autophagy, scientific efforts should clarify boundaries between "self-eating" autophagy to provide energy building substrates and autophagy with a self-destructive consequence. Questions like "how autophagy markers contribute to MFB status" remain hugely unanswered. Similar questions in metabolism and autophagy also apply to epigenetics. Many epigenomic targets are druggable and several epigenomic drugs yield a beneficial response in MFB and fibrosis. Hence, understanding how DNA methylation and histone modification control HSC transdifferentiation could substantially improve the prospects of better therapeutic interventions. Furthermore, questions also arise on possible crosstalks or feedback loops or overarching control by potential regulators that are chiefly responsible for MFB physiology. These may include transcription factors, microRNAs, long non-coding RNAs, and other regulators of the genome. Hence, it is yet unclear under which circumstances certain regulators switch on/off metabolism, autophagy, or epigenetic modifiers in MFB (**Figure 1**). Ultimately, there are currently no established metabolic, autophagy, or epigenetics markers in MFB. Dissecting these uncharted territories will substantially open new windows for therapeutic interventions in MFB-mediated fibrosis.

CONCLUSION

In the light of evolving molecular insights, metabolism, autophagy, and epigenetics are critical players in HSC activation and MFB functions. Currently available data lead us to propose that transcriptional and epigenetic controls likely coordinate metabolism and autophagy in HSC to MFB transdifferentiation (**Figure 1**). In the context of the discussed molecular processes, more studies are required to deepen understanding of MFB origin and function in liver fibrogenesis. We suggest that ongoing and future MFB research should interrogate the relevance of key metabolic enzymes, autophagy markers, and epigenetic modifiers, including but not limited to those mentioned here and already investigated in cancer (Claus and Lübbert, 2003; Cheong et al., 2012; Popovic and Licht, 2012; Galluzzi et al., 2013; Helin and Dhanak, 2013; **Table 1**). We also recommend that researchers should critically consider the time points selected for MFB studies since unforeseen switch from activation to quiescence or vice versa could obscure molecular details. In the end, a broad-spectrum integration of cutting edge tools that enable simultaneous measurements, such as "omics" technologies, will enable better understanding of MFB and further expose novel regulators or biomarkers of MFB activity. We conclude that a detailed understanding of metabolism, autophagy and epigenetics in liver MFB will inspire a new frontier in the development of antifibrotic therapy.

AUTHOR CONTRIBUTIONS

ZCN, HA, and YL conceived and wrote the manuscript, while SW, SD, and YL provided comments, revised, and corrected the manuscript.

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REFERENCES

Adachi, M., Osawa, Y., Uchinami, H., Kitamura, T., Accili, D., and Brenner, D. A. (2007). The forkhead transcription factor FoxO1 regulates proliferation and transdifferentiation of hepatic stellate cells. *Gastroenterology* 132, 1434–1446. doi: 10.1053/j.gastro.2007.01.033

Aher, J. S., Khan, S., Jain, S., Tikoo, K., and Jena, G. (2015). Valproate ameliorates thioacetamide-induced fibrosis by hepatic stellate cell inactivation. *Hum. Exp. Toxicol.* 34, 44–55. doi: 10.1177/0960327114531992

Alhouayek, M., and Muccioli, G. G. (2014). COX-2-derived endocannabinoid metabolites as novel inflammatory mediators. *Trends Pharmacol. Sci.* 35, 284–292. doi: 10.1016/j.tips.2014.03.001

Aoyama, T., Paik, Y. H., Watanabe, S., Laleu, B., Gaggini, F., Fioraso-Cartier, L., et al. (2012). Nicotinamide adenine dinucleotide phosphate oxidase in experimental liver fibrosis: GKT137831 as a novel potential therapeutic agent. *Hepatology* 56, 2316–2327. doi: 10.1002/hep.25938

Atta, H. M. (2015). Reversibility and heritability of liver fibrosis: implications for research and therapy. *World J. Gastroenterol.* 21, 5138–5148. doi: 10.3748/wjg.v21.i17.5138

Austinat, M., Dunsch, R., Wittekind, C., Tannapfel, A., Gebhardt, R., and Gaunitz, F. (2008). Correlation between beta-catenin mutations and expression of Wnt-signaling target genes in hepatocellular carcinoma. *Mol. Cancer* 7:21. doi: 10.1186/1476-4598-7-21

Bampton, E. T., Goemans, C. G., Niranjan, D., Mizushima, N., and Tolkovsky, A. M. (2005). The dynamics of autophagy visualized in live cells: from autophagosome formation to fusion with endo/lysosomes. *Autophagy* 1, 23–36. doi: 10.4161/auto.1.1.1495

Barden, A. O., Goler, A. S., Humphreys, S. C., Tabatabaei, S., Lochner, M., Ruepp, M. D., et al. (2015). Tracking individual membrane proteins and their biochemistry: The power of direct observation. *Neuropharmacology* 98, 22–30. doi: 10.1016/j.neuropharm.2015.05.003

Bataller, R., and Brenner, D. A. (2005). Liver fibrosis. *J. Clin. Invest.* 115, 209–218. doi: 10.1172/JCI24282

Bazinet, R. P., and Layé, S. (2014). Polyunsaturated fatty acids and their metabolites in brain function and disease. *Nat. Rev. Neurosci.* 15, 771–785. doi: 10.1038/nrn3820

Bentaib, A., De Tullio, P., Chneiweiss, H., Hermans, E., Junier, M. P., and Leprince, P. (2014). Metabolic reprogramming in transformed mouse cortical astrocytes: a proteomic study. *J. Proteomics* 113C, 292–314. doi: 10.1016/j.jprot.2014.09.019

Berger, S. L., Kouzarides, T., Shiekhattar, R., and Shilatifard, A. (2009). An operational definition of epigenetics. *Genes Dev.* 23, 781–783. doi: 10.1101/gad.1787609

Bergner, S., Vatsyayan, P., and Matysik, F. M. (2013). Recent advances in high resolution scanning electrochemical microscopy of living cells–a review. *Anal. Chim. Acta* 775, 1–13. doi: 10.1016/j.aca.2012.12.042

Bian, E. B., Huang, C., Wang, H., Chen, X. X., Zhang, L., Lv, X. W., et al. (2014). Repression of Smad7 mediated by DNMT1 determines hepatic stellate cell activation and liver fibrosis in rats. *Toxicol. Lett.* 224, 175–185. doi: 10.1016/j.toxlet.2013.10.038

Bianchini, F., Giannoni, E., Serni, S., Chiarugi, P., and Calorini, L. (2012). 22: 6n-3 DHA inhibits differentiation of prostate fibroblasts into myofibroblasts and tumorigenesis. *Br. J. Nutr.* 108, 2129–2137. doi: 10.1017/S0007114512000359

Blaner, W. S., O'Byrne, S. M., Wongsiriroj, N., Kluwe, J., D'Ambrosio, D. M., Jiang, H., et al. (2009). Hepatic stellate cell lipid droplets: a specialized lipid droplet for retinoid storage. *Biochim. Biophys. Acta* 1791, 467–473. doi: 10.1016/j.bbalip.2008.11.001

Bode, J. G., Peters-Regehr, T., Gressner, A. M., and Häussinger, D. (1998). De novo expression of glutamine synthetase during transformation of hepatic stellate cells into myofibroblast-like cells. *Biochem. J.* 335(Pt 3), 697–700. doi: 10.1042/bj3350697

Brenner, C., Galluzzi, L., Kepp, O., and Kroemer, G. (2013). Decoding cell death signals in liver inflammation. *J. Hepatol.* 59, 583–594. doi: 10.1016/j.jhep.2013.03.033

Brindle, K. M. (2015). Imaging metabolism with hyperpolarized. (13)C-labeled cell substrates. *J. Am. Chem. Soc.* 137, 6418–6427. doi: 10.1021/jacs.5b03300

Buckley, C. D., Gilroy, D. W., and Serhan, C. N. (2014). Proresolving lipid mediators and mechanisms in the resolution of acute inflammation. *Immunity* 40, 315–327. doi: 10.1016/j.immuni.2014.02.009

Cadoret, A., Ovejero, C., Terris, B., Souil, E., Lévy, L., Lamers, W. H., et al. (2002). New targets of beta-catenin signaling in the liver are involved in the glutamine metabolism. *Oncogene* 21, 8293–8301. doi: 10.1038/sj.onc.1206118

Carey, B. W., Finley, L. W., Cross, J. R., Allis, C. D., and Thompson, C. B. (2015). Intracellular α-ketoglutarate maintains the pluripotency of embryonic stem cells. *Nature* 518, 413–416. doi: 10.1038/nature13981

Cat, B., Stuhlmann, D., Steinbrenner, H., Alili, L., Holtkötter, O., Sies, H., et al. (2006). Enhancement of tumor invasion depends on transdifferentiation of skin fibroblasts mediated by reactive oxygen species. *J. Cell Sci.* 119(Pt 13), 2727–2738. doi: 10.1242/jcs.03011

Cedar, H., and Bergman, Y. (2009). Linking DNA methylation and histone modification: patterns and paradigms. *Nat. Rev. Genet.* 10, 295–304. doi: 10.1038/nrg2540

Chan, E. C., Peshavariya, H. M., Liu, G. S., Jiang, F., Lim, S. Y., and Dusting, G. J. (2013). Nox4 modulates collagen production stimulated by transforming growth factor β1 *in vivo* and *in vitro*. *Biochem. Biophys. Res. Commun.* 430, 918–925. doi: 10.1016/j.bbrc.2012.11.138

Chen, J., Shearer, G. C., Chen, Q., Healy, C. L., Beyer, A. J., Nareddy, V. B., et al. (2011). Omega-3 fatty acids prevent pressure overload-induced cardiac fibrosis through activation of cyclic GMP/protein kinase G signaling in cardiac fibroblasts. *Circulation* 123, 584–593. doi: 10.1161/CIRCULATIONAHA.110.971853

Chen, Y., Choi, S. S., Michelotti, G. A., Chan, I. S., Swiderska-Syn, M., Karaca, G. F., et al. (2012). Hedgehog controls hepatic stellate cell fate by regulating metabolism. *Gastroenterology* 143, 1319-29.e1–1319-29.e11. doi: 10.1053/j.gastro.2012.07.115

Cheong, H., Lu, C., Lindsten, T., and Thompson, C. B. (2012). Therapeutic targets in cancer cell metabolism and autophagy. *Nat. Biotechnol.* 30, 671–678. doi: 10.1038/nbt.2285

Chéreau, R., Tønnesen, J., and Nägerl, U. V. (2015). STED microscopy for nanoscale imaging in living brain slices. *Methods* 88, 57–66. doi: 10.1016/j.ymeth.2015.06.006

Cho, S. W., Kim, S., Kim, Y., Kweon, J., Kim, H. S., Bae, S., et al. (2014). Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. *Genome Res.* 24, 132–141. doi: 10.1101/gr.162339.113

Choi, A. M., Ryter, S. W., and Levine, B. (2013). Autophagy in human health and disease. *N. Engl. J. Med.* 368, 651–662. doi: 10.1056/NEJMra1205406

Chong, L. W., Hsu, Y. C., Lee, T. F., Lin, Y., Chiu, Y. T., Yang, K. C., et al. (2015). Fluvastatin attenuates hepatic steatosis-induced fibrogenesis in rats through inhibiting paracrine effect of hepatocyte on hepatic stellate cells. *BMC Gastroenterol.* 15:22. doi: 10.1186/s12876-015-0248-8

Claus, R., and Lübbert, M. (2003). Epigenetic targets in hematopoietic malignancies. *Oncogene* 22, 6489–6496. doi: 10.1038/sj.onc.1206814

Colmenero, J., Bataller, R., Sancho-Bru, P., Bellot, P., Miquel, R., Moreno, M., et al. (2007). Hepatic expression of candidate genes in patients with alcoholic hepatitis: correlation with disease severity. *Gastroenterology* 132, 687–697. doi: 10.1053/j.gastro.2006.12.036

Crider, B. J., Risinger, G. M. Jr, Haaksma, C. J., Howard, E. W., and Tomasek, J. J. (2011). Myocardin-related transcription factors A and B are key regulators of

TGF-β1-induced fibroblast to myofibroblast differentiation. *J. Invest. Dermatol.* 131, 2378–2385. doi: 10.1038/jid.2011.219

Cucoranu, I., Clempus, R., Dikalova, A., Phelan, P. J., Ariyan, S., Dikalov, S., et al. (2005). NAD(P)H oxidase 4 mediates transforming growth factor-beta1-induced differentiation of cardiac fibroblasts into myofibroblasts. *Circ. Res.* 97, 900–907. doi: 10.1161/01.RES.0000187457.24338.3D

DeBerardinis, R. J., Mancuso, A., Daikhin, E., Nissim, I., Yudkoff, M., Wehrli, S., et al. (2007). Beyond aerobic glycolysis: transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. *Proc. Natl. Acad. Sci. U.S.A.* 104, 19345–19350. doi: 10.1073/pnas.0709747104

De Minicis, S., Seki, E., Oesterreicher, C., Schnabl, B., Schwabe, R. F., and Brenner, D. A. (2008). Reduced nicotinamide adenine dinucleotide phosphate oxidase mediates fibrotic and inflammatory effects of leptin on hepatic stellate cells. *Hepatology* 48, 2016–2026. doi: 10.1002/hep.22560

De Minicis, S., Seki, E., Uchinami, H., Kluwe, J., Zhang, Y., Brenner, D. A., et al. (2007). Gene expression profiles during hepatic stellate cell activation in culture and *in vivo*. *Gastroenterology* 132, 1937–1946. doi: 10.1053/j.gastro.2007.02.033

Ding, N., Yu, R. T., Subramaniam, N., Sherman, M. H., Wilson, C., Rao, R., et al. (2013). A vitamin D receptor/SMAD genomic circuit gates hepatic fibrotic response. *Cell* 153, 601–613. doi: 10.1016/j.cell.2013.03.028

Dooley, S., Hamzavi, J., Breitkopf, K., Wiercinska, E., Said, H. M., Lorenzen, J., et al. (2003). Smad7 prevents activation of hepatic stellate cells and liver fibrosis in rats. *Gastroenterology* 125, 178–191. doi: 10.1016/S0016-5085(03)00666-8

Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicol Pathol.* 35, 495–516. doi: 10.1080/01926230701320337

Espinosa-Diez, C., Miguel, V., Mennerich, D., Kietzmann, T., Sánchez-Pérez, P., Cadenas, S., et al. (2015). Antioxidant responses and cellular adjustments to oxidative stress. *Redox Biol.* 6, 183–197. doi: 10.1016/j.redox.2015.07.008

Filomeni, G., De Zio, D., and Cecconi, F. (2015). Oxidative stress and autophagy: the clash between damage and metabolic needs. *Cell Death Differ.* 22, 377–388. doi: 10.1038/cdd.2014.150

Freigang, S., Ampenberger, F., Weiss, A., Kanneganti, T. D., Iwakura, Y., Hersberger, M., et al. (2013). Fatty acid-induced mitochondrial uncoupling elicits inflammasome-independent IL-1α and sterile vascular inflammation in atherosclerosis. *Nat. Immunol.* 14, 1045–1053. doi: 10.1038/ni.2704

Fu, Y., Zheng, S., Lu, S. C., and Chen, A. (2008). Epigallocatechin-3-gallate inhibits growth of activated hepatic stellate cells by enhancing the capacity of glutathione synthesis. *Mol. Pharmacol.* 73, 1465–1473. doi: 10.1124/mol.107.040634

Gabbiani, G. (2003). The myofibroblast in wound healing and fibrocontractive diseases. *J. Pathol.* 200, 500–503. doi: 10.1002/path.1427

Galluzzi, L., Kepp, O., Vander Heiden, M. G., and Kroemer, G. (2013). Metabolic targets for cancer therapy. *Nat. Rev. Drug Discov.* 12, 829–846. doi: 10.1038/nrd4145

Galluzzi, L., Pietrocola, F., Levine, B., and Kroemer, G. (2014). Metabolic control of autophagy. *Cell* 159, 1263–1276. doi: 10.1016/j.cell.2014.11.006

Gimeno-Bayón, J., López-López, A., Rodríguez, M. J., and Mahy, N. (2014). Glucose pathways adaptation supports acquisition of activated microglia phenotype. *J. Neurosci. Res.* 92, 723–731. doi: 10.1002/jnr.23356

Glenisson, W., Castronovo, V., and Waltregny, D. (2007). Histone deacetylase 4 is required for TGFbeta1-induced myofibroblastic differentiation. *Biochim. Biophys. Acta* 1773, 1572–1582. doi: 10.1016/j.bbamcr.2007.05.016

Götze, S., Schumacher, E. C., Kordes, C., and Häussinger, D. (2015). Epigenetic changes during hepatic stellate cell activation. *PLoS ONE* 10:e0128745. doi: 10.1371/journal.pone.0128745

Green, D. R., and Levine, B. (2014). To be or not to be? How selective autophagy and cell death govern cell fate. *Cell.* 157, 65–75. doi: 10.1016/j.cell.2014.02.049

Guido, C., Whitaker-Menezes, D., Capparelli, C., Balliet, R., Lin, Z., Pestell, R. G., et al. (2012). Metabolic reprogramming of cancer-associated fibroblasts by TGF-β drives tumor growth: connecting TGF-β signaling with "Warburg-like" cancer metabolism and L-lactate production. *Cell Cycle* 11, 3019–3035. doi: 10.4161/cc.21384

Guo, W., Shan, B., Klingsberg, R. C., Qin, X., and Lasky, J. A. (2009). Abrogation of TGF-beta1-induced fibroblast-myofibroblast differentiation by histone deacetylase inhibition. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 297, L864–L870. doi: 10.1152/ajplung.00128.2009

Hanahan, D., and Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell* 144, 646–674. doi: 10.1016/j.cell.2011.02.013

Hanley, B., Dijane, J., Fewtrell, M., Grynberg, A., Hummel, S., Junien, C., et al. (2010). Metabolic imprinting, programming and epigenetics – a review of present priorities and future opportunities. *Br. J. Nutr.* 104(Suppl. 1), S1–S25. doi: 10.1017/S0007114510003338

Hazra, S., Xiong, S., Wang, J., Rippe, R. A., Krishna, V., Chatterjee, K., et al. (2004). Peroxisome proliferator-activated receptor gamma induces a phenotypic switch from activated to quiescent hepatic stellate cells. *J. Biol. Chem.* 279, 11392–11401. doi: 10.1074/jbc.M310284200

Hecker, L., Logsdon, N. J., Kurundkar, D., Kurundkar, A., Bernard, K., Hock, T., et al. (2014). Reversal of persistent fibrosis in aging by targeting Nox4-Nrf2 redox imbalance. *Sci. Transl. Med.* 6, 231ra47. doi: 10.1126/scitranslmed.3008182

Helin, K., and Dhanak, D. (2013). Chromatin proteins and modifications as drug targets. *Nature* 502, 480–488. doi: 10.1038/nature12751

Henderson, N. C., Mackinnon, A. C., Farnworth, S. L., Poirier, F., Russo, F. P., Iredale, J. P., et al. (2006). Galectin-3 regulates myofibroblasts activation and hepatic fibrosis. *Proc. Natl. Acad. Sci. U.S.A.* 103, 5060–5065. doi: 10.1073/pnas.0511167103

Hernández-Gea, V., Ghiassi-Nejad, Z., Rozenfeld, R., Gordon, R., Fiel, M. I., Yue, Z., et al. (2012). Autophagy releases lipid that promotes fibrogenesis by activated hepatic stellate cells in mice and in human tissues. *Gastroenterology* 142, 938–946. doi: 10.1053/j.gastro.2011.12.044

Hiller, K., and Metallo, C. M. (2013). Profiling metabolic networks to study cancer metabolism. *Curr. Opin. Biotechnol.* 24, 60–68. doi: 10.1016/j.copbio.2012.11.001

Hsieh, W. C., Mackinnon, A. C., Lu, W. Y., Jung, J., Boulter, L., Henderson, N. C., et al. (2015). Galectin-3 regulates hepatic progenitor cell expansion during liver injury. *Gut* 64, 312–321. doi: 10.1136/gutjnl-2013-306290

Hung, T. M., Yuan, R. H., Huang, W. P., Chen, Y. H., Lin, Y. C., Lin, C. W., et al. (2015). Increased autophagy markers are associated with ductular reaction during the development of cirrhosis. *Am. J. Pathol.* 185, 2454–2467. doi: 10.1016/j.ajpath.2015.05.010

Hurley, J. H., and Schulman, B. A. (2014). Atomistic autophagy: the structures of cellular self-digestion. *Cell* 157, 300–311. doi: 10.1016/j.cell.2014.01.070

Iacobini, C., Menini, S., Ricci, C., Blasetti Fantauzzi, C., Scipioni, A., Salvi, L., et al. (2011). Galectin-3 ablation protects mice from diet-induced NASH: a major scavenging role for galectin-3 in liver. *J. Hepatol.* 54, 975–983. doi: 10.1016/j.jhep.2010.09.020

Iwaisako, K., Jiang, C., Zhang, M., Cong, M., Moore-Morris, T. J., Park, T. J., et al. (2014). Origin of myofibroblasts in the fibrotic liver in mice. *Proc. Natl. Acad. Sci. U.S.A.* 111, E3297–E3305. doi: 10.1073/pnas.1400062111

Jain, M., Rivera, S., Monclus, E. A., Synenki, L., Zirk, A., Eisenbart, J., et al. (2013). Mitochondrial reactive oxygen species regulate transforming growth factor-β signaling. *J. Biol. Chem.* 288, 770–777. doi: 10.1074/jbc.M112.431973

Jamal, M., Khan, F. A., Da, L., Habib, Z., Dai, J., and Cao, G. (2015). Keeping CRISPR/Cas on-Target. *Curr. Issues Mol. Biol.* 20, 1–20.

Jiang, J. X., Chen, X., Hsu, D. K., Baghy, K., Serizawa, N., Scott, F., et al. (2012). Galectin-3 modulates phagocytosis-induced stellate cell activation and liver fibrosis *in vivo*. *Am. J. Physiol. Gastrointest. Liver Physiol.* 302, G439–G446. doi: 10.1152/ajpgi.00257.2011

Jiang, Y., Wang, S., Zhao, Y., Lin, C., Zhong, F., Jin, L., et al. (2015). Histone H3K9 demethylase JMJD1A modulates hepatic stellate cells activation and liver fibrosis by epigenetically regulating peroxisome proliferator-activated receptor γ. *FASEB J.* 29, 1830–1841. doi: 10.1096/fj.14-251751

Kang, N., Shah, V. H., and Urrutia, R. (2015). Membrane-to-nucleus signals and epigenetic mechanisms for myofibroblastic activation and desmoplastic stroma: potential therapeutic targets for liver metastasis? *Mol. Cancer Res.* 13, 604–612. doi: 10.1158/1541-7786.MCR-14-0542

Karner, C. M., Esen, E., Okunade, A. L., Patterson, B. W., and Long, F. (2015). Increased glutamine catabolism mediates bone anabolism in response to WNT signaling. *J. Clin. Invest.* 125, 551–562. doi: 10.1172/JCI78470

Kelly, B., and O'Neill, L. A. (2015). Metabolic reprogramming in macrophages and dendritic cells in innate immunity. *Cell Res.* 25, 771–784. doi: 10.1038/cr.2015.68

Klein, S., Klösel, J., Schierwagen, R., Körner, C., Granzow, M., Huss, S., et al. (2012). Atorvastatin inhibits proliferation and apoptosis, but induces senescence in

hepatic myofibroblasts and thereby attenuates hepatic fibrosis in rats. *Lab. Invest.* 92, 1440–1450. doi: 10.1038/labinvest.2012.106

Klionsky, D. J., Abdalla, F. C., Abeliovich, H., Abraham, R. T., Acevedo-Arozena, A., Adeli, K., et al. (2012). Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy* 8, 445–544. doi: 10.4161/auto.19496

Kluwe, J., Wongsiriroj, N., Troeger, J. S., Gwak, G. Y., Dapito, D. H., Pradere, J. P., et al. (2011). Absence of hepatic stellate cell retinoid lipid droplets does not enhance hepatic fibrosis but decreases hepatic carcinogenesis. *Gut* 60, 1260–1268. doi: 10.1136/gut.2010.209551

Kordes, C., Sawitza, I., and Häussinger, D. (2008). Canonical Wnt signaling maintains the quiescent stage of hepatic stellate cells. *Biochem. Biophys. Res. Commun.* 367, 116–123. doi: 10.1016/j.bbrc.2007.12.085

LaFountaine, J. S., Fathe, K., and Smyth, H. D. (2015). Delivery and therapeutic applications of gene editing technologies ZFNs, TALENs, and CRISPR/Cas9. *Int. J. Pharm.* 494, 180–194. doi: 10.1016/j.ijpharm.2015.08.029

Lan, T., Kisseleva, T., and Brenner, D. A. (2015). Deficiency of NOX1 or NOX4 prevents liver inflammation and fibrosis in mice through inhibition of hepatic stellate cell activation. *PLoS ONE* 10:e0129743. doi: 10.1371/journal.pone.0129743

Laufer, B. I., and Singh, S. M. (2015). Strategies for precision modulation of gene expression by epigenome editing: an overview. *Epigenetics Chromatin* 8, 34. doi: 10.1186/s13072-015-0023-7

Lee, K. W., Thiyagarajan, V., Sie, H. W., Cheng, M. F., Tsai, M. J., Chia, Y. C., et al. (2014). Synergistic effect of natural compounds on the fatty acid-induced autophagy of activated hepatic stellate cells. *J. Nutr. Biochem.* 25, 903–913. doi: 10.1016/j.jnutbio.2014.04.001

Lee, S. H., Zhao, Y. Z., Park, E. J., Che, X. H., Seo, G. S., and Sohn, D. H. (2011). 2′,4′,6′-Tris(methoxymethoxy) chalcone induces apoptosis by enhancing Fas-ligand in activated hepatic stellate cells. *Eur. J. Pharmacol.* 658, 9–15. doi: 10.1016/j.ejphar.2011.01.067

Lee, T. F., Mak, K. M., Rackovsky, O., Lin, Y. L., Kwong, A. J., Loke, J. C., et al. (2010). Downregulation of hepatic stellate cell activation by retinol and palmitate mediated by adipose differentiation-related protein. (ADRP). *J. Cell Physiol.* 223, 648–657. doi: 10.1002/jcp.22063

Lee, Y. A., Wallace, M. C., and Friedman, S. L. (2015). Pathobiology of liver fibrosis: a translational success story. *Gut* 64, 830–841. doi: 10.1136/gutjnl-2014-306842

Levine, B., Mizushima, N., and Virgin, H. W. (2011). Autophagy in immunity and inflammation. *Nature* 469, 323–335. doi: 10.1038/nature09782

Li, L. C., Li, J., and Gao, J. (2014). Functions of galectin-3 and its role in fibrotic diseases. *J. Pharmacol. Exp. Ther.* 351, 336–343. doi: 10.1124/jpet.114.218370

Li, Y. H., Woo, S. H., Choi, D. H., and Cho, E. H. (2015). Succinate causes α-SMA production through GPR91 activation in hepatic stellate cells. *Biochem. Biophys. Res. Commun.* 463, 853–858. doi: 10.1016/j.bbrc.2015.06.023

Lian, N., Jiang, Y., Zhang, F., Jin, H., Lu, C., Wu, X., et al. (2015). Curcumin regulates cell fate and metabolism by inhibiting hedgehog signaling in hepatic stellate cells. *Lab. Invest.* 95, 790–803. doi: 10.1038/labinvest.2015.59

Liu, N., He, S., Ma, L., Ponnusamy, M., Tang, J., Tolbert, E., et al. (2013). Blocking the class I histone deacetylase ameliorates renal fibrosis and inhibits renal fibroblast activation via modulating TGF-beta and EGFR signaling. *PLoS ONE.* 8:e54001. doi: 10.1371/journal.pone.0054001

Lleo, A., Zhang, W., Zhao, M., Tan, Y., Bernuzzi, F., Zhu, B., et al. (2015). DNA methylation profiling of the X chromosome reveals an aberrant demethylation on CXCR3 promoter in primary biliary cirrhosis. *Clin. Epigenetics* 7, 61. doi: 10.1186/s13148-015-0098-9

Lodder, J., Denaës, T., Chobert, M. N., Wan, J., El-Benna, J., Pawlotsky, J. M., et al. (2015). Macrophage autophagy protects against liver fibrosis in mice. *Autophagy* 11, 1280–1292. doi: 10.1080/15548627.2015.1058473

Lu, S. C. (2013). Glutathione synthesis. *Biochim. Biophys. Acta* 1830, 3143–3153. doi: 10.1016/j.bbagen.2012.09.008

Lua, I., James, D., Wang, J., Wang, K. S., and Asahina, K. (2014). Mesodermal mesenchymal cells give rise to myofibroblasts, but not epithelial cells, in mouse liver injury. *Hepatology* 60, 311–322. doi: 10.1002/hep.27035

Maeda, N., Kawada, N., Seki, S., Arakawa, T., Ikeda, K., Iwao, H., et al. (2003). Stimulation of proliferation of rat hepatic stellate cells by galectin-1 and galectin-3 through different intracellular signaling pathways. *J. Biol. Chem.* 278, 18938–18944. doi: 10.1074/jbc.M209673200

Maekawa, Y., Ishifune, C., Tsukumo, S., Hozumi, K., Yagita, H., and Yasutomo, K. (2015). Notch controls the survival of memory CD4+ T cells by regulating glucose uptake. *Nat. Med.* 21, 55–61. doi: 10.1038/nm.3758

Mallat, A., Lodder, J., Teixeira-Clerc, F., Moreau, R., Codogno, P., and Lotersztajn, S. (2014). Autophagy: a multifaceted partner in liver fibrosis. *Biomed Res. Int.* 2014:869390. doi: 10.1155/2014/869390

Manickam, N., Patel, M., Griendling, K. K., Gorin, Y., and Barnes, J. L. (2014). RhoA/Rho kinase mediates TGF-β1-induced kidney myofibroblast activation through Poldip2/Nox4-derived reactive oxygen species. *Am. J. Physiol. Renal Physiol.* 307, F159–F171. doi: 10.1152/ajprenal.00546.2013

Mann, D. A. (2014). Epigenetics in liver disease. *Hepatology* 60, 1418–1425. doi: 10.1002/hep.27131

Mann, J., Oakley, F., Akiboye, F., Elsharkawy, A., Thorne, A. W., and Mann, D. A. (2007). Regulation of myofibroblast transdifferentiation by DNA methylation and MeCP2: implications for wound healing and fibrogenesis. *Cell Death Differ.* 14, 275–285. doi: 10.1038/sj.cdd.4401979

Mannaerts, I., Eysackers, N., Onyema, O. O., Van Beneden, K., Valente, S., Mai, A., et al. (2013). Class II HDAC inhibition hampers hepatic stellate cell activation by induction of microRNA-29. *PLoS ONE* 8:e55786. doi: 10.1371/journal.pone.0055786

Mannaerts, I., Nuytten, N. R., Rogiers, V., Vanderkerken, K., van Grunsven, L. A., and Geerts, A. (2010). Chronic administration of valproic acid inhibits activation of mouse hepatic stellate cells *in vitro* and *in vivo*. *Hepatology* 51, 603–614. doi: 10.1002/hep.23334

Marrone, G., Maeso-Díaz, R., García-Cardena, G., Abraldes, J. G., García-Pagán, J. C., Bosch, J., et al. (2015). KLF2 exerts antifibrotic and vasoprotective effects in cirrhotic rat livers: behind the molecular mechanisms of statins. *Gut* 64, 1434–1443. doi: 10.1136/gutjnl-2014-308338

Marrone, G., Russo, L., Rosado, E., Hide, D., García-Cardeña, G., García-Pagán, J. C., et al. (2013). The transcription factor KLF2 mediates hepatic endothelial protection and paracrine endothelial-stellate cell deactivation induced by statins. *J. Hepatol.* 58, 98–103. doi: 10.1016/j.jhep.2012.08.026

Martínez-Martínez, E., Jurado-López, R., Valero-Muñoz, M., Bartolomé, M. V., Ballesteros, S., Luaces, M., et al. (2014). Leptin induces cardiac fibrosis through galectin-3, mTOR and oxidative stress: potential role in obesity. *J. Hypertens.* 32, 1104–1114; discussion 1114. doi: 10.1097/HJH.0000000000000149

Martinez-Outschoorn, U. E., Prisco, M., Ertel, A., Tsirigos, A., Lin, Z., Pavlides, S., et al. (2011). Ketones and lactate increase cancer cell "stemness," driving recurrence, metastasis and poor clinical outcome in breast cancer: achieving personalized medicine via metabolo-genomics. *Cell Cycle* 10, 1271–1286. doi: 10.4161/cc.10.8.15330

Mederacke, I., Hsu, C. C., Troeger, J. S., Huebener, P., Mu, X., Dapito, D. H., et al. (2013). Fate tracing reveals hepatic stellate cells as dominant contributors to liver fibrosis independent of its aetiology. *Nat. Commun.* 4:2823. doi: 10.1038/ncomms3823

Metallo, C. M., Gameiro, P. A., Bell, E. L., Mattaini, K. R., Yang, J., Hiller, K., et al. (2012). Reductive glutamine metabolism by IDH1 mediates lipogenesis under hypoxia. *Nature* 481, 380–384. doi: 10.1038/nature10602

Miller, J. D., Weiss, R. M., Serrano, K. M., Brooks, R. M. II, Berry, C. J., Zimmerman, K., et al. (2009). Lowering plasma cholesterol levels halts progression of aortic valve disease in mice. *Circulation* 119, 2693–2701. doi: 10.1161/CIRCULATIONAHA.108.834614

Mishra, R., and Simonson, M. S. (2008). Oleate induces a myofibroblast-like phenotype in mesangial cells. *Arterioscler. Thromb. Vasc. Biol.* 28, 541–547. doi: 10.1161/ATVBAHA.107.157339

Mullen, A. R., Wheaton, W. W., Jin, E. S., Chen, P. H., Sullivan, L. B., Cheng, T., et al. (2012). Reductive carboxylation supports growth in tumour cells with defective mitochondria. *Nature* 481, 385–388. doi: 10.1038/nature10642

Muzzey, D., and van Oudenaarden, A. (2009). Quantitative time-lapse fluorescence microscopy in single cells. *Annu. Rev. Cell Dev. Biol.* 25, 301–327. doi: 10.1146/annurev.cellbio.042308.113408

Nowicki, S., and Gottlieb, E. (2015). Oncometabolites: tailoring our genes. *FEBS J.* 282, 2796–2805. doi: 10.1111/febs.13295

O'Connor, J. W., and Gomez, E. W. (2013). Cell adhesion and shape regulate TGF-beta1-induced epithelial-myofibroblast transition via MRTF-A signaling. *PLoS ONE* 8:e83188. doi: 10.1371/journal.pone.0083188

O'Connor, J. W., Riley, P. N., Nalluri, S. M., Ashar, P. K., and Gomez, E. W. (2015). Matrix rigidity mediates TGFβ1-induced epithelial-myofibroblast transition by

controlling cytoskeletal organization and MRTF-A localization. *J. Cell. Physiol.* 230, 1829–1839. doi: 10.1002/jcp.24895

Ono, A., Utsugi, M., Masubuchi, K., Ishizuka, T., Kawata, T., Shimizu, Y., et al. (2009). Glutathione redox regulates TGF-beta-induced fibrogenic effects through Smad3 activation. *FEBS Lett.* 583, 357–362. doi: 10.1016/j.febslet.2008.12.021

Page, A., Paoli, P. P., Hill, S. J., Howarth, R., Wu, R., Kweon, S. M., et al. (2015). Alcohol directly stimulates epigenetic modifications in hepatic stellate cells. *J. Hepatol.* 62, 388–397. doi: 10.1016/j.jhep.2014.09.033

Pallett, L. J., Gill, U. S., Quaglia, A., Sinclair, L. V., Jover-Cobos, M., Schurich, A., et al. (2015). Metabolic regulation of hepatitis B immunopathology by myeloid-derived suppressor cells. *Nat. Med.* 21, 591–600. doi: 10.1038/nm.3856

Pannem, R. R., Dorn, C., Hellerbrand, C., and Massoumi, R. (2014). Cylindromatosis gene CYLD regulates hepatocyte growth factor expression in hepatic stellate cells through interaction with histone deacetylase 7. *Hepatology* 60, 1066–1081. doi: 10.1002/hep.27209

Park, K. C., Park, J. H., Jeon, J. Y., Kim, S. Y., Kim, J. M., Lim, C. Y., et al. (2014). A new histone deacetylase inhibitor improves liver fibrosis in BDL rats through suppression of hepatic stellate cells. *Br. J. Pharmacol.* 171, 4820–4830. doi: 10.1111/bph.12590

Park, M., Kim, Y. H., Woo, S. Y., Lee, H. J., Yu, Y., Kim, H. S., et al. (2015). Tonsil-derived mesenchymal stem cells ameliorate CCl4-induced liver fibrosis in mice via autophagy activation. *Sci. Rep.* 5:8616. doi: 10.1038/srep08616

Pellicoro, A., Ramachandran, P., Iredale, J. P., and Fallowfield, J. A. (2014). Liver fibrosis and repair: immune regulation of wound healing in a solid organ. *Nat. Rev. Immunol.* 14, 181–194. doi: 10.1038/nri3623

Perry, C. G., Kane, D. A., Lanza, I. R., and Neufer, P. D. (2013). Methods for assessing mitochondrial function in diabetes. *Diabetes* 62, 1041–1053. doi: 10.2337/db12-1219

Popova, E. N., Pletjushkina, O. Y., Dugina, V. B., Domnina, L. V., Ivanova, O. Y., Izyumov, D. S., et al. (2010). Scavenging of reactive oxygen species in mitochondria induces myofibroblast differentiation. *Antioxid. Redox Signal.* 13, 1297–1307. doi: 10.1089/ars.2009.2949

Popovic, R., and Licht, J. D. (2012). Emerging epigenetic targets and therapies in cancer medicine. *Cancer Discov.* 2, 405–413. doi: 10.1158/2159-8290.CD-12-0076

Portela, A., and Esteller, M. (2010). Epigenetic modifications and human disease. *Nat. Biotechnol.* 28, 1057–1068. doi: 10.1038/nbt.1685

Porter, K. E., Turner, N. A., O'Regan, D. J., Balmforth, A. J., and Ball, S. G. (2004). Simvastatin reduces human atrial myofibroblast proliferation independently of cholesterol lowering via inhibition of RhoA. *Cardiovasc. Res.* 61, 745–755. doi: 10.1016/j.cardiores.2003.11.032

Prosser, C. C., Yen, R. D., and Wu, J. (2006). Molecular therapy for hepatic injury and fibrosis: where are we? *World J. Gastroenterol.* 12, 509–515. doi: 10.3748/wjg.v12.i4.509

Qin, L., and Han, Y. P. (2010). Epigenetic repression of matrix metalloproteinases in myofibroblastic hepatic stellate cells through histone deacetylases 4: implication in tissue fibrosis. *Am. J. Pathol.* 177, 1915–1928. doi: 10.2353/ajpath.2010.100011

Qu, X., Zhang, X., Yao, J., Song, J., Nikolic-Paterson, D. J., and Li, J. (2012). Resolvins E1 and D1 inhibit interstitial fibrosis in the obstructed kidney via inhibition of local fibroblast proliferation. *J. Pathol.* 228, 506–519. doi: 10.1002/path.4050

Rautou, P. E., Mansouri, A., Lebrec, D., Durand, F., Valla, D., and Moreau, R. (2010). Autophagy in liver diseases. *J. Hepatol.* 53, 1123–1134. doi: 10.1016/j.jhep.2010.07.006

Reddy, A. T., Lakshmi, S. P., Zhang, Y., and Reddy, R. C. (2014). Nitrated fatty acids reverse pulmonary fibrosis by dedifferentiating myofibroblasts and promoting collagen uptake by alveolar macrophages. *FASEB J.* 28, 5299–5310. doi: 10.1096/fj.14-256263

Rinella, M. E. (2015). Nonalcoholic fatty liver disease: a systematic review. *JAMA* 313, 2263–2273. doi: 10.1001/jama.2015.5370

Rodrigues, T. B., Serrao, E. M., Kennedy, B. W., Hu, D. E., Kettunen, M. I., and Brindle, K. M. (2014). Magnetic resonance imaging of tumor glycolysis using hyperpolarized 13C-labeled glucose. *Nat. Med.* 20, 93–97. doi: 10.1038/nm.3416

Rombouts, K., Kisanga, E., Hellemans, K., Wielant, A., Schuppan, D., and Geerts, A. (2003). Effect of HMG-CoA reductase inhibitors on proliferation and protein synthesis by rat hepatic stellate cells. *J. Hepatol.* 38, 564–572. doi: 10.1016/S0168-8278(03)00051-5

Ryall, J. G., Dell'Orso, S., Derfoul, A., Juan, A., Zare, H., Feng, X., et al. (2015). The NAD(+)-dependent SIRT1 deacetylase translates a metabolic switch into regulatory epigenetics in skeletal muscle stem cells. *Cell Stem Cell.* 16, 171–183. doi: 10.1016/j.stem.2014.12.004

Sampson, N., Berger, P., and Zenzmaier, C. (2014). Redox signaling as a therapeutic target to inhibit myofibroblast activation in degenerative fibrotic disease. *Biomed. Res. Int.* 2014:131737. doi: 10.1155/2014/131737

Sancho, P., Mainez, J., Crosas-Molist, E., Roncero, C., Fernández-Rodriguez, C. M., Pinedo, F., et al. (2012). NADPH oxidase NOX4 mediates stellate cell activation and hepatocyte cell death during liver fibrosis development. *PLoS ONE* 7:e45285. doi: 10.1371/journal.pone.0045285

Sanders, Y. Y., Hagood, J. S., Liu, H., Zhang, W., Ambalavanan, N., and Thannickal, V. J. (2014). Histone deacetylase inhibition promotes fibroblast apoptosis and ameliorates pulmonary fibrosis in mice. *Eur. Respir. J.* 43, 1448–1458. doi: 10.1183/09031936.00095113

Savolainen, E. R., Leo, M. A., Timpl, R., and Lieber, C. S. (1984). Acetaldehyde and lactate stimulate collagen synthesis of cultured baboon liver myofibroblasts. *Gastroenterology* 87, 777–787.

Schmidt, A., Braeuning, A., Ruck, P., Seitz, G., Armeanu-Ebinger, S., Fuchs, J., et al. (2011). Differential expression of glutamine synthetase and cytochrome P450 isoforms in human hepatoblastoma. *Toxicology* 281, 7–14. doi: 10.1016/j.tox.2011.01.006

Schuetze, K. B., McKinsey, T. A., and Long, C. S. (2014). Targeting cardiac fibroblasts to treat fibrosis of the heart: focus on HDACs. *J. Mol. Cell. Cardiol.* 70, 100–107. doi: 10.1016/j.yjmcc.2014.02.015

Seki, E., and Schwabe, R. F. (2015). Hepatic inflammation and fibrosis: functional links and key pathways. *Hepatology* 61, 1066–1079. doi: 10.1002/hep.27332

Serhan, C. N. (2014). Pro-resolving lipid mediators are leads for resolution physiology. *Nature* 510, 92–101. doi: 10.1038/nature13479

Shaker, M. E., Ghani, A., Shiha, G. E., Ibrahim, T. M., and Mehal, W. Z. (2013). Nilotinib induces apoptosis and autophagic cell death of activated hepatic stellate cells via inhibition of histone deacetylases. *Biochim. Biophys. Acta* 1833, 1992–2003. doi: 10.1016/j.bbamcr.2013.02.033

Shi, S. Y., Lu, S. Y., Sivasubramaniyam, T., Revelo, X. S., Cai, E. P., Luk, C. T., et al. (2015). DJ-1 links muscle ROS production with metabolic reprogramming and systemic energy homeostasis in mice. *Nat. Commun.* 6:7415. doi: 10.1038/ncomms8415

Shull, A. Y., Noonepalle, S. K., Lee, E. J., Choi, J. H., and Shi, H. (2015). Sequencing the cancer methylome. *Methods Mol. Biol.* 1238, 627–651. doi: 10.1007/978-1-4939-1804-1_33

Singh, R., Kaushik, S., Wang, Y., Xiang, Y., Novak, I., Komatsu, M., et al. (2009). Autophagy regulates lipid metabolism. *Nature* 458, 1131–1135. doi: 10.1038/nature07976

Sirtori, C. R. (2014). The pharmacology of statins. *Pharmacol. Res.* 88, 3–11. doi: 10.1016/j.phrs.2014.03.002

Sisson, T. H., Ajayi, I. O., Subbotina, N., Dodi, A. E., Rodansky, E. S., Chibucos, L. N., et al. (2015). Inhibition of myocardin-related transcription factor/serum response factor signaling decreases lung fibrosis and promotes mesenchymal cell apoptosis. *Am. J. Pathol.* 185, 969–986. doi: 10.1016/j.ajpath.2014.12.005

Son, J., Lyssiotis, C. A., Ying, H., Wang, X., Hua, S., Ligorio, M., et al. (2013). Glutamine supports pancreatic cancer growth through a KRAS-regulated metabolic pathway. *Nature* 496, 101–105. doi: 10.1038/nature12040

Sosulski, M. L., Gongora, R., Danchuk, S., Dong, C., Luo, F., and Sanchez, C. G. (2015). Deregulation of selective autophagy during aging and pulmonary fibrosis: the role of TGFβ1. *Aging Cell.* 14, 774–783. doi: 10.1111/acel.12357

Sullivan, L. B., Martinez-Garcia, E., Nguyen, H., Mullen, A. R., Dufour, E., Sudarshan, S., et al. (2013). The proto-oncometabolite fumarate binds glutathione to amplify ROS-dependent signaling. *Mol. Cell* 51, 236–248. doi: 10.1016/j.molcel.2013.05.003

Swiderska-Syn, M., Syn, W. K., Xie, G., Krüger, L., Machado, M. V., Karaca, G., et al. (2014). Myofibroblastic cells function as progenitors to regenerate murine livers after partial hepatectomy. *Gut* 63, 1333–1344. doi: 10.1136/gutjnl-2013-305962

Takeshita, Y., Takamura, T., Honda, M., Kita, Y., Zen, Y., Kato, K., et al. (2014). The effects of ezetimibe on non-alcoholic fatty liver disease and glucose metabolism:

a randomised controlled trial. *Diabetologia* 57, 878–890. doi: 10.1007/s00125-013-3149-9

Tang, J., Fang, F., Miller, D. F., Pilrose, J. M., Matei, D., Huang, T. H., et al. (2015). Global DNA methylation profiling technologies and the ovarian cancer methylome. *Methods Mol. Biol.* 1238, 653–675. doi: 10.1007/978-1-4939-1804-1_34

Testerink, N., Ajat, M., Houweling, M., Brouwers, J. F., Pully, V. V., van Manen, H. J., et al. (2012). Replacement of retinyl esters by polyunsaturated triacylglycerol species in lipid droplets of hepatic stellate cells during activation. *PLoS ONE* 7:e34945. doi: 10.1371/journal.pone.0034945

Thoen, L. F., Guimarães, E. L., Dollé, L., Mannaerts, I., Najimi, M., Sokal, E., et al. (2011). A role for autophagy during hepatic stellate cell activation. *J. Hepatol.* 55, 1353–1360. doi: 10.1016/j.jhep.2011.07.010

Tian, W., Hao, C., Fan, Z., Weng, X., Qin, H., Wu, X., et al. (2015). Myocardin related transcription factor A programs epigenetic activation of hepatic stellate cells. *J. Hepatol.* 62, 165–174. doi: 10.1016/j.jhep.2014.07.029

Tobar, N., Toyos, M., Urra, C., Méndez, N., Arancibia, R., Smith, P. C., et al. (2014). c-Jun N terminal kinase modulates NOX-4 derived ROS production and myofibroblasts differentiation in human breast stromal cells. *BMC Cancer* 14:640. doi: 10.1186/1471-2407-14-640

Tomita, K., Teratani, T., Suzuki, T., Shimizu, M., Sato, H., Narimatsu, K., et al. (2014). Free cholesterol accumulation in hepatic stellate cells: mechanism of liver fibrosis aggravation in nonalcoholic steatohepatitis in mice. *Hepatology* 59, 154–169. doi: 10.1002/hep.26604

Trebicka, J., Hennenberg, M., Odenthal, M., Shir, K., Klein, S., Granzow, M., et al. (2010). Atorvastatin attenuates hepatic fibrosis in rats after bile duct ligation via decreased turnover of hepatic stellate cells. *J. Hepatol.* 53, 702–712. doi: 10.1016/j.jhep.2010.04.025

Trostchansky, A., and Rubbo, H. (2008). Nitrated fatty acids: mechanisms of formation, chemical characterization, and biological properties. *Free Radic. Biol. Med.* 44, 1887–1896. doi: 10.1016/j.freeradbiomed.2008.03.006

Untergasser, G., Gander, R., Lilg, C., Lepperdinger, G., Plas, E., and Berger, P. (2005). Profiling molecular targets of TGF-beta1 in prostate fibroblast-to-myofibroblast transdifferentiation. *Mech. Ageing Dev.* 126, 59–69. doi: 10.1016/j.mad.2004.09.023

Vander Heiden, M. G., Cantley, L. C., and Thompson, C. B. (2009). Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* 324, 1029–1033. doi: 10.1126/science.1160809

Velasquez, L. S., Sutherland, L. B., Liu, Z., Grinnell, F., Kamm, K. E., Schneider, J. W., et al. (2013). Activation of MRTF-A-dependent gene expression with a small molecule promotes myofibroblast differentiation and wound healing. *Proc. Natl. Acad. Sci. U.S.A.* 110, 16850–16855. doi: 10.1073/pnas.1316764110

Vozenin-Brotons, M. C., Sivan, V., Gault, N., Renard, C., Geffrotin, C., Delanian, S., et al. (2001). Antifibrotic action of Cu/Zn SOD is mediated by TGF-beta1 repression and phenotypic reversion of myofibroblasts. *Free Radic. Biol. Med.* 30, 30–42. doi: 10.1016/S0891-5849(00)00431-7

Vyas-Read, S., Wang, W., Kato, S., Colvocoresses-Dodds, J., Fifadara, N. H., Gauthier, T. W., et al. (2014). Hyperoxia induces alveolar epithelial-to-mesenchymal cell transition. *Am. J. Physiol. Lung Cell Mol. Physiol.* 306, L326–L340. doi: 10.1152/ajplung.00074.2013

Wang, W., Zhao, C., Zhou, J., Zhen, Z., Wang, Y., and Shen, C. (2013). Simvastatin ameliorates liver fibrosis via mediating nitric oxide synthase in rats with non-alcoholic steatohepatitis-related liver fibrosis. *PLoS ONE* 8:e76538. doi: 10.1371/journal.pone.0076538

Williams, M. J., Clouston, A. D., and Forbes, S. J. (2014). Links between hepatic fibrosis, ductular reaction, and progenitor cell expansion. *Gastroenterology* 146, 349–356. doi: 10.1053/j.gastro.2013.11.034

Witt, W., Büttner, P., Jannasch, A., Matschke, K., and Waldow, T. (2014). Reversal of myofibroblastic activation by polyunsaturated fatty acids in valvular interstitial cells from aortic valves. Role of RhoA/G-actin/MRTF signalling. *J. Mol. Cell Cardiol.* 74, 127–138. doi: 10.1016/j.yjmcc.2014.05.008

Xie, N., Tan, Z., Banerjee, S., Cui, H., Ge, J., Liu, R. M., et al. (2015). Glycolytic reprogramming in myofibroblast differentiation and lung fibrosis. *Am. J. Respir. Crit. Care Med.* 192, 1462–1474. doi: 10.1164/rccm.201504-0780OC

Xu, J., Cong, M., Park, T. J., Scholten, D., Brenner, D. A., and Kisseleva, T. (2015). Contribution of bone marrow-derived fibrocytes to liver fibrosis. *Hepatobiliary Surg. Nutr.* 4, 34–47. doi: 10.3978/j.issn.2304-3881

Xu, L., Hui, A. Y., Albanis, E., Arthur, M. J., O'Byrne, S. M., Blaner, W. S., et al. (2005). Human hepatic stellate cell lines, LX-1 and LX-2: new tools for analysis of hepatic fibrosis. *Gut* 54, 142–151. doi: 10.1136/gut.2004.042127

Xu, W., Yang, H., Liu, Y., Yang, Y., Wang, P., Kim, S. H., et al. (2011). Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of α-ketoglutarate-dependent dioxygenases. *Cancer Cell* 19, 17–30. doi: 10.1016/j.ccr.2010.12.014

Xu, Z. Z., Zhang, L., Liu, T., Park, J. Y., Berta, T., Yang, R., et al. (2010). Resolvins RvE1 and RvD1 attenuate inflammatory pain via central and peripheral actions. *Nat Med.* 16, 592–597, 1p following 597. doi: 10.1038/nm.2123

Yan, C., Zhou, L., and Han, Y. P. (2008). Contribution of hepatic stellate cells and matrix metalloproteinase 9 in acute liver failure. *Liver Int.* 28, 959–971. doi: 10.1111/j.1478-3231.2008.01775.x

Yang, J. I., Yoon, J. H., Bang, Y. J., Lee, S. H., Lee, S. M., Byun, H. J., et al. (2010). Synergistic antifibrotic efficacy of statin and protein kinase C inhibitor in hepatic fibrosis. *Am. J. Physiol. Gastrointest. Liver Physiol.* 298, G126–G132. doi: 10.1152/ajpgi.00299.2009

Yang, J. J., Tao, H., Huang, C., Shi, K. H., Ma, T. T., Bian, E. B., et al. (2013a). DNA methylation and MeCP2 regulation of PTCH1 expression during rats hepatic fibrosis. *Cell Signal.* 25, 1202–1211. doi: 10.1016/j.cellsig.2013.01.005

Yang, M. D., Chiang, Y. M., Higashiyama, R., Asahina, K., Mann, D. A., Mann, J., et al. (2012). Rosmarinic acid and baicalin epigenetically derepress peroxisomal proliferator-activated receptor γ in hepatic stellate cells for their antifibrotic effect. *Hepatology* 55, 1271–1281. doi: 10.1002/hep.24792

Yang, Y., Wang, Z., Yang, H., Wang, L., Gillespie, S. R., Wolosin, J. M., et al. (2013b). TRPV1 potentiates TGFβ-induction of corneal myofibroblast development through an oxidative stress-mediated p38-SMAD2 signaling loop. *PLoS ONE* 8:e77300. doi: 10.1371/journal.pone.0077300

Zamboni, N., Fendt, S. M., Rühl, M., and Sauer, U. (2009). (13)C-based metabolic flux analysis. *Nat. Protoc.* 4, 878–892. doi: 10.1038/nprot.2009.58

Zhang, J., Nuebel, E., Wisidagama, D. R., Setoguchi, K., Hong, J. S., Van Horn, C. M., et al. (2012). Measuring energy metabolism in cultured cells, including human pluripotent stem cells and differentiated cells. *Nat. Protoc.* 7, 1068–1085. doi: 10.1038/nprot.2012.048

Zhang, M., Ye, Y., Wang, F., Zhu, J., Zhao, Q., Zheng, Y., et al. (2014). Liver myofibroblasts up-regulate monocyte CD163 expression via PGE2 during hepatitis B induced liver failure. *J. Transl. Med.* 12:60. doi: 10.1186/1479-5876-12-60

Zhang, X., Liu, H., Hock, T., Thannickal, V. J., and Sanders, Y. Y. (2013). Histone deacetylase inhibition downregulates collagen 3A1 in fibrotic lung fibroblasts. *Int. J. Mol. Sci.* 14, 19605–19617. doi: 10.3390/ijms141019605

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REVIEW
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The role of miRNAs in stress-responsive hepatic stellate cells during liver fibrosis

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The progression of liver fibrosis and cirrhosis is associated with the persistence of an injury causing agent, leading to changes in the extracellular environment and a disruption of the cellular homeostasis of liver resident cells. Recruitment of inflammatory cells, apoptosis of hepatocytes, and changes in liver microvasculature are some examples of changing cellular environment that lead to the induction of stress responses in nearby cells. During liver fibrosis, the major stresses include hypoxia, oxidative stress, and endoplasmic reticulum stress. When hepatic stellate cells (HSCs) are subjected to such stress, they modulate fibrosis progression by induction of their activation toward a myofibroblastic phenotype, or by undergoing apoptosis, and thus helping fibrosis resolution. It is widely accepted that microRNAs are import regulators of gene expression, both during normal cellular homeostasis, as well as in pathologic conditions. MicroRNAs are short RNA sequences that regulate the gene expression by mRNA destabilization and inhibition of mRNA translation. Specific microRNAs have been identified to play a role in the activation process of HSCs on the one hand and in stress-responsive pathways on the other hand in other cell types (**Table 2**). However, so far there are no reports for the involvement of miRNAs in the different stress responses linked to HSC activation. Here, we review briefly the major stress response pathways and propose several miRNAs to be regulated by these stress responsive pathways in activating HSCs, and discuss their potential specific pro-or anti-fibrotic characteristics.

Keywords: miRNAs, hepatic stellate cells, fibrosis, ER stress, hypoxia, oxidative stress

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Introduction

Liver fibrosis is the pathological condition of the liver resulting from sustained wound healing in response to chronic liver injury. Multiple factors can lead to such injury, including genetic (the accumulation of misfolded alpha1-antitrypsin), cholestatic (sclerosing cholangitis), metabolic (non-alcoholic fatty liver disease and non-alcoholic steatohepatitis), drug induced (paracetamol-intoxication and alcohol) and viral diseases (hepatitis B and C) (Friedman, 2003; Wallace et al., 2008). Liver fibrosis can eventually progress toward cirrhosis, which is characterized by the loss of endothelial fenestrations, excessive scar formation in the space of Disse, and the presence of vascularized fibrotic septa. These distortions of liver architecture and subsequent cellular homeostasis lead to impaired organ function, ascites, encephalopathy, variceal hemorrhage, portal hypertension and the development of hepatocellular carcinoma (Schuppan and Afdhal, 2008).

Role of miRNAs during Hepatic Stellate Cell Activation

One of the key features in the development of liver fibrosis is the augmenting presence of myofibroblasts in the liver. Myofibroblasts are characterized by their stellate shape, the expression of some specific proteins, such as alpha-smooth muscle actin (α-SMA), and the excessive production of extracellular matrix proteins, including fibronectin and collagen type I, III, and IV. Hepatic stellate cells (HSCs) transdifferentiate upon injury into myofibroblasts, and can be considered as the major origin of myofibroblasts (Mederacke et al., 2013). During initiation and progression of the liver fibrosis process, the liver is subjected to various kinds of stress including hypoxia (Nath and Szabo, 2012), oxidative stress (Parola and Robino, 2001), and endoplasmic reticulum (ER) stress (Li et al., 2015). HSCs will respond by activating into myofibroblasts, which is characterized by a change in gene (Jiang et al., 2006; De Minicis et al., 2007) and microRNA expression (Guo et al., 2009a), as reviewed in He et al. (2012a); Huang et al. (2014a) and Coll et al. (2015). Numerous detailed reports on gene expression changes during HSC activation are available, but information regarding their regulation by specific miRNAs remains rather vague.

MiRNAs are short non-protein coding RNA sequences of 20–23 nucleotides that are evolutionary conserved and are encoded in the genome. The human genome is supposed to encode for approximately 1000 miRNAs, which can be expressed in an ubiquitous or a tissue/cell-type specific way (Lee, 2013), and each of these miRNAs is thought to have a great range of potential targets, thus indicating its importance in gene regulation (Bartel and Chen, 2004). MiRNA-encoding genes are transcribed by RNA polymerase II, with the generation of primary miRNA, which will then be processed in the nucleus by activity of a microprocessor complex, named Drosha. The activity of this Drosha containing complex leads to the production of a hairpin-shaped premature miRNA defined by a length of approximately 70 nucleotides and the presence of a stem-loop structure (Lee et al., 2003; Gregory et al., 2004). Correctly processed premature miRNAs are then bound by Exportin-5 in a Ran guanosine triphosphate (RanGTP)-dependent manner, leading to the transport of these pre-miRNAs toward the cytoplasm (Lund et al., 2004). In the cytoplasm, the pre-miRNAs undergo processing by Dicer, another ribonuclease III enzyme, resulting in the production of double stranded RNA (dsRNA) of 20–23 nucleotides (Bernstein et al., 2001). In this double stranded nucleotide-complex, a mature miRNA strand, known as the guide strand, and a miRNA* strand, known as the passenger strand can be identified. The mature miRNA strand will be loaded into the Argonaute 2 (Ago2)-containing RNA-induced silencing complex (RISC), which is the effector of miRNA-mediated activities (Gregory et al., 2005). It is believed that the RISC complex can cause down-regulation of gene expression through 2 mechanisms; by an inhibition of mRNA translation or by reducing the mRNA stability and thus facilitating the degradation (**Figure 1**) (Bagga et al., 2005; Orban and Izaurralde, 2005; Pillai et al., 2005).

Since the discovery of miRNAs in 1993 (Wightman et al., 1993), researchers continuously tried to evoke the role of miRNAs in cellular homeostasis and in development of pathological conditions, including liver fibrosis. There are many miRNAs expressed during, and described to be involved in, HSC activation (**Table 1**), making them the topic of concise reviews (He et al., 2012a; Huang et al., 2014a). Here, we only briefly highlight some key miRNAs to illustrate the possible roles a miRNA could have in quiescent or activated HSCs. When evaluating these miRNA-studies it is important to keep in mind that although many miRNAs are conserved among eukaryotic organisms, it is possible that they do not display the same expression patterns in specific (pathological) processes, and thus can display interspecies differences in expression (Ha et al., 2008).

miR-29

miR-29 is the first and most thoroughly investigated miRNA-family in HSCs. miR-29a, miR-29b, and miR-29c are all down-regulated during the *in vitro* activation of isolated rat and mouse HSCs, and in liver biopsies from patients with advanced liver fibrosis. This down-regulation is promoted by transforming growth factor-β (TGF-β) and factors like inflammatory signals including lipopolysaccharide (LPS) and nuclear factor kappa B (NF-κB) (Roderburg et al., 2011). The miR-29 family is of importance for HSC activation, as they can bind to 3′-UTR collagen types I and IV (Kwiecinski et al., 2011). Consequently, miR-29 overexpression in HSCs reduces Collagen I and IV synthesis (Roderburg et al., 2011) and maintenance of the quiescent morphology (Sekiya et al., 2011). In addition to collagen targeting, PDGF-C and IGF-I are identified as targets of miR-29, with PDGF-C having pro-mitogenic and migratory capacities, and IGF-I being an important mitogenic factor when present in an autocrine manner in combination with PDGF-BB (Kwiecinski et al., 2012). In support with these findings, miR-29a/b levels were found to decrease in CCl_4-treated male mice. Interestingly, female mice do not show this decrease, most likely due to differences in E2, which can induce miR-29a/b levels (Zhang et al., 2012). Not only collagen production, but also other aspects of HSC activation such as inflammatory response and cell proliferation can be regulated by miRNAs such as is the case for miR-146a and miR-16, respectively.

miR-146

miR-146 is also down-regulated during TGF-β-induced HSC activation (He et al., 2012b), while overexpression of miR-146a in HSCs leads to up-regulation of tissue inhibitor of metalloproteinase 3 (TIMP-3) and down-regulation of IL-6 mRNA (Maubach et al., 2011). In another study, overexpression of miR-146a lead to inhibition of proliferation of activated HSCs. This would be the result of direct binding to the promoter region of the SMAD4 mRNA, which regulates TGF-β1-mediated gene expression, thus leaving the cell insensitive to TGF-β1 stimulation (He et al., 2012b), demonstrating its importance in the inflammatory response, and its link with liver fibrosis. In addition, miR-146a is known to have a role in the inflammatory response during liver reperfusion injury, as it negatively regulates IL-1 receptor-associated kinase 1 (IRAK1)

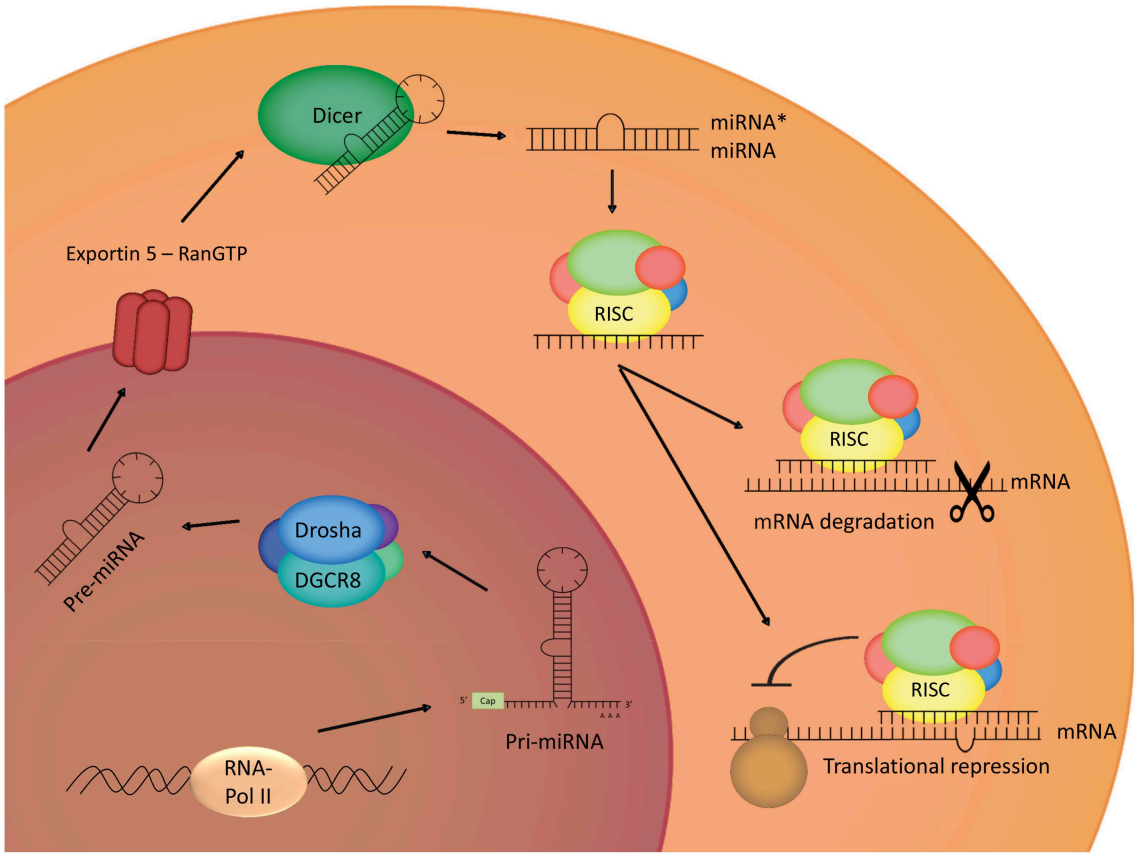

FIGURE 1 | MiRNA biogenesis. Transcription of the genes coding for miRNAs leads to the generation of primary miRNAs, which will be cleaved in the nucleus by Drosha, a ribonuclease III complex. The produced ribonucleic structure is called premature miRNA, and will be transported to the cytoplasm by Exportin 5, where it will undergo cleaving by Dicer, another ribonuclease III enzyme. One strain of the double-stranded obtained structure will integrate in the RISC-complex, leading to translational repression, or degradation of the target mRNA.

and Toll-like receptor-associated factor 6 (TRAF6), leading to a decrease in pro-inflammatory cytokine production, and by inhibiting the pro-inflammatory NF-κB pathway (Jiang et al., 2014). MiR-126 represents another miRNA that can regulate the NF-κB pathway by suppressing the expression of NF-κB inhibitor alpha (IκBα), thus leading to NF-κB activation (Feng et al., 2015).

MiR-16

miR-16 is another down-regulated miRNA during HSC activation. This miRNA has been shown to inhibit the expression of Cyclin D1, an important regulator of the cell cycle pathway. Expression levels of miR-16 and Cyclin D1 are inversely correlated in activating HSCs. Overexpression of this miRNA in activated HSCs leads to accumulation of the cells in the G0/G1-phase or G0/G1 to S-phase of cell cycle progression (Guo et al., 2009c). In HSCs, miR-16 also acts as an anti-apoptotic regulator in HSCs, by inhibition of B-cell lymphoma 2 (Bcl-2) translation, a known anti-apoptotic gene, leading to the enhanced expression levels of the underlying caspase-pathway consisting of caspases 3, 8, and 9, and thus induction of apoptosis (Guo et al., 2009b).

Function of Stress-Responsive Pathways and Possible Contribution of miRNAs during HSC Activation

As mentioned before, HSCs will undergo an activation process in the presence of different (fibrogenic) stimuli like liver injury, paracrine stimulation and autocrine regulation. This activation changes the quiescent fat storing cells into fibrogenic, proliferative and contractile myofibroblasts characterized by their expression of abundant intracellular filaments like α-SMA and vimentin, secretion of ECM including collagen type I and III and fibronectin and their high contractility (Kisseleva and Brenner, 2013). The contribution of stress response pathways in liver fibrosis, cirrhosis and to the HSC activation is generally accepted (Parola and Robino, 2001; Nath and Szabo, 2012; Li et al., 2015), but cannot be interpreted as a simple cause and consequence reaction. As literature mainly describes the contribution of hypoxia (Nath and Szabo, 2012), oxidative stress (Parola and Robino, 2001), and ER stress (Li et al., 2015) pathways during liver fibrosis and cirrhosis progression (**Figure 2**), we will focus on these three pathways.

TABLE 1 | Significantly regulated miRNAs during HSC-activation.

References	Up-regulated	Down-regulated
MiRNAs REGULATED DURING HSC ACTIVATION		
Guo et al., 2009b	miR−29c*, −138, −140, **−143**, **−193**, −207, −325 − 5p, −328, −349, −501, −872, −874	miR−15, **−16**, −20*b* − 3p, −92b, −122, **−126**, **−146a**, −341, −375
Ji et al., 2009	miR-27a, −27b, −30a, −30c, −30d, −130a, −130b, −450, −455	miR-9, **−19b**, −301, −520b, −520c, −721
Maubach et al., 2011	Let-7b, −7c, −7e, miR-**125b**, −21, −22, −31, −132, **−143**, **−145**, **−152**, **−199a**, −210, **−214**, **−221**, **−222**	Let-7f, miR**−10a**, **−16**, −26b, **−29a**, −30*a* − 5p, −30b, −30c, −30d, −99a, −122a, −125a, **−126**, **−146a**, **−150**, −151*, −181a, **−192**, **−194**, **−195**, −207, −296, **−335**, −422b, −483
Chen et al., 2011	miR-31, −34b, **−34c**, **−125b−5p**, **−143**, **−145**, **−152**, **−193**, **−199a −5p**, −199a−3p, **−214**, −218, **−221**, **−222**, −301a,−345−5p, −425	miR-**10a−5p**, −101a, **−126**, −126*, −139−5p, **−150**, **−192**, **−195**, **−335**, −338, −378*, −450a, −497, −877
Lakner et al., 2012	miR**−34c**, −184, **−221**	miR**−16**, −19a, **−19b**, **−29a**, −29c, −92a, **−150**, **−194**

*Summary of published data regarding microRNA microarray profiling of activating primary rat HSCs. MiRNAs which display an overlap in different published data sets are displayed in bold. *Mature miRNA derived from the 5′ arm of the precursor RNA also known as passenger strand.*

Specific stress-related genes can be quickly switched on and off in presence or absence of environmental stress-inducing factors and this can be mediated by miRNAs (Babar et al., 2008; Leung and Sharp, 2010) (**Table 2**, right panel). So far there are no reports describing the functionality of specific miRNAs in these stress response pathways of activating HSCs during liver fibrosis. However, assumptions about miRNAs forming the link in stress-responsive HSCs (**Table 2**) and their potential functions in these conditions can be made based on the available data and will be discussed here. We should keep in mind that the presence or lack of overlap in miRNA expression pattern can be due to cell-type and species-specificity and is no proof for actual involvement of the miRNA in stress responsive HSCs, and should be elucidated in future research.

Hypoxia Regulated miRNAs

In the process of liver fibrosis and cirrhosis, hypoxia in the liver cells can be due to disruption of the normal hepatic blood flow, damage of the microvasculature, and excessive deposition of extracellular matrix in the sinusoidal space (Copple et al., 2006). Cellular hypoxia leads to the activation of several Hypoxia Inducible Factors (HIFs), a family of transcriptional factors that work as key regulators for the maintenance of cellular homeostasis when confronted with low oxygen levels (Paternostro et al., 2010). At normal cellular oxygen levels, the oxygen-dependent hypoxia inducible factor HIF-1α (HIF-1α) is hydroxylated by members of the prolyl hydroxylase family (PHD), leading to the rapid degradation of this protein. Decrease of the cellular oxygen levels leads to loss of function of PHD, and subsequent accumulation and translocation of HIF-1α/HIF-2α to the nucleus. In the nucleus, the functional HIF transcription factor complex is formed consisting of HIF-α, HIF-1β and some hypoxic responsive elements (Semenza, 2007). HIF regulates certain processes such as angiogenesis, iron metabolism, glycolysis, and pH control (Jiang et al., 1996; Rosmorduc et al., 1999; Moon et al., 2009). Hypoxic conditions lead to activation of the HSC cell line LX-2 as illustrated by an up-regulation of α-SMA and collagen I protein levels, possibly through activation of the Smad/TGF-β pathway (Shi et al., 2007). HIF is proposed as a main regulator of hypoxia-mediated HSC activation, since it can act as a regulator and stimulator of profibrogenic mediators such as platelet-derived growth factor (PDGF) A and B, plasminogen activator inhibitor-1, and vascular epithelial growth factor (VEGF) (Forsythe et al., 1996; Moon et al., 2009; Wang et al., 2013). The essential role of HIF-1α during hypoxia-induced HSC activation was confirmed *in vitro* by inhibition of HSC-activation due to silencing of HIF-1α (Wang et al., 2013), and the reduced expression of activation genes in HIF-1α-deficient HSCs undergoing hypoxia (Copple et al., 2011). *In vivo* experiments using bile duct ligated (BDL) Hif-1α-deficient and control mice, showed less fibrosis in Hif-1α-deficient mice, as observed by lower levels of α-SMA and type I collagen, thus further indicating its importance during liver fibrosis (Moon et al., 2009).

MiRNAs can act down-stream and up-stream of the HIF pathway. For example, miR-210 expression is directly regulated by HIF-1α as it can bind to the hypoxia responsive element (HRE) located up-stream of the transcription start site of miR-210, leading to its enhanced transcription (Huang et al., 2009). It is suggested, that HIF-2α would mediate miR-210 expression in the absence of HIF-1α, also by interaction with consensus HREs in the miR-210 promoter region (Zhang et al., 2009). MiR-210 effects a broad variety of cellular processes such as fine-tuning cell proliferation by targeting e2f transcription factor 3 (E2f3) (Giannakakis et al., 2008) and MNT, a known MYC antagonist, and a member of the Myc/Max/Mad network (Zhang et al., 2009) while regulating apoptosis by controlling expression of the pro-apoptotic FLICE-associated huge protein (FLASH)/caspase-8-associated protein 2 (Casp8ap2) (Kim et al., 2009). Genes such as Nptx1, Rad52, Acvr1b, Fgrl, Hoxa1, and Hoxa9 associated with pathways like angiogenesis, tumor invasion, regulation of the mitochondrial metabolism, and DNA damage repair were also found to be miR-210 targets (Fasanaro et al., 2009; Huang et al., 2009). The hypoxia-induced up-regulation of miR-210 in various cancer cell lines (Huang et al., 2009) displays an overlap with its

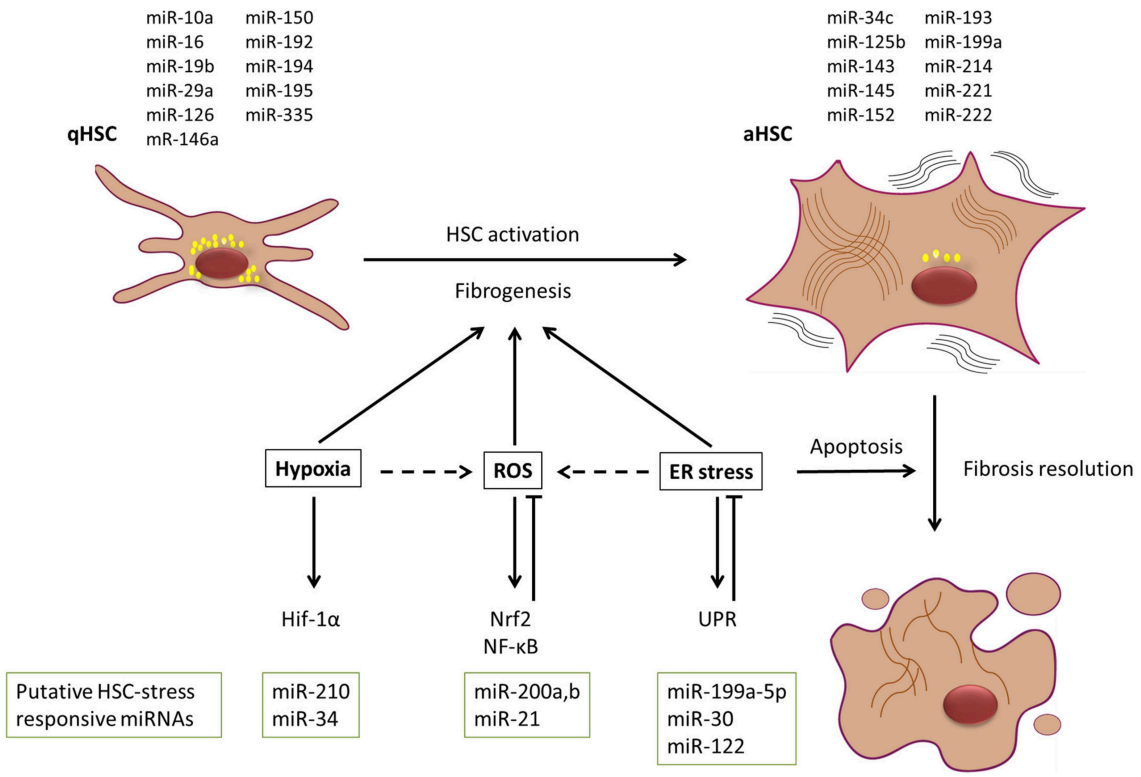

FIGURE 2 | Dynamic contribution of stress stimuli and miRNAs to liver fibrosis progression and resolution. HSCs are major contributors to the myofibroblastic cell pool in the fibrotic liver. In the presence of various activation stimuli, HSCs will undergo a myofibroblastic transdifferentiation process toward an activated state, which is characterized by a change in miRNA and mRNA expression pattern. It is widely accepted that the presence of hypoxia, oxidative stress (ROS), and endoplasmic reticulum (ER) stress most likely supports this activation process. However, ER stress could have a potential dual role in the process, as it can also lead to induction of apoptosis in activated HSCs, and thus could contribute to resolution of fibrosis. Simplified representation of some of the signaling cascades and potential miRNAs involved in these stress responses are given. MiRNAs depicted above the HSCs have been reported to be enriched in either qHSC or aHSCs. Putative HSC-stress responsive miRNAs that are discussed in the text are depicted below the signaling cascades.

enhanced expression during the activation process of HSCs, thus suggesting a potential role of this miRNA in hypoxia-mediated HSC activation.

Another potential link in hypoxia-mediated regulation of HSC activation is presented by miR-31. MiR-31 is up-regulated in both *in vivo* and *in vitro* activated rat HSCs (Maubach et al., 2011). This was confirmed in humans, where miR-31 was not changed in whole liver samples of fibrotic livers, but an increased expression of miR-31 was detected in HSCs during fibrogenesis. Functional studies showed repression of HSC activation by miR-31 inhibition, while miR-31 overexpression revealed its promoting role in cell migration (Hu et al., 2015). Interestingly it has been suggested that the biological function of miR-31 in activating HSCs would be obtained through its effect on Factor-inhibiting HIF-1(FIH) (Mahon et al., 2001; Hu et al., 2015). In head and neck carcinoma, miR-31 negatively regulates the expression of FIH and can thus regulate the expression of FIH in a hypoxia-independent manner (Liu et al., 2010). In cancer models this miRNA is up-regulated under hypoxic conditions (Hebert et al., 2007), suggesting a very complicated and diverse functionality of miR-31 during the reach for cellular homeostasis. Previous research identified a direct link between the increased nuclear levels of HIF-1α protein and an increased activated status of HSCs in a hypoxic environment. HIF-1α has an indirect activating effect on the expression of pro-fibrogenic genes such as TGF-β, IL-6 and CTGF (Copple et al., 2011; Wang et al., 2013). The exact role of miR-31 in this hypoxia-induced HSC activation remains to be elucidated. We speculate on two possible scenarios that are perhaps not exclusive. Due to pro-activating signals from surrounding liver cells, HSCs will up-regulate miR-31 expression, leading to inhibition of FIH function, and thus enhanced HIF-1α expression, thereby favoring HSC activation in normoxic conditions. Hypoxic regions appear in the liver due to injury, what could favor the (further) induction of miR-31 expression, boosting the already enhanced HIF-1α expression, further leading to progression or maintenance of HSC activation.

Oxidative Stress Regulated miRNAs

Cells in aerobic organisms have a continuous balance between the production of pro-oxidants, such as reactive oxygen species (ROS), and anti-oxidants. When a cell is subjected to oxidative

TABLE 2 | Potential miRNAs involved in stress responsive HSC activation.

miRNAs INVOLVED IN HSC ACTIVATION				STRESS RESPONSIVE miRNAs IN OTHER CELL TYPES				
miRNA	**Expression during *in vitro* HSC activation**	**Species**	**References**	**Stress**	**Expression during stress**	**Cell type**	**Challenge or treatment**	**References**
miR-214	Up-regulated	Rat, mouse	Maubach et al., 2011; Iizuka et al., 2012	Hypoxia	Up-regulated	Squamous cell carcinoma-cell line	1% oxygen for 1 h or 5% oxygen for 8 h	Hebert et al., 2007
miR-15b	Down-regulated	Rat	Guo et al., 2009b	Hypoxia	Down-regulated	CNE cells: a human naso-pharyngeal carcinoma cell line	Deferoxamine Mesylate	Hua et al., 2006
miR-422b	Down-regulated	Rat	Maubach et al., 2011	Hypoxia	Down-regulated	Squamous cell carcinoma-cell line	1% O_2 for 1 h or 5% O_2 for 8 h	Hebert et al., 2007
miR-125b	Up-regulated	Rat	Maubach et al., 2011; Chen et al., 2011	Hypoxia	Up-regulated	Colon and breast cancer cell lines	Culture in 0.2% O_2	Kulshreshtha et al., 2007
miR-101a	Down-regulated	Rat, mouse	Chen et al., 2011; Tu et al., 2014a	Hypoxia	Down-regulated	Neonatal rat cardiofibroblasts	Culture in 2% O_2	Zhao et al., 2015
miR-27a	Up-regulated	Rat	Ji et al., 2009	Hypoxia	Up-regulated	Colon-, breast-, human bladder-, and human colon-cancer cell lines	Culture in 3% O_2, $CoCl_2$	Kulshreshtha et al., 2007; Xu et al., 2014
miR-195	Down-regulated	Rat	Maubach et al., 2011	Hypoxia	Down-regulated	Chondrocytes	Culture in 5% O_2	Bai et al., 2015
miR-210	Up-regulated	Rat	Maubach et al., 2011	Hypoxia	Up-regulated	Pancreatic, breast, head and neck, lung, colon, renal cell lines	2% O_2 for 24 h	Huang et al., 2009
miR-31	Up-regulated	Rat	Maubach et al., 2011	Hypoxia	Up-regulated	Squamous cell carcinoma-cell line	1% O_2 for 1 h or 5% O_2 for 8 h	Hebert et al., 2007
miR-9	Down-regulated	Rat	Ji et al., 2009	Oxidative stress	Down-regulated	ARPE-19: human retinal pigment cells	4-hydroxynonenal and tert-butyl hydroperoxide	Yoon et al., 2014
miR-92a	Down-regulated	Rat	Lakner et al., 2012	Oxidative stress	Down-regulated	TK6: human lymphoblast cell line, endothelial cells (HUVEC)	Irradiation, H_2O_2	Chaudhry et al., 2013; Zhang et al., 2014
miR-21	Up-regulated	Rat	Maubach et al., 2011	Oxidative stress	Up-regulated	Neonatal cardiomyocytes	H_2O_2	Wei et al., 2014
miR-200a	Up-regulated	HSC-T6 cell line	Sun et al., 2014	Oxidative stress	Up-regulated	Mouse fibroblasts	H_2O_2	Mateescu et al., 2011
miR-199a-5p	Up-regulated	Rat	Maubach et al., 2011	ER stress	Up-regulated	Human hepatocyte line	Thapsigargin and deoxycholic acid	Dai et al., 2013
miR-30a	Up-regulated	Rat	Ji et al., 2009	ER stress	Down-regulated	Neonatal rat ventricular cells and rat aorta vascular smooth muscle cells	H_2O_2	Chen et al., 2014
miR-122	Down-regulated	Rat	Guo et al., 2009b	ER stress	Down-regulated	Huh7, HepG2 cell lines	Thapsigargin	Yang et al., 2011
miR-30c−2*	Down-regulated	Rat	Ji et al., 2009	ER stress	Up-regulated	NIH-3T3 fibroblasts	Tunicamycin and thapsigargin	Byrd et al., 2012
miR-34a	Up-regulated	Rat	Chen et al., 2011	ER stress	Down-regulated	Mouse embryonic fibroblasts	Brefeldin A	Upton et al., 2012
miR-455	Up-regulated	Rat	Ji et al., 2009	ER stress	Down-regulated	Neonatal rat ventricular myocytes	Tunicamycin	Belmont et al., 2012
miR-181a	Up-regulated	Human HSC cell line	Zheng et al., 2015	ER stress	Down-regulated	Various cell lines	Thapsigargin treatment	Su et al., 2013

*MiRNAs which display an overlap in expression profile between activating HSCs and in specific stress responses are displayed in green, those with a contradictory expression profile are displayed in red. *Mature miRNA derived from the 5′ arm of the precursor RNA also known as passenger strand.*

stress, this normal balance fades by excessive production of pro-oxidants. Various types of ROS are known, such as the singlet molecular oxygen, hydrogen peroxide and the hydrogen radical, which all have a specific half-life and mechanism of action (Sies, 1991).

There are several possible sources of ROS in the cell. Mitochondria, the main site of oxygen consumption in aerobic cells, are the main producers of ROS derived mainly through the leakage of electrons and formation of superoxide (Guarente, 2008). Cytochrome P450 (CYP) acts in the detoxification of metabolic as well as xenobiotic compounds by means of oxidation (Aubert et al., 2011) making it also an important source of ROS. Specifically the form CYP2E1, which is highly expressed in hepatocytes, has been demonstrated to be a key source of ROS in the liver (Poli, 2000). Another major source of ROS in several cell types and HSCs is nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) (De Minicis and Brenner, 2007; Sergey, 2011).

Oxidative stress and the subsequent decreased levels of anti-oxidants during liver fibrosis has been shown for a broad variety of etiologies (Poli, 2000). ROS are produced by various cell types, but it is thought that the major contributors of ROS production in this pathology are apoptotic hepatocytes. HSCs express a non-phagocytic form of NADPH oxidase, which presents a basal level of activity, producing constitutively low levels of ROS and increasing production upon different stimuli (Bataller et al., 2003). NADPH oxidase of HSCs is activated upon phagocytosis of these apoptotic bodies of hepatocytes (Shan-Shan et al., 2006). Furthermore, NADPH oxidase-generated ROS in HSCs is also induced by advanced glycation end-products (AGEs) which are products of a non-enzymatic reaction of sugars with molecules such as proteins, lipids and nucleic acids that accumulate in diseases related to the metabolic syndrome (Yan et al., 2010). Liver fibrosis is correlated with accumulation of systemic AGEs and ROS in HSCs has been show to participate during the development of liver diseases (Šebeková et al., 2002; Hyogo et al., 2007; Guimarães et al., 2010).

Activated Kupffer cells and neutrophils are also described as important producers of ROS during early stages of liver fibrosis (Kisseleva and Brenner, 2007). The most important result of oxidative stress is lipid peroxidation. As example, liver fibrosis caused by excessive alcohol intake leads to injury of the different liver cell types and consecutive excessive oxidation of polyunsaturated membrane lipids due to enhanced generation of ROS due to the elevated levels of cytochrome CYP2E1 (Nieto et al., 1999). The products of such lipid peroxidation could further catalyze the progression of fibrosis by activation of the production of collagen α2 (I) in HSCs in a paracrine manner (Bedossa et al., 1994). Furthermore, exposure of HSCs to ROS can promote their proliferation and invasiveness. It is thought that it would obtain these effects by an induction of MMP-2 expression, and the enhancement of MT1-MMP and TIMP-2 protein levels, in an ERK1/2 and PI3K dependent manner (Galli et al., 2005).

Several miRNAs have already been linked to the regulation of the oxidative stress pathway, including members of the miR-200 family. From this miRNA-family, especially miR-200c has been shown to display an increased expression after cellular exposure to H_2O_2. This miRNA would lead to down-regulation of zinc finger E-box binding homeobox 1 (Zfhx1a, aka Zeb1, or TCF8), a transcriptional repressor, both on mRNA and protein level, leading to cellular senescence and inhibition of cell proliferation. Interestingly, an inhibitory loop was found between miR-200c and Zeb1, as the promoter region of miR-200c contains two conserved Zeb1 binding sites (Magenta et al., 2011). MiR-200c can also regulate apoptosis, as it inhibits the translation of FAS associated phosphatase (FAP-1) mRNA. Decreased expression of FAP-1 leads to a greater sensitivity to CD95-mediated apoptosis (Schickel et al., 2010). Some of the other identified targets of miR-200c include Moesin (MSN), Fibronectin 1 (FN1), and Rho GTPase activating protein 19 (ARHGAP19), important regulators of the migratory and invasive capacity of cancer cells (Howe et al., 2011). Another miRNA associated with oxidative stress is miR-21. Cells exposed to ROS would up-regulate miR-21, which can directly interact with the 3′UTR of the programmed cell death 4 (PDCD4) gene, a known tumor suppressor and apoptosis-regulator, thereby preventing cell death. Oxidative stress mediated up-regulation of miR-21 can be induced by NF-κB activation through five NF-κB binding sites in the 5' miR-21 promoter region (Tu et al., 2014b; Wei et al., 2014). Up-regulation of miR-21 would be a down-stream effect of NADPH oxidase activity (Dattaroy et al., 2015), as this induces NF-κB translocation to the nucleus (Yao et al., 2007) and its subsequent binding to the miR-21 promotor (Sheedy et al., 2010). This enhanced expression of miR-21 also leads to a suppression of SMAD7 expression and therefore favors assembly of SMAD2/3-SMAD4 heterodimers, a crucial event in the pro-fibrogenic TGF-β signaling pathway (Dattaroy et al., 2015).

A potential link in oxidative stress-induced HSC activation could be represented by miR-200a, which is down-regulated during the process of liver fibrosis in rat, and in TGF-β1-mediated activation of a rat HSC cell line (Sun et al., 2014). MiR-200a also regulates proliferation of these activating HSCs, shown by an accumulation of cells in the G0/G1 phase upon miR-200a overexpression. Targets of miR-200a include pro-fibrogenic factors TGF-β2 and β-catenin (Sun et al., 2014). Another important miRNA-200a target gene is Kelch-like ECH-associated protein 1 (Keap1), which negatively regulates the stability of nuclear factor-erythroid-2-related factor 2 (Nrf2), a known regulator of the expression of antioxidants involved in the protection against oxidative damage (Yang et al., 2014). While no information is available for miR-200c, in rat, miR-200a seems to be down-regulated upon HSC activation while during liver fibrosis progression in human and mouse, miR-200a and miR-200b undergo a significant up-regulation (Murakami et al., 2011). This is in line with the up-regulation in expression of the miR-200 family after induction of oxidative stress in mouse fibroblasts where miR-200a can target p38α mitogen-activated protein kinase (MAPK) (Mateescu et al., 2011), which is downstream of the oxidative stress stimulus, and leads to an inhibition of cell division (Kurata, 2000). Despite opposing expression patterns observed in different species, the involvement of miR-200a in both HSC activation and oxidative stress response is clear. It is therefore tempting to speculate that miR-200a could participate in the anti-oxidant response of HSCs during liver injury.

Endoplasmic Reticulum Stress Regulated miRNAs

The generation of mediators that lead to a perturbation of the ER homeostasis can be evoked by various stimuli associated with the initiation or progression of the liver fibrosis process, such as repeated cycles of ischemia and reperfusion due to distorted hepatic flow, genetic mutations of proteins involved in ER constitution and function, excessive exposure to certain drugs (paracetamol, ethanol), obesity-linked enhanced presence of lipids, and viral infections (HCV, HBV). These stimuli can lead to oxidative stress, formation of protein aggregates, altered membrane lipid-composition, and hyperhomocysteinemia with resulting N-homocysteinylation, all leading to the dysfunction of the ER, and accumulation of unfolded and misfolded proteins (Malhi and Kaufman, 2011). Cells will try to counteract this accumulation of misfolded proteins by diverse mechanisms such as the unfolded protein response (UPR). The activation of the UPR pathway, due to ER-resident stress sensors such as ATF-6, IRE1, and PERK (Asselah et al., 2010), will lead to an enhanced and more stringent folding and degradation of proteins in the ER, and an overall diminishment of protein synthesis. When the UPR fails to diminish the ER stress, the cells go into apoptosis. Persistent ER stress has several consequences including the excessive energy depletion due to the enhanced utilization of energy for translocation of misfolded proteins; ASK1/JNK mediated signaling leading to activation of caspases, and the activation of the pro-apoptotic pathway of CHOP/GADD153 transcription factor, which all direct the cell toward apoptosis (Xu et al., 2005). It will also lead to the release of the stored calcium in the ER, which affects mitochondria; moreover it will lead to the induction of oxidative stress, activation of the pro-inflammatory NF-κB pathway and apoptosis of the cell. ER stress will also lead to translocation and activation of SREBP, causing an enhanced synthesis of lipids such as fatty acids and cholesterol, and an enhanced cellular uptake of lipoproteins (Ji and Kaplowitz, 2006; Ji, 2008).

Cultured HSCs, which are known to be relatively apoptosis-insensitive, have been shown to undergo apoptosis in response to persistent ER stress due to an increase of the amount of intracellular calcium, and activation of JNK/p38 MAPK and Calpain/Caspase pathways (Huang et al., 2014b). Activation of the latter pathway can be explained by the decrease of Calpastatin expression, which works as an inhibitor of the pro-apoptotic Calpain. During the activation of HSCs, Calpastatin levels become elevated, leading to the desensitization of the HSCs toward apoptotic stimuli. ER-stress mediated decrease of Calpastatin expression can thus lead to higher Calpain levels, and consequent sensitization toward apoptotic stimuli (De Minicis et al., 2012). The fibrosis counteracting effect of ER stress was further supported by the decrease in α-SMA and Col1a1-expression in ER-stress responsive activating HSCs (Huang et al., 2014b). However, it is found that when HSCs are exposed to oxidative stress-induced ER stress, the UPR will lead to the up-regulation of different pathways leading to enhanced autophagy and consequent HSC activation *in vitro* (Hernandez-Gea et al., 2013). All described ER stress could thus be considered as a complex mechanism of fibrosis regulation, with a possible stimulatory role in HSC activation and a possible role in fibrosis resolution due to its pro-apoptotic effects in activated HSCs.

The role of miRNAs during ER-stress remains largely unknown. One of the miRNAs that has been studied in this process is miR-199a-5p, which displays an up-regulation in hepatocytes undergoing ER stress. This miRNA would have several ER-stress related targets including the chaperone protein GRP78 (which is also known as Bip and HSPA5), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1α (IRE1α), with the latter two being UPR transducers. As IRE1α activated ER stress can induce cell death, activation of miR-199a-5p, and thus subsequent down-regulation of IRE1α, would work as a rescue mechanism to prevent the induction of apoptosis. *In silico* target prediction identified DNA-damage regulated autophagy modulator 1 (DRAM1) and cyclin-dependent kinase inhibitor 1B (p27), both pro-apoptotic genes, as additional potential targets of miR-199a-5p, thus further underlining its pro-survival role (Dai et al., 2013). miR-199a-5p could also have some effect on cell proliferation, as it has been shown to target frizzles type 7 receptor (FZD7), and thus regulates the expression of its downstream genes including β-catenin, Jun, Cyclin D1, and Myc (Song et al., 2014). A second class of miRNAs linked with ER stress includes members of the miR-30 family, which are being down-regulated due to this specific stress responsive pathway. This miRNA family contains six members (from a to e), which contain all an identical seed sequence motif, but are located at different sites of the genome. GRP78 is targeted by miR-30a, which further underlines the importance of this miRNA in this stress response. Knockdown of miR-30 in cardiac cells identified ATF6, CHOP, and caspase-12 as indirect targets of this miRNA, thus revealing its role in regulation of cell death (Chen et al., 2014).

MiR-122 could perhaps represent a regulator of ER-stress-modulated HSC activation. MiR-122 is described as liver-specific and the most abundant miRNA in the liver (Lagos-Quintana et al., 2002). It has been shown that miR-122 is down-regulated in total liver samples during the progression of liver disease in mouse, rat (Li et al., 2013) and human (Padgett et al., 2009), and this down-regulation was furthermore observed in activating HSCs (Li et al., 2013). Overexpression of this miRNA in LX-2 cells leads to a decrease in cell proliferation and maturation of Col1a1, most likely through regulation of P4HA1 by miR-122. The expression of P4HA1 is up-regulated during fibrosis progression, and encodes a component of prolyl 4-hydroxylase, which is necessary for collagen maturation (Li et al., 2013). Overexpression of miR-122 in LX2 further identified FN1, which is involved in the assembly of collagen fibrils, and serum response factor (SRF) as direct targets, and confirmed its inhibitory effect on TGF-β-induced HSC activation (Zeng et al., 2015). Further target identification studies in hepatocytes identified mitogen-activated protein kinase kinase kinase 3 (MAP3K3), which plays a role in cell survival and proliferation, the intermediate filament vimentin, and HIF-1α (Csak et al., 2015). MiR-122 inhibition in hepatoma cells suggests a role in the UPR. Moreover its inhibition leads to an up-regulation of the 26S proteasome non-ATPase regulatory subunit 10 (PMSD10), which can enhance the protein

folding-capacity and thus promoting recovery, by up-regulation of GRP78. MiR-122 would have this effect on PMSD10 in an indirect manner through targeting of cyclin dependent kinase 4 (CDk4) which interacts with PMSD10. Other miR-122 targets include the ER stress chaperones calreticulin (CALR), ER protein 29 (ERP29) and SET nuclear oncogene (SET), which help in the correct folding of malfunctional proteins (Yang et al., 2011). Taken together, even though miR-122 is not abundantly expressed in HSCs, it is tempting to speculate that down-regulation of miR-122 is involved in the UPR in HSCs.

Discussion

MiRNAs have been proposed as key regulators of gene expression and dysregulated patterns of miRNA expression were observed in various diseases (Tufekci et al., 2014), including the progression of liver fibrosis and cirrhosis (Wang et al., 2012; Xin et al., 2014). Studying miRNAs is very popular and raised a lot of expectations in their use as biomarkers for diseases and therapeutic interventions using miRNA mimics and antagomirs. Unfortunately, so far this has not turned out to be easy, partly because of their cell type-specific and species-specific activity and wide range of targets.

Diagnosis of liver fibrosis could be facilitated by identification of blood-circulating biomarkers representative for HSC activation, as the current golden standard for diagnosis remains the invasive and harmful liver biopsy (Piccinino et al., 1986; Friedman, 2003). Circulating miRNAs, both protein-bound and packaged into extracellular vesicles (Turchinovich et al., 2011), have been proposed as such a potential biomarker, and various research groups already tried to identify circulating miRNAs that could be linked with progression and regression of liver disease (Roderburg and Luedde, 2014). To date, this has not yet led to a diagnostic protocol that is used in clinic. It is tempting to speculate that perhaps stress-responsive miRNAs of activating HSCs secreted in the blood could also be used as a liquid biopsy to document the stress present in the liver.

We discussed several miRNAs with a potential role in stress-mediated regulation of HSC activation. Experimental validation of these suggested links between stress-related miRNAs and HSCs should address a number of issues. First, are specific miRNAs dysregulated in HSCs in response to specific stress signals and does this lead to an imbalance of the cellular homeostasis and consequent HSC apoptosis or activation? *In vivo*, paracrine stimulation of quiescent HSCs by stress-undergoing surrounding cells is likely to create a warning for the quiescent cell, leading to its activation and reducing its responsiveness to more stress-signals. Secondly, responding to stress is necessary to counteract short term challenges to restore cell homeostasis. Thus the question is, whether there are miRNAs that specifically respond to prolonged stresses present in the fibrotic liver, and if so, could a targeted mimic/antagomir approach inhibit HSC activation or promote HSC apoptosis or inactivation?

In conclusion, HSC activation *in vivo* can be seen as a very complicated and multifactorial process in which hypoxia (Cannito et al., 2014), oxidative stress (Poli, 2000), and ER stress (Malhi and Kaufman, 2011) are surely involved. This suggests a potential role for stress-related miRNAs during HSC activation and disease development and opens perspectives for new therapeutic approaches.

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References

Asselah, T., Bieche, I., Mansouri, A., Laurendeau, I., Cazals-Hatem, D., Feldmann, G., et al. (2010). *In vivo* hepatic endoplasmic reticulum stress in patients with chronic hepatitis C. *J. Pathol.* 221, 264–274. doi: 10.1002/path.2703

Aubert, J., Begriche, K., Knockaert, L., Robin, M. A., and Fromenty, B. (2011). Increased expression of cytochrome P450 2E1 in nonalcoholic fatty liver disease: mechanisms and pathophysiological role. *Clin. Res. Hepatol. Gastroenterol.* 35, 630–637. doi: 10.1016/j.clinre.2011.04.015

Babar, I. A., Slack, F. J., and Weidhaas, J. B. (2008). miRNA modulation of the cellular stress response. *Future Oncol.* 4, 289–298. doi: 10.2217/14796694.4.2.289

Bagga, S., Bracht, J., Hunter, S., Massirer, K., Holtz, J., Eachus, R., et al. (2005). Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. *Cell* 122, 553–563. doi: 10.1016/j.cell.2005.07.031

Bai, R., Zhao, A. Q., Zhao, Z. Q., Liu, W. L., and Jian, D. M. (2015). MicroRNA-195 induced apoptosis in hypoxic chondrocytes by targeting hypoxia-inducible factor 1 alpha. *Eur. Rev. Med. Pharmacol. Sci.* 19, 545–551.

Bartel, D. P., and Chen, C. Z. (2004). Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. *Nat. Rev. Genet.* 5, 396–400. doi: 10.1038/nrg1328

Bataller, R., Schwabe, R. F., Choi, Y. H., Yang, L., Paik, Y. H., Lindquist, J., et al. (2003). NADPH oxidase signal transduces angiotensin II in hepatic stellate cells and is critical in hepatic fibrosis. *J. Clin. Invest.* 112, 1383–1394. doi: 10.1172/JCI18212

Bedossa, P., Houglum, K., Trautwein, C., Holstege, A., and Chojkier, M. (1994). Stimulation of collagen alpha 1(I) gene expression is associated with lipid peroxidation in hepatocellular injury: a link to tissue fibrosis? *Hepatology* 19, 1262–1271. doi: 10.1002/hep.1840190527

Belmont, P. J., Chen, W. J., Thuerauf, D. J., and Glembotski, C. C. (2012). Regulation of microRNA expression in the heart by the ATF6 branch of the ER stress response. *J. Mol. Cell. Cardiol.* 52, 1176–1182. doi: 10.1016/j.yjmcc.2012.01.017

Bernstein, E., Caudy, A. A., Hammond, S. M., and Hannon, G. J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 409, 363–366. doi: 10.1038/35053110

Byrd, A. E., Aragon, I. V., and Brewer, J. W. (2012). MicroRNA-30c-2* limits expression of proadaptive factor XBP1 in the unfolded protein response. *J. Cell Biol.* 196, 689–698. doi: 10.1083/jcb.201201077

Cannito, S., Paternostro, C., Busletta, C., Bocca, C., Colombatto, S., Miglietta, A., et al. (2014). Hypoxia, hypoxia-inducible factors and fibrogenesis in chronic liver diseases. *Histol. Histopathol.* 29, 33–44.

Chaudhry, M. A., Omaruddin, R. A., Brumbaugh, C. D., Tariq, M. A., and Pourmand, N. (2013). Identification of radiation-induced microRNA transcriptome by next-generation massively parallel sequencing. *J. Radiat. Res.* 54, 808–822. doi: 10.1093/jrr/rrt014

Chen, C., Wu, C. Q., Zhang, Z. Q., Yao, D. K., and Zhu, L. (2011). Loss of expression of miR-335 is implicated in hepatic stellate cell migration and activation. *Exp. Cell Res.* 317, 1714–1725. doi: 10.1016/j.yexcr.2011.05.001

Chen, M., Ma, G., Yue, Y., Wei, Y., Li, Q., Tong, Z., et al. (2014). Downregulation of the miR-30 family microRNAs contributes to endoplasmic reticulum stress in cardiac muscle and vascular smooth muscle cells. *Int. J. Cardiol.* 173, 65–73. doi: 10.1016/j.ijcard.2014.02.007

Coll, M., Taghdouini, A. E., Perea, L., Mannaerts, I., Vila-Casadesús, M., Blaya, D., et al. (2015). Integrative miRNA and gene expression profiling analysis of human quiescent hepatic stellate cells. *Sci. Rep.* 5:11549. doi: 10.1038/srep11549

Copple, B. L., Bai, S., Burgoon, L. D., and Moon, J. O. (2011). Hypoxia-inducible factor-1alpha regulates the expression of genes in hypoxic hepatic stellate cells important for collagen deposition and angiogenesis. *Liver Int.* 31, 230–244. doi: 10.1111/j.1478-3231.2010.02347.x

Copple, B. L., Roth, R. A., and Ganey, P. E. (2006). Anticoagulation and inhibition of nitric oxide synthase influence hepatic hypoxia after monocrotaline exposure. *Toxicology* 225, 128–137. doi: 10.1016/j.tox.2006.05.016

Csak, T., Bala, S., Lippai, D., Satishchandran, A., Catalano, D., Kodys, K., et al. (2015). microRNA-122 regulates hypoxia-inducible factor-1 and vimentin in hepatocytes and correlates with fibrosis in diet-induced steatohepatitis. *Liver Int.* 35, 532–541. doi: 10.1111/liv.12633

Dai, B. H., Geng, L., Wang, Y., Sui, C. J., Xie, F., Shen, R. X., et al. (2013). microRNA-199a-5p protects hepatocytes from bile acid-induced sustained endoplasmic reticulum stress. *Cell Death Dis.* 4, e604. doi: 10.1038/cddis.2013.134

Dattaroy, D., Pourhoseini, S., Das, S., Alhasson, F., Seth, R. K., Nagarkatti, M., et al. (2015). Micro-RNA 21 inhibition of SMAD7 enhances fibrogenesis via leptin-mediated NADPH oxidase in experimental and human nonalcoholic steatohepatitis. *Am. J. Physiol. Gastrointest. Liver Physiol.* 308, G298–G312. doi: 10.1152/ajpgi.00346.2014

De Minicis, S., and Brenner, D. A. (2007). NOX in liver fibrosis. *Arch. Biochem. Biophys.* 462, 266–272. doi: 10.1016/j.abb.2007.04.016

De Minicis, S., Candelaresi, C., Agostinelli, L., Taffetani, S., Saccomanno, S., Rychlicki, C., et al. (2012). Endoplasmic Reticulum stress induces hepatic stellate cell apoptosis and contributes to fibrosis resolution. *Liver Int.* 32, 1574–1584. doi: 10.1111/j.1478-3231.2012.02860.x

De Minicis, S., Seki, E., Uchinami, H., Kluwe, J., Zhang, Y., Brenner, D. A., et al. (2007). Gene expression profiles during hepatic stellate cell activation in culture and *in vivo*. *Gastroenterology* 132, 1937–1946. doi: 10.1053/j.gastro.2007.02.033

Fasanaro, P., Greco, S., Lorenzi, M., Pescatori, M., Brioschi, M., Kulshreshtha, R., et al. (2009). An integrated approach for experimental target identification of hypoxia-induced miR-210. *J. Biol. Chem.* 284, 35134–35143. doi: 10.1074/jbc.M109.052779

Feng, X., Tan, W., Cheng, S., Wang, H., Ye, S., Yu, C., et al. (2015). Upregulation of microRNA-126 in hepatic stellate cells may affect pathogenesis of liver fibrosis through the NF-kappaB pathway. *DNA Cell Biol.* 34, 470–480. doi: 10.1089/dna.2014.2760

Forsythe, J. A., Jiang, B. H., Iyer, N. V., Agani, F., Leung, S. W., Koos, R. D., et al. (1996). Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol. Cell. Biol.* 16, 4604–4613.

Friedman, S. L. (2003). Liver fibrosis – from bench to bedside. *J. Hepatol.* 38, 38–53. doi: 10.1016/S0168-8278(02)00429-4

Galli, A., Svegliati-Baroni, G., Ceni, E., Milani, S., Ridolfi, F., Salzano, R., et al. (2005). Oxidative stress stimulates proliferation and invasiveness of hepatic stellate cells via a MMP2-mediated mechanism. *Hepatology* 41, 1074–1084. doi: 10.1002/hep.20683

Giannakakis, A., Sandaltzopoulos, R., Greshock, J., Liang, S., Huang, J., Hasegawa, K., et al. (2008). miR-210 links hypoxia with cell cycle regulation and is deleted in human epithelial ovarian cancer. *Cancer Biol. Ther.* 7, 255–264. doi: 10.4161/cbt.7.2.5297

Gregory, R. I., Chendrimada, T. P., Cooch, N., and Shiekhattar, R. (2005). Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. *Cell* 123, 631–640. doi: 10.1016/j.cell.2005.10.022

Gregory, R. I., Yan, K. P., Amuthan, G., Chendrimada, T., Doratotaj, B., Cooch, N., et al. (2004). The microprocessor complex mediates the genesis of microRNAs. *Nature* 432, 235–240. doi: 10.1038/nature03120

Guarente, L. (2008). Mitochondria–a nexus for aging, calorie restriction, and sirtuins? *Cell* 132, 171–176. doi: 10.1016/j.cell.2008.01.007

Guimarães, E. L. M., Empsen, C., Geerts, A., and van Grunsven, L. A. (2010). Advanced glycation end products induce production of reactive oxygen species via the activation of NADPH oxidase in murine hepatic stellate cells. *J. Hepatol.* 52, 389–397. doi: 10.1016/j.jhep.2009.12.007

Guo, C. J., Pan, Q., Cheng, T., Jiang, B., Chen, G. Y., and Li, D. G. (2009a). Changes in microRNAs associated with hepatic stellate cell activation status identify signaling pathways. *FEBS J.* 276, 5163–5176. doi: 10.1111/j.1742-4658.2009.07213.x

Guo, C. J., Pan, Q., Jiang, B., Chen, G. Y., and Li, D. G. (2009c). Effects of upregulated expression of microRNA-16 on biological properties of culture-activated hepatic stellate cells. *Apoptosis* 14, 1331–1340. doi: 10.1007/s10495-009-0401-3

Guo, C. J., Pan, Q., Li, D. G., Sun, H., and Liu, B. W. (2009b). miR-15b and miR-16 are implicated in activation of the rat hepatic stellate cell: an essential role for apoptosis. *J. Hepatol.* 50, 766–778. doi: 10.1016/j.jhep.2008.11.025

Ha, M., Pang, M., Agarwal, V., and Chen, Z. J. (2008). Interspecies regulation of microRNAs and their targets. *Biochim. Biophys. Acta* 1779, 735–742. doi: 10.1016/j.bbagrm.2008.03.004

He, Y., Huang, C., Sun, X., Long, X. R., Lv, X. W., and Li, J. (2012b). MicroRNA-146a modulates TGF-beta1-induced hepatic stellate cell proliferation by targeting SMAD4. *Cell. Signal.* 24, 1923–1930. doi: 10.1016/j.cellsig.2012.06.003

He, Y., Huang, C., Zhang, S.-P., Sun, X., Long, X.-R., and Li, J. (2012a). The potential of microRNAs in liver fibrosis. *Cell. Signal.* 24, 2268–2272. doi: 10.1016/j.cellsig.2012.07.023

Hebert, C., Norris, K., Scheper, M. A., Nikitakis, N., and Sauk, J. J. (2007). High mobility group A2 is a target for miRNA-98 in head and neck squamous cell carcinoma. *Mol. Cancer* 6, 5. doi: 10.1186/1476-4598-6-5

Hernandez-Gea, V., Hilscher, M., Rozenfeld, R., Lim, M. P., Nieto, N., Werner, S., et al. (2013). Endoplasmic reticulum stress induces fibrogenic activity in hepatic stellate cells through autophagy. *J. Hepatol.* 59, 98–104. doi: 10.1016/j.jhep.2013.02.016

Howe, E. N., Cochrane, D. R., and Richer, J. K. (2011). Targets of miR-200c mediate suppression of cell motility and anoikis resistance. *Breast Cancer Res.* 13, R45. doi: 10.1186/bcr2867

Hu, J., Chen, C., Liu, Q., Liu, B., Song, C., Zhu, S., et al. (2015). The role of miR-31/FIH1 pathway in TGFbeta-induced liver fibrosis. *Clin. Sci.* 129, 305–317. doi: 10.1042/CS20140012

Hua, Z., Lv, Q., Ye, W., Wong, C. K., Cai, G., Gu, D., et al. (2006). MiRNA-directed regulation of VEGF and other angiogenic factors under hypoxia. *PLoS ONE* 1:e116. doi: 10.1371/journal.pone.0000116

Huang, J., Yu, X., Fries, J. W., Zhang, L., and Odenthal, M. (2014a). MicroRNA function in the profibrogenic interplay upon chronic liver disease. *Int. J. Mol. Sci.* 15, 9360–9371. doi: 10.3390/ijms15069360

Huang, X., Ding, L., Bennewith, K. L., Tong, R. T., Welford, S. M., Ang, K. K., et al. (2009). Hypoxia-inducible mir-210 regulates normoxic gene expression involved in tumor initiation. *Mol. Cell* 35, 856–867. doi: 10.1016/j.molcel.2009.09.006

Huang, Y., Li, X., Wang, Y., Wang, H., Huang, C., and Li, J. (2014b). Endoplasmic reticulum stress-induced hepatic stellate cell apoptosis through calcium-mediated JNK/P38 MAPK and Calpain/Caspase-12 pathways. *Mol. Cell. Biochem.* 394, 1–12. doi: 10.1007/s11010-014-2073-8

Hyogo, H., Yamagishi, S.-i., Iwamoto, K., Arihiro, K., Takeuchi, M., Sato, T., et al. and Tazuma, S. (2007). Elevated levels of serum advanced glycation end products in patients with non-alcoholic steatohepatitis. *J. Gastroenterol. Hepatol.* 22, 1112–1119. doi: 10.1111/j.1440-1746.2007.04943.x

Iizuka, M., Ogawa, T., Enomoto, M., Motoyama, H., Yoshizato, K., Ikeda, K., et al. (2012). Induction of microRNA-214-5p in human and rodent liver fibrosis. *Fibrogenesis Tissue Repair* 5, 12. doi: 10.1186/1755-1536-5-12

Ji, C. (2008). Dissection of endoplasmic reticulum stress signaling in alcoholic and non-alcoholic liver injury. *J. Gastroenterol. Hepatol.* 23(Suppl. 1), S16–S24. doi: 10.1111/j.1440-1746.2007.05276.x

Ji, C., and Kaplowitz, N. (2006). ER stress: can the liver cope? *J. Hepatol.* 45, 321–333. doi: 10.1016/j.jhep.2006.06.004

Ji, J., Zhang, J., Huang, G., Qian, J., Wang, X., and Mei, S. (2009). Over-expressed microRNA-27a and 27b influence fat accumulation and cell proliferation

during rat hepatic stellate cell activation. *FEBS Lett.* 583, 759–766. doi: 10.1016/j.febslet.2009.01.034

Jiang, B. H., Semenza, G. L., Bauer, C., and Marti, H. H. (1996). Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O2 tension. *Am. J. Physiol.* 271, C1172–C1180.

Jiang, F., Parsons, C. J., and Stefanovic, B. (2006). Gene expression profile of quiescent and activated rat hepatic stellate cells implicates Wnt signaling pathway in activation. *J. Hepatol.* 45, 401–409. doi: 10.1016/j.jhep.2006.03.016

Jiang, W., Kong, L., Ni, Q., Lu, Y., Ding, W., Liu, G., et al. (2014). miR-146a ameliorates liver ischemia/reperfusion injury by suppressing IRAK1 and TRAF6. *PLoS ONE* 9:e101530. doi: 10.1371/journal.pone.0101530

Kim, H. W., Haider, H. K., Jiang, S., and Ashraf, M. (2009). Ischemic preconditioning augments survival of stem cells via miR-210 expression by targeting caspase-8-associated protein 2. *J. Biol. Chem.* 284, 33161–33168. doi: 10.1074/jbc.M109.020925

Kisseleva, T., and Brenner, D. A. (2007). Role of hepatic stellate cells in fibrogenesis and the reversal of fibrosis. *J. Gastroenterol. Hepatol.* 22(Suppl. 1), S73–S78. doi: 10.1111/j.1440-1746.2006.04658.x

Kisseleva, T., and Brenner, D. A. (2013). Inactivation of myofibroblasts during regression of liver fibrosis. *Cell Cycle* 12, 381–382. doi: 10.4161/cc.23549

Kulshreshtha, R., Ferracin, M., Wojcik, S. E., Garzon, R., Alder, H., Agosto-Perez, F. J., et al. (2007). A microRNA signature of hypoxia. *Mol. Cell. Biol.* 27, 1859–1867. doi: 10.1128/MCB.01395-06

Šebeková, K. N., Kupèová, V., Schinzel, R., and Heidland, A. (2002). Markedly elevated levels of plasma advanced glycation end products in patients with liver cirrhosis – amelioration by liver transplantation. *J. Hepatol.* 36, 66–71. doi: 10.1016/S0168-8278(01)00232-X

Kurata, S. (2000). Selective activation of p38 MAPK cascade and mitotic arrest caused by low level oxidative stress. *J. Biol. Chem.* 275, 23413–23416. doi: 10.1074/jbc.C000308200

Kwiecinski, M., Elfimova, N., Noetel, A., Tox, U., Steffen, H. M., Hacker, U., et al. (2012). Expression of platelet-derived growth factor-C and insulin-like growth factor I in hepatic stellate cells is inhibited by miR-29. *Lab. Invest.* 92, 978–987. doi: 10.1038/labinvest.2012.70

Kwiecinski, M., Noetel, A., Elfimova, N., Trebicka, J., Schievenbusch, S., Strack, I., et al. (2011). Hepatocyte growth factor (HGF) inhibits collagen I and IV synthesis in hepatic stellate cells by miRNA-29 induction. *PLoS ONE* 6:e24568. doi: 10.1371/journal.pone.0024568

Lagos-Quintana, M., Rauhut, R., Yalcin, A., Meyer, J., Lendeckel, W., and Tuschl, T. (2002). Identification of tissue-specific microRNAs from mouse. *Curr. Biol.* 12, 735–739. doi: 10.1016/S0960-9822(02)00809-6

Lakner, A. M., Steuerwald, N. M., Walling, T. L., Ghosh, S., Li, T., McKillop, I. H., et al. (2012). Inhibitory effects of microRNA 19b in hepatic stellate cell-mediated fibrogenesis. *Hepatology* 56, 300–310. doi: 10.1002/hep.25613

Lee, H. J. (2013). Exceptional stories of microRNAs. *Exp. Biol. Med.* 238, 339–343. doi: 10.1258/ebm.2012.012251

Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., et al. (2003). The nuclear RNase III Drosha initiates microRNA processing. *Nature* 425, 415–419. doi: 10.1038/nature01957

Leung, A. K., and Sharp, P. A. (2010). MicroRNA functions in stress responses. *Mol. Cell* 40, 205–215. doi: 10.1016/j.molcel.2010.09.027

Li, J., Ghazwani, M., Zhang, Y., Lu, J., Li, J., Fan, J., et al. (2013). miR-122 regulates collagen production via targeting hepatic stellate cells and suppressing P4HA1 expression. *J. Hepatol.* 58, 522–528. doi: 10.1016/j.jhep.2012.11.011

Li, X., Wang, Y., Wang, H., Huang, C., Huang, Y., and Li, J. (2015). Endoplasmic reticulum stress is the crossroads of autophagy, inflammation, and apoptosis signaling pathways and participates in liver fibrosis. *Inflamm. Res.* 64, 1–7. doi: 10.1007/s00011-014-0772-y

Liu, C. J., Tsai, M. M., Hung, P. S., Kao, S. Y., Liu, T. Y., Wu, K. J., et al. (2010). miR-31 ablates expression of the HIF regulatory factor FIH to activate the HIF pathway in head and neck carcinoma. *Cancer Res.* 70, 1635–1644. doi: 10.1158/0008-5472.CAN-09-2291

Lund, E., Guttinger, S., Calado, A., Dahlberg, J. E., and Kutay, U. (2004). Nuclear export of microRNA precursors. *Science* 303, 95–98. doi: 10.1126/science.1090599

Magenta, A., Cencioni, C., Fasanaro, P., Zaccagnini, G., Greco, S., Sarra-Ferraris, G., et al. (2011). miR-200c is upregulated by oxidative stress and induces endothelial cell apoptosis and senescence via ZEB1 inhibition. *Cell Death Differ.* 18, 1628–1639. doi: 10.1038/cdd.2011.42

Mahon, P. C., Hirota, K., and Semenza, G. L. (2001). FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. *Genes Dev.* 15, 2675–2686. doi: 10.1101/gad.924501

Malhi, H., and Kaufman, R. J. (2011). Endoplasmic reticulum stress in liver disease. *J. Hepatol.* 54, 795–809. doi: 10.1016/j.jhep.2010.11.005

Mateescu, B., Batista, L., Cardon, M., Gruosso, T., de Feraudy, Y., Mariani, O., et al. (2011). miR-141 and miR-200a act on ovarian tumorigenesis by controlling oxidative stress response. *Nat. Med.* 17, 1627–1635. doi: 10.1038/nm.2512

Maubach, G., Lim, M. C., Chen, J., Yang, H., and Zhuo, L. (2011). miRNA studies in *in vitro* and *in vivo* activated hepatic stellate cells. *World J. Gastroenterol.* 17, 2748–2773. doi: 10.3748/wjg.v17.i22

Mederacke, I., Hsu, C. C., Troeger, J. S., Huebener, P., Mu, X., Dapito, D. H., et al. (2013). Fate tracing reveals hepatic stellate cells as dominant contributors to liver fibrosis independent of its aetiology. *Nat. Commun.* 4, 2823. doi: 10.1038/ncomms3823

Moon, J. O., Welch, T. P., Gonzalez, F. J., and Copple, B. L. (2009). Reduced liver fibrosis in hypoxia-inducible factor-1alpha-deficient mice. *Am. J. Physiol. Gastrointest. Liver Physiol.* 296, G582–G592. doi: 10.1152/ajpgi.90368.2008

Murakami, Y., Toyoda, H., Tanaka, M., Kuroda, M., Harada, Y., Matsuda, F., et al. (2011). The progression of liver fibrosis is related with overexpression of the miR-199 and 200 families. *PLoS ONE* 6:e16081. doi: 10.1371/journal.pone.0016081

Nath, B., and Szabo, G. (2012). Hypoxia and hypoxia inducible factors: diverse roles in liver diseases. *Hepatology* 55, 622–633. doi: 10.1002/hep.25497

Nieto, N., Friedman, S. L., Greenwel, P., and Cederbaum, A. I. (1999). CYP2E1-mediated oxidative stress induces collagen type, I., expression in rat hepatic stellate cells. *Hepatology* 30, 987–996. doi: 10.1002/hep.510300433

Orban, T. I., and Izaurralde, E. (2005). Decay of mRNAs targeted by RISC requires XRN1, the Ski complex, and the exosome. *RNA* 11, 459–469. doi: 10.1261/rna.7231505

Padgett, K. A., Lan, R. Y., Leung, P. C., Lleo, A., Dawson, K., Pfeiff, J., et al. (2009). Primary biliary cirrhosis is associated with altered hepatic microRNA expression. *J. Autoimmun.* 32, 246–253. doi: 10.1016/j.jaut.2009.02.022

Parola, M., and Robino, G. (2001). Oxidative stress-related molecules and liver fibrosis. *J. Hepatol.* 35, 297–306. doi: 10.1016/S0168-8278(01)00142-8

Paternostro, C., David, E., Novo, E., and Parola, M. (2010). Hypoxia, angiogenesis and liver fibrogenesis in the progression of chronic liver diseases. *World J. Gastroenterol.* 16, 281–288. doi: 10.3748/wjg.v16.i3.281

Piccinino, F., Sagnelli, E., Pasquale, G., and Giusti, G. (1986). Complications following percutaneous liver biopsy. A multicentre retrospective study on 68,276 biopsies. *J. Hepatol.* 2, 165–173. doi: 10.1016/S0168-8278(86)80075-7

Pillai, R. S., Bhattacharyya, S. N., Artus, C. G., Zoller, T., Cougot, N., Basyuk, E., et al. (2005). Inhibition of translational initiation by Let-7 MicroRNA in human cells. *Science* 309, 1573–1576. doi: 10.1126/science.1115079

Poli, G. (2000). Pathogenesis of liver fibrosis: role of oxidative stress. *Mol. Aspects Med.* 21, 49–98. doi: 10.1016/S0098-2997(00)00004-2

Roderburg, C., and Luedde, T. (2014). Circulating microRNAs as markers of liver inflammation, fibrosis and cancer. *J. Hepatol.* 61, 1434–1437. doi: 10.1016/j.jhep.2014.07.017

Roderburg, C., Urban, G. W., Bettermann, K., Vucur, M., Zimmermann, H., Schmidt, S., et al. (2011). Micro-RNA profiling reveals a role for miR-29 in human and murine liver fibrosis. *Hepatology* 53, 209–218. doi: 10.1002/hep.23922

Rosmorduc, O., Wendum, D., Corpechot, C., Galy, B., Sebbagh, N., Raleigh, J., et al. (1999). Hepatocellular hypoxia-induced vascular endothelial growth factor expression and angiogenesis in experimental biliary cirrhosis. *Am. J. Pathol.* 155, 1065–1073. doi: 10.1016/S0002-9440(10)65209-1

Schickel, R., Park, S. M., Murmann, A. E., and Peter, M. E. (2010). miR-200c regulates induction of apoptosis through CD95 by targeting FAP-1. *Mol. Cell* 38, 908–915. doi: 10.1016/j.molcel.2010.05.018

Schuppan, D., and Afdhal, N. H. (2008). Liver cirrhosis. *Lancet* 371, 838–851. doi: 10.1016/S0140-6736(08)60383-9

Sekiya, Y., Ogawa, T., Yoshizato, K., Ikeda, K., and Kawada, N. (2011). Suppression of hepatic stellate cell activation by microRNA-29b. *Biochem. Biophys. Res. Commun.* 412, 74–79. doi: 10.1016/j.bbrc.2011.07.041

Semenza, G. L. (2007). Hypoxia-inducible factor 1 (HIF-1) pathway. *Sci. STKE* 2007:cm8. doi: 10.1126/stke.4072007cm8

Sergey, D. (2011). Cross talk between mitochondria and NADPH oxidases. *Free Radic. Biol. Med.* 51, 1289–1301. doi: 10.1016/j.freeradbiomed.2011.06.033

Shan-Shan, Z., Joy, X. J., Jian, W., Charles, H., Scott, L. F., Mark, A. Z., et al. (2006). Phagocytosis of apoptotic bodies by hepatic stellate cells induces NADPH oxidase and is associated with liver fibrosis *in vivo*. *Hepatology* 43, 435–443. doi: 10.1002/hep.21093

Sheedy, F. J., E., Palsson-McDermott, Hennessy, E. J., Martin, C., O'Leary, J. J., Ruan, Q., et al. (2010). Negative regulation of TLR4 via targeting of the proinflammatory tumor suppressor PDCD4 by the microRNA miR-21. *Nat. Immunol.* 11, 141–147. doi: 10.1038/ni.1828

Shi, Y. F., Fong, C. C., Zhang, Q., Cheung, P. Y., Tzang, C. H., Wu, R. S., et al. (2007). Hypoxia induces the activation of human hepatic stellate cells LX-2 through TGF-beta signaling pathway. *FEBS Lett.* 581, 203–210. doi: 10.1016/j.febslet.2006.12.010

Sies, H. (1991). Oxidative stress: from basic research to clinical application. *Am. J. Med.* 91, 31S–38S. doi: 10.1016/0002-9343(91)90281-2

Song, J., Gao, L., Yang, G., Tang, S., Xie, H., Wang, Y., et al. (2014). MiR-199a regulates cell proliferation and survival by targeting FZD7. *PLoS ONE* 9:e110074. doi: 10.1371/journal.pone.0110074

Su, S. F., Chang, Y. W., Andreu-Vieyra, C., Fang, J. Y., Yang, Z., Han, B., et al. (2013). miR-30d, miR-181a and miR-199a-5p cooperatively suppress the endoplasmic reticulum chaperone and signaling regulator GRP78 in cancer. *Oncogene* 32, 4694–4701. doi: 10.1038/onc.2012.483

Sun, X., He, Y., Ma, T. T., Huang, C., Zhang, L., and Li, J. (2014). Participation of miR-200a in TGF-beta1-mediated hepatic stellate cell activation. *Mol. Cell. Biochem.* 388, 11–23. doi: 10.1007/s11010-013-1895-0

Tu, H., Sun, H., Lin, Y., Ding, J., Nan, K., Li, Z., et al. (2014b). Oxidative stress upregulates PDCD4 expression in patients with gastric cancer via miR-21. *Curr. Pharm. Des.* 20, 1917–1923. doi: 10.2174/13816128113199990547

Tu, X., Zhang, H., Zhang, J., Zhao, S., Zheng, X., Zhang, Z., et al. (2014a). MicroRNA-101 suppresses liver fibrosis by targeting the TGFbeta signalling pathway. *J. Pathol.* 234, 46–59. doi: 10.1002/path.4373

Tufekci, K. U., Oner, M. G., Meuwissen, R. L., and Genc, S. (2014). The role of microRNAs in human diseases. *Methods Mol. Biol.* 1107, 33–50. doi: 10.1007/978-1-62703-748-8_3

Turchinovich, A., Weiz, L., Langheinz, A., and Burwinkel, B. (2011). Characterization of extracellular circulating microRNA. *Nucleic Acids Res.* 39, 7223–7233. doi: 10.1093/nar/gkr254

Upton, J. P., Wang, L., Han, D., Wang, E. S., Huskey, N. E., Lim, L., et al. (2012). IRE1alpha cleaves select microRNAs during ER stress to derepress translation of proapoptotic Caspase-2. *Science* 338, 818–822. doi: 10.1126/science.1226191

Wallace, K., Burt, A. D., and Wright, M. C. (2008). Liver fibrosis. *Biochem. J.* 411, 1–18. doi: 10.1042/BJ20071570

Wang, X. W., Heegaard, N. H., and Orum, H. (2012). MicroRNAs in liver disease. *Gastroenterology* 142, 1431–1443. doi: 10.1053/j.gastro.2012.04.007

Wang, Y., Huang, Y., Guan, F., Xiao, Y., Deng, J., Chen, H., et al. (2013). Hypoxia-inducible factor-1alpha and MAPK co-regulate activation of hepatic stellate cells upon hypoxia stimulation. *PLoS ONE* 8:e74051. doi: 10.1371/journal.pone.0074051

Wei, C., Li, L., Kim, I. K., Sun, P., and Gupta, S. (2014). NF-kappaB mediated miR-21 regulation in cardiomyocytes apoptosis under oxidative stress. *Free Radic. Res.* 48, 282–291. doi: 10.3109/10715762.2013.865839

Wightman, B., Ha, I., and Ruvkun, G. (1993). Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in *C. elegans*. *Cell* 75, 855–862. doi: 10.1016/0092-8674(93)90530-4

Xin, X., Zhang, Y., Liu, X., Xin, H., Cao, Y., and Geng, M. (2014). MicroRNA in hepatic fibrosis and cirrhosis. *Front. Biosci.* 19, 1418–1424. doi: 10.2741/4292

Xu, C., Bailly-Maitre, B., and Reed, J. C. (2005). Endoplasmic reticulum stress: cell life and death decisions. *J. Clin. Invest.* 115, 2656–2664. doi: 10.1172/JCI26373

Xu, Y., Zhou, M., Wang, J., Zhao, Y., Li, S., Zhou, B., et al. (2014). Role of microRNA-27a in down-regulation of angiogenic factor AGGF1 under hypoxia associated with high-grade bladder urothelial carcinoma. *Biochim. Biophys. Acta* 1842, 712–725. doi: 10.1016/j.bbadis.2014.01.007

Yan, S. F., Ramasamy, R., and Schmidt, A. M. (2010). The RAGE axis: a fundamental mechanism signaling danger to the vulnerable vasculature. *Circ. Res.* 106, 842–853. doi: 10.1161/CIRCRESAHA.109.212217

Yang, F., Zhang, L., Wang, F., Wang, Y., Huo, X. S., Yin, Y. X., et al. (2011). Modulation of the unfolded protein response is the core of microRNA-122-involved sensitivity to chemotherapy in hepatocellular carcinoma. *Neoplasia* 13, 590–600. doi: 10.1593/neo.11422

Yang, J. J., Tao, H., Hu, W., Liu, L. P., Shi, K. H., Deng, Z. Y., et al. (2014). MicroRNA-200a controls Nrf2 activation by target Keap1 in hepatic stellate cell proliferation and fibrosis. *Cell. Signal.* 26, 2381–2389. doi: 10.1016/j.cellsig.2014.07.016

Yao, H., Yang, S. R., Kode, A., Rajendrasozhan, S., Caito, S., Adenuga, D., et al. (2007). Redox regulation of lung inflammation: role of NADPH oxidase and NF-kappaB signalling. *Biochem. Soc. Trans.* 35, 1151–1155. doi: 10.1042/BST0351151

Yoon, C., Kim, D., Kim, S., Park, G. B., Hur, D. Y., Yang, J. W., et al. (2014). MiR-9 regulates the post-transcriptional level of VEGF165a by targeting SRPK-1 in ARPE-19 cells. *Graefes Arch. Clin. Exp. Ophthalmol.* 252, 1369–1376. doi: 10.1007/s00417-014-2698-z

Zeng, C., Wang, Y. L., Xie, C., Sang, Y., Li, T. J., Zhang, M., et al. (2015). Identification of a novel TGF-beta-miR-122-fibronectin 1/serum response factor signaling cascade and its implication in hepatic fibrogenesis. *Oncotarget* 6, 12224–12233. doi: 10.1074/jbc.M111.314922

Zhang, L., Zhou, M., Qin, G., Weintraub, N. L., and Tang, Y. (2014). MiR-92a regulates viability and angiogenesis of endothelial cells under oxidative stress. *Biochem. Biophys. Res. Commun.* 446, 952–958. doi: 10.1016/j.bbrc.2014.03.035

Zhang, Y., Wu, L., Wang, Y., Zhang, M., Li, L., Zhu, D., et al. (2012). Protective role of estrogen-induced miRNA-29 expression in carbon tetrachloride-induced mouse liver injury. *J. Biol. Chem.* 287, 14851–14862. doi: 10.1074/jbc.M111.314922

Zhang, Z., Sun, H., Dai, H., Walsh, R. M., Imakura, M., Schelter, J., et al. (2009). MicroRNA miR-210 modulates cellular response to hypoxia through the MYC antagonist MNT. *Cell Cycle* 8, 2756–2768. doi: 10.4161/cc.8.17.9387

Zhao, X., Wang, K., Liao, Y., Zeng, Q., Li, Y., Hu, F., et al. (2015). MicroRNA-101a inhibits cardiac fibrosis induced by hypoxia via targeting TGFbetaRI on cardiac fibroblasts. *Cell. Physiol. Biochem.* 35, 213–226. doi: 10.1159/000369689

Zheng, J., Wu, C., Xu, Z., Xia, P., Dong, P., Chen, B., et al. (2015). Hepatic stellate cell is activated by microRNA-181b via PTEN/Akt pathway. *Mol. Cell. Biochem.* 398, 1–9. doi: 10.1007/s11010-014-2199-8

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REVIEW
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The Role of NADPH Oxidases (NOXs) in Liver Fibrosis and the Activation of Myofibroblasts

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Chronic liver injury, resulted from different etiologies (e.g., virus infection, alcohol abuse, nonalcoholic steatohepatitis (NASH) and cholestasis) can lead to liver fibrosis characterized by the excess accumulation of extracellular matrix (ECM) proteins (e.g., type I collagen). Hepatic myofibroblasts that are activated upon liver injury are the key producers of ECM proteins, contributing to both the initiation and progression of liver fibrosis. Hepatic stellate cells (HSCs) and to a lesser extent, portal fibroblast, are believed to be the precursor cells that give rise to hepatic myofibroblasts in response to liver injury. Although, much progress has been made toward dissecting the lineage origin of myofibroblasts, how these cells are activated and become functional producers of ECM proteins remains incompletely understood. Activation of myofibroblasts is a complex process that involves the interactions between parenchymal and non-parenchymal cells, which drives the phenotypic change of HSCs from a quiescent stage to a myofibroblastic and active phenotype. Accumulating evidence has suggested a critical role of NADPH oxidase (NOX), a multi-component complex that catalyzes reactions from molecular oxygen to reactive oxygen species (ROS), in the activation process of hepatic myofibroblasts. NOX isoforms, including NOX1, NOX2 and NOX4, and NOX-derived ROS, have all been implicated to regulate HSC activation and hepatocyte apoptosis, both of which are essential steps for initiating liver fibrosis. This review highlights the importance of NOX isoforms in hepatic myofibroblast activation and the progression of liver fibrosis, and also discusses the therapeutic potential of targeting NOXs for liver fibrosis and associated hepatic diseases.

Keywords: NADPH oxidase (NOX), liver fibrosis, myofibroblasts, reactive oxygen species (ROS), hepatic stellate cells (HSCs), hepatocytes

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INTRODUCTION

The main causes of hepatic fibrosis are chronic hepatitis B and C infection, autoimmune and biliary diseases, alcoholic steatohepatitis (ASH) and, increasingly, nonalcoholic steatohepatitis (NASH) (Bataller and Brenner, 2005). Liver fibrosis results from a sustained wound healing process in response to chronic liver injuries, and is characterized by accumulation of excessive extracellular matrix (ECM) proteins. Prolonged and excessive buildup of ECM proteins leads to pronounced distortion of hepatic vascular architecture due to formation of the fibrous scar, which promotes subsequent hepatocyte regeneration and hepatic endothelial dysfunction

(Friedman, 2000). These processes facilitate the transition from liver fibrosis to cirrhosis, which may ultimately progress to more serious complications, such as portal hypertension due to increased resistance to portal blood flow, spontaneous bacterial peritonitis, and hepatic encephalopathy. Liver fibrosis is reversible, whereas cirrhosis, the end-stage consequence of fibrosis, is often irreversible and results in liver failure or the development of hepatocellular carcinoma (HCC) and death unless liver transplantation is done (Tsochatzis et al., 2014). Thus, it is of utmost importance to investigate the molecular and cellular mechanisms involved in the fibrogenic processes in order to design novel therapeutic interventions for liver fibrosis.

The major source of excessive ECM and fibrogenic mediators, such as collagen, is myofibroblasts. Recent studies indicate that the origin of myofibroblasts is liver intrinsic, and activated hepatic stellate cells (HSCs) and portal fibroblasts are believed to be the main precursors that give rise to hepatic myofibroblasts (Brenner et al., 2012). Upon liver injury, HSCs and portal fibroblasts undergo dramatic phonotypical changes by acquiring profibrogenic properties. In the normal liver, quiescent HSCs positive for adipocytes markers (PPARγ, SREBP-1c, and leptin) are the major cell type responsible for vitamin A storage (Bataller and Brenner, 2005). Upon activation by fibrogenic cytokines such as TGF-β1, angiotensin II, and leptin, quiescent HSCs trans-differentiate into myofibroblasts, possessing the properties of contractile, proinflammatory, and profibrogenic (Friedman, 2000). Activated HSCs express myogenic markers, such as α smooth muscle actin, c-myc, and myocyte enhancer factor–2 (Bataller and Brenner, 2005).

Accumulating clinical and pre-clinical data suggest that chronic liver injury results in the generation of oxidative stress, which disrupts lipids, proteins and DNA, induces necrosis/apoptosis of hepatocytes and amplifies the inflammatory response. Moreover, reactive oxygen species (ROS) mediate the progression of hepatic fibrosis by stimulating the production of profibrogenic mediators from Kupffer cells and circulating inflammatory cells and by directly activating HSCs to induce their trans-differentiation into myofibroblasts (Sánchez-Valle et al., 2012). Emerging evidence indicate that the nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs) are sources of ROS, which play crucial roles in the progression of hepatic fibrosis (Aoyama et al., 2012; Paik et al., 2014). Seven NOXs isoforms have been identified in mammals so far. The major NOX isoforms expressed in the liver are NOX1, NOX2, and NOX4. NOX2 was the first discovered NOX in phagocytes, which plays important role in inflammation and host immune defense. HSCs and hepatocytes express NOX1, NOX2, and NOX4. It becomes increasingly clear that NOX-dependent ROS production is not limited to phagocytes because NOX enzymes are widely expressed and active in many different cell types from varies of tissues and organs. This review will focus on summarizing the roles of NOX isoforms that are distinctly expressed in different cell types in the liver.

NADPH OXIDASES

ROS are defined as oxygen radicals, including reactive molecules, such as peroxide, superoxide, hydroxide, and singlet oxygen. In physiological conditions, ROS are generated during normal oxygen metabolism and play important roles in maintaining cellular homeostasis by orchestrating host defense, cell growth and signaling. However, ROS can also rapidly accumulate in large quantities during oxidative stress when cells encounter either endogenous or exogenous challenges. This, if not properly controlled, might lead to adverse cellular events, including irreversible cellular damage and death which may ultimately results in tissue damage and organ dysfunction (Devasagayam et al., 2004). ROS mediated oxidative stress is strongly associated with varieties of human diseases, including Parkinson's (Smeyne and Smeyne, 2013), Alzheimer (Aliev et al., 2014), cardiovascular (Robert and Robert, 2014), immunological (De Deken et al., 2014), pulmonary (Wong et al., 2013), renal (Ozbek, 2012), as well as liver diseases (Jaeschke, 2011).

In chronic liver diseases, pathological insults, such as ischemia-reperfusion, cholestasis or drug toxicity, induce hepatocyte death, which activates immune cells and promotes HSC transdifferentiation into collagen-producing myofibroblasts, which ultimately drives the development of hepatic fibrosis and cirrhosis. ROS accumulation in hepatocytes can cause cell death, which release damage-associated molecular patterns (DAMPs) that stimulates liver resident Kupffer cells and newly recruited immune cells to produce profibrogenic mediators. ROS is vital for HSC activation, resulting in the initiation of fibrosis. In the liver, several cellular machineries can generate ROS, including the mitochondrial respiratory chain, cytochrome P450 (CYP) family members, peroxisomes, xanthine oxidase, and NADPH oxidases. NADPH oxidase that produces ROS was first discovered in phagocytes, referred as $gp91^{phox}$ (also known as NOX2), and serves as an important inflammatory mediator against invading bacteria. Recently, other NOX2 like molecules have been identified in various tissues. Due to the sequential and functional similarities of these enzymes to NOX2, these enzymes, together with NOX2 are collectively referred to as the NOX family. The NOX family genes encode proteins responsible for a transmembrane electron transport chain containing a flavocytochrome b, which transfers electrons donated by NADPH across biological membranes to form superoxide (O_2^-) and hydrogen peroxide (H_2O_2) from molecular oxygen (Cross and Segal, 2004). Seven NOX family members have been identified so far, including NOX1, NOX2 (formerly known as $gp91^{phox}$), NOX3, NOX4, NOX5, and dual oxidase Duox proteins (DUOX1 and DUOX2).

The phagocytic NOX (NOX2) core enzyme comprises several different subunits that interact with each other to form an active enzyme complex, including NOX2 ($gp91^{phox}$), $p40^{phox}$ (*PHOX* for phagocyte oxidase), $p47^{phox}$, $p67^{phox}$, $p22^{phox}$, Rac2, and Rap1A, which is responsible for superoxide production upon agonist stimulation. In the resting stage, two integral membrane proteins—gp91phox and p22phox, form a large heterodimeric subunit flavocytochrome b_{558} (cyt b_{558}). Three of the regulatory

proteins, $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$ form a complex in the cytosol (Groemping and Rittinger, 2005; Sumimoto et al., 2005). Upon stimulation (e.g., exposure of cells to microorganisms or inflammatory mediators), $p40^{phox}$ is highly phosphorylated, resulting in the entire cytosolic complex translocation to plasma membrane and association with flavocytochrome b_{558}. The whole NOX complex activation also requires the association of two low-molecular-weight guanine nucleotide-binding proteins, Rac2 GTPase and Rap1A (Diebold and Bokoch, 2001). Then the activated complex transfers electrons from the cytosolic NADPH to oxygen on the luminal or extracellular region (Koga et al., 1999). The expression of NOX2 is induced by interferon-γ (IFN-γ) through a transcription factor protein complex, called hematopoiesis-associated factor (HAF1), which is comprised of PU.1, interferon regulatory factor 1 (IRF-1), and interferon consensus sequence-binding protein (ICSBP) (Eklund et al., 1998).

NOX1 is identified as the first homolog of NOX2, and shares 60% amino-acid identity with NOX2 (Suh et al., 1999). NOX1 is widely expressed in many cell types, such as vascular smooth muscle cells (VSMCs), endothelial cells, astrocytes, and microglia. In liver, NOX1 is expressed in HSCs, ECs, and hepatocytes. However, the subcellular localization of NOX1 remains nebulous. It was suggested that NOX1 is a plasma membrane protein, and potentially resides in caveolin 1-containing lipid rafts (Hilenski et al., 2004; Zuo et al., 2005). Similar to NOX2, the activation of NOX1 also requires regulatory subunits, known as NOX organizer 1 (NOXO1) and NOX activator 1 (NOXA1), which are homologs of $p47^{phox}$ and $p67^{phox}$, respectively (Bánfi et al., 2003; Cheng and Lambeth, 2004). In addition, $p22^{phox}$ and Rac GTPase are also required for NOX1 activation. Expression of NOX1 is also highly regulated. Its mRNA is induced by the growth factors including platelet-derived growth factor (PDGF), and angiotensin and phorbol esters (Suh et al., 1999; Lassègue et al., 2001).

NOX4, which is first discovered in kidney, shares 39% sequence homology with NOX2 (Geiszt et al., 2000). Its activity requires direct interaction with $p22^{phox}$, but independent of the interaction with any cytosolic regulatory subunits (Ambasta et al., 2004). Moreover, Poldip2, a polymerase delta-interacting protein, has been shown to be associated with $p22^{phox}$, which ultimately increases NOX4 enzymatic activity in VSMCs (Lyle et al., 2009). Similar to NOX1, NOX4 expression can also be regulated by angiotensin II. Moreover, TGFβ is also a potent regulator of NOX4 mRNA (Sturrock et al., 2006; Bondi et al., 2010).

ORIGINS AND ACTIVATION OF HEPATIC MYOFIBROBLASTS

Chronic liver injury of all etiologies can promote liver fibrosis, a wound healing process whose hallmark is the formation of fibrous scar constituted by ECM. The main producer of extracellular matrix proteins in the liver is myofibroblast, a terminally differentiated cell type that plays a critical role in wound healing and connective tissue remodeling. Not only possessing the ECM synthesizing features of fibroblasts, myofibroblast also has the contractile functions similar to the smooth muscle cells (Hinz et al., 2012). Under the self-limiting and homeostatic tissue repair processes, such as wound healing, myofibroblasts are induced and differentiated from their precursors, migrate to the site of injury, function to produce ECM proteins to contract the wound, and finally undergo apoptosis once injury is resolved. However, these processes can become uncontrolled when the myofibroblasts activities become excessive and persist due to the inability to undergo apoptosis, for example. This will lead to overwhelming ECM deposition, resulting in fibrosis and eventually cirrhosis (Watsky et al., 2010). In addition to the normal tissue repair and wound healing responses, myofibroblasts also contribute to regeneration, inflammation, angiogenesis, and stromal reaction during tumorigenesis. Although, myofibroblasts differ from fibroblasts by their ability of the former to de novo synthesize of α-smooth muscle actin (α-SMA), this is not an absolute requirement to define a cell as myofibroblast. Instead, the most reliable features of myofibroblasts are secretion of extracellular matrix, development of adhesion structures, and formation of contractile bundles (Hinz, 2010). Several novel markers of myofibroblasts, such as endosialin for tumor-associated myofibroblasts (Christian et al., 2008), P311 for hypertrophic scar myofibroblasts (Tan et al., 2010), and integrin α11β1 for human corneal myofibroblasts (Carracedo et al., 2010), have been recently identified. However, none of these markers are specific for myofibroblasts, and they play distinct roles in various types of fibroblasts, therefore affecting myofibroblasts differentiation in a tissue- and context- dependent manner. Nonetheless, reliable and unique markers for myofibroblasts remain to be defined.

Myofibroblasts are absent in healthy liver, but they are induced and activated from their precursor cells in response to hepatic injury. Although, the origin of myofibroblasts is yet unclear, three possible sources of myofibroblasts precursors in the liver have been proposed. The first possible source is the group of resident cells from the mesodermal origin that can potentially become myofibroblasts. This includes HSCs, portal fibroblasts, smooth muscle cells, and fibroblast around the central veins, which are different from hepatocytes, Kupffer cells, and sinusoidal endothelial cells. The second group of possible precursors of myofibroblasts are hepatocytes, cholangiocytes, and endothelial cells that can undergo epithelial or endothelial mesenchymal transition (EMT). However, several fate tracing and genetic labeling studies argued that hepatocytes or cholangiocytes did not undergo EMT in liver fibrosis models (Humphreys et al., 2010; Scholten et al., 2010; Taura et al., 2010). As for renal fibrosis, recent two studies argued that renal epithelial cells can undergo EMT, relaying signals to the interstitium to promote myofibroblast differentiation and fibrogenesis rather than directly giving rise to myofibroblasts population (Grande et al., 2015; Lovisa et al., 2015). Finally, bone marrow (BM)-derived cells consisting of fibrocytes and circulating mesenchymal cells can migrate into fibrotic liver tissue, transform into myofibroblasts and may contribute in the progression of liver fibrosis (Russo et al., 2006). Thus, these cells could also be possible precursors of myofibroblasts. However, a recent study using bone marrow (BM) chimeric mice

reconstituted from transgenic collagen reporter mice suggested that BM cells had negligible contribution in collagen production during hepatic fibrosis (Higashiyama et al., 2009).

Among different mesenchymal cell types, the vitamin A-containing lipocytes (HSCs) capable of producing type III collagen was the first identified myofibroblast precursor in the liver (Kent et al., 1976). Since then, much focus has been put on HSCs to identify the origin of myofibroblasts. Upon liver injury, HSCs are activated, and converted from quiescent vitamin-A rich cells to proliferative, fibrogenic, and contractile myofibroblasts (Friedman, 2008). HSCs are regarded as the "warehouse" of retinoid droplets that exhibit blue-green autofluorescence when excited by UV light. However, cells that are absent of retinoid droplets are distinct from HSCs, which undergo a PDGF-mediated conversion into myofibroblasts (Kinnman et al., 2003). These cells are thought to be portal fibroblasts that are accumulated around bile ducts, and might play a critical role in the early stage of bile duct ligation (BDL) induced fibrosis (Tuchweber et al., 1996). Moreover, liver fibrosis seems to develop predominantly from the portal area and progress from there, irrespectively of the underlying etiology. Therefore the role of portal fibroblasts in the development of fibrosis may be more important than generally assumed.

NOXS IN HSC ACTIVATION

Upon liver injury, quiescent HSC become activated. The activation process is characterized by the loss of vitamin-A containing droplets, *de novo* synthesis of a-SMA, collagen and ECM proteins, and increased contractile and cell survival. The activation of HSC is a complex process, which involves the contribution of extracellular stimuli and different cell types, including parenchymal cells, immune cells. NOX proteins and NOX-derived ROS play a key role during HSC activation (**Figure 1**). ROS are produced in defined cellular compartments, but diffuse throughout the cell (e.g., superoxide) or across the plasma membranes (e.g., H_2O_2). ROS, when present at low levels, could serve as secondary messengers in response to a variety of cellular stimuli. For instance, it has been shown that low amount of hydrogen peroxide (H_2O_2) can act as second messenger that plays a critical role in the initiation and amplification of signaling during lymphocyte activation (Reth, 2002). In contrast, high level of ROS can be toxic and may lead to cell death. Although, low levels of ROS promote HSC to produce collagen and proliferate, while high-level toxic amount of ROS can induce death of HSCs (Novo et al., 2006).

NOXs are highly upregulated in patients with organ fibrosis, such as heart, lung, kidney, and pancreas. In consistence with their role in human fibrosis, NOX-derived ROS is also essential for multiple organ fibrosis in mice (Paik et al., 2006). In addition, during HSC activation, NOX mediates a number of fibrogenic responses induced by different agonists, including Ang II, PDGF, leptin, and TGFβ. Moreover, phagocytosis of apoptotic bodies by HSC leads to NOX activation and procollagen α1 (I) expression (Zhan et al., 2006). The expression of NOX isoforms is different among different types of liver resident cells. Kupffer cells only express phagocytic NOX2, whereas hepatocytes and HSC express various NOX isoforms, including phagocytic NOX2 and non-phagocytic NOX1, NOX4, DUOX1, and DUOX2. Endothelial cells mainly express NOX1, NOX2, and NOX4. Upon liver injury, NOX isoforms in HSC are strongly upregulated when quiescent HSC become activated myofibroblasts (Paik et al., 2011; Aoyama et al., 2012). HSCs can also fine-tune ROS production by expressing the regulatory subunits of NOX complexes, including $p22^{phox}$, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, NOXO1, NOXOA1, and Rac1. It has been shown that the p47phox regulatory subunit is induced in HSCs activated upon BDL-mediated fibrosis (De Minicis et al.,

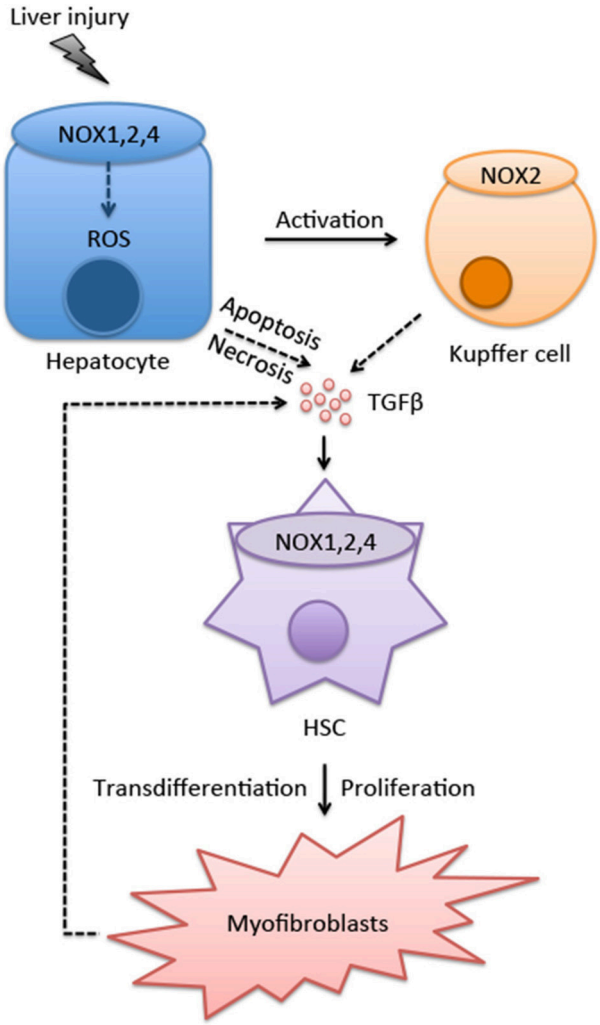

FIGURE 1 | The role of NOXs in myofibroblasts activation. The interactions between hepatocytes, Kupffer cells and HSCs promotes myofibroblast activation. Various NOX isoforms expressed in different cell types in the liver play crucial roles during this process. After exposure to hepatic insults, such as ischaemia/reperfusion (IR) injuries, alcohol abuse, viral infection, hyper-nutrition, and cholestasis, ROS is produced through NOXs in hepatocytes. Increased oxidative stress also induces hepatocyte apoptosis/necrosis, resulting in release of DAMPs that activate Kupffer cells. Injured hepatocytes and activated Kupffer cells secrete proinflammatory and profibrogenic cytokine TGFβ, which promotes the differentiation of HSCs into myofibroblasts (see text for further details).

2010). At resting stage, human HSCs express low levels of both catalytic and regulatory NOX components, including NOX1, NOX2, and $p47^{phox}$. However, these NOX subunits are highly upregulated in HSCs from patients with fibrotic liver diseases (Bataller et al., 2003). Moreover, NOX1 and NOX4 protein levels were increased in human livers with cirrhosis compared with normal controls (Lan et al., 2015).

NOX4 IN HSCS

A complex network involving paracrine/autocrine signals in parenchymal and nonparenchymal cells is required for HSCs activation and differentiation into myofibroblasts. Accumulating evidence has suggested that NOXs are the key mediators in HSC activation, which promotes hepatic fibrosis. TGFβ is the most potent regulator promoting collagen production and α-SMA expression in myofibroblasts from various organs, including liver (Gressner et al., 2002), kidney (Desmoulière et al., 2003), lung (Hardie et al., 2009), and heart (Kuwahara et al., 2002). During the initiation and progression of liver fibrosis, TGFβ plays a crucial role in regulating HSC activation, as well as inducing hepatocyte apoptosis, which leads to the secretion of cytokines, chemokines and microparticles that are critical for HSC and Kupffer cell activation. A number of studies have shown that NOX4 is essential for TGFβ-induced myofibroblast activation and fibrogenic responses such as collagen production in different organs, including lung (Hecker et al., 2009), kidney (Bondi et al., 2010), heart (Cucoranu et al., 2005; Chan et al., 2013), and prostate (Sampson et al., 2011). Moreover, although NOX4 can be induced by TGFβ in several different organs (Cucoranu et al., 2005; Sturrock et al., 2006; Bondi et al., 2010; Boudreau et al., 2012), the mechanism involved is controversial among different organs. In kidney and lung myofibroblasts, TGFβ induces NOX4 expression and ROS generation through the classical Smad2/3 pathway (Sturrock et al., 2006; Bondi et al., 2010), whereas NOX4 ugregulation is upstream of Smad2/3 activation in cardiac myofibroblasts (Cucoranu et al., 2005). In liver fibrosis, TGFβ induces NOX activity and ROS production during HSC activation, which plays key role in hepatic myofibroblasts activation (Proell et al., 2007). In BDL- or CCl4-mediated liver fibrosis, NOX4 expression and its activity are upregulated via a TGFβ-Smad3 dependent manner in HSCs (Jiang et al., 2012; Sancho et al., 2012). In addition, NOX4 expression correlates with the fibrotic scores in patients with hepatitis C virus infection or NASH (Sancho et al., 2012; Bettaieb et al., 2015). ROS production and the expression of fibrogenic markers are dramatically reduced in HSCs deficient in NOX4 (Jiang et al., 2012). Moreover, experiments using siRNA against NOX4 attenuated HSC activation, and more importantly, knocking down NOX4 in activated myofibroblasts could reverse the fibrotic phenotypes. Knocking down NOX4 in activated HSCs decreased the expression a-SMA and collagen production with no influence on TGFβ1 expression and phosphorylation of Smad2/3. These indicate that NOX4 activation and the following ROS production are downstream of TGFβ-Smad2/3 signaling pathway (Sancho et al., 2012).

Patients with hepatic fibrosis as a result of various chronic liver injuries, including viral infection, toxin, metabolic disorders, alcohol abuse, and cholestasis, have a breach in gut barrier function. This leads to compromised intestinal permeability that allows the entry of bacteria-derived components (e.g., LPS and CpG-containing DNAs through portal circulation and eventually into the liver, where they activates liver immune cells via acting on toll-like receptors (TLRs) (Seki and Brenner, 2008; Yang and Seki, 2012). TLRs are a group of pattern recognition receptors that recognize their cognate ligands with as either pathogen-associated molecular patterns (PAMPs) or DAMPs. Although, both murine and human HSCs express multiple TLRs (Wang et al., 2009), they respond poorly to TLR ligands, such as Gram-positive bacterial products peptidoglycan (PGN) and lipoteichoic acid (LTA) (Paik et al., 2006). Upon ligand engagement, TLRs are activated and transduce signals through downstream adaptor molecules MyD88 and TRIF to induce the expression of proinflammatory cytokines and chemokines. These inflammatory mediators then recruit KCs and circulating monocytes/macrophages, which produce TGFβ1 to drive the differentiation of HSC into myofibroblast (Seki and Brenner, 2008; Aoyama et al., 2010). Although, the LPS-TLR4 axis is crucial for hepatic fibrogenesis and liver fibrosis is dramatically attenuated in germ-free mice (Seki et al., 2007), the roles of NOX and ROS have not been extensively studied in the context of regulating LPS-TLR4 mediated inflammatory or fibrogenic responses in HSCs. In macrophages, NOX inhibitor DPI or siRNA against $p22^{phox}$ significantly decreased LPS-TLR4-mediated activation of endoplasmic reticulum (ER)-stress sensor kinase IRE1α and its downstream target, the transcription factor XBP1 (Martinon et al., 2010). Moreover, it has been shown that the C-terminal domains of NOX4 and TLR4 directly interact with each other in HEK293T cells (Park et al., 2004). Consistently, in human aortic endothelial cell (HAECs), overexpression of the C-terminal region of NOX4 inhibited nuclear factor-kappaB (NF- κB) activation in response to LPS. NOX4 downregulation using siRNA resulted in reduced ROS production and less expression of adhesion molecule (ICAM-1) and chemokines such as CXCL8 and MCP-1 in response to LPS (Park et al., 2006). Therefore, NOX4 and NOX4-mediated ROS generation may regulate LPS induced NF-κB activation and its downstream signaling pathway in HSC activation and profibrogenic effects of myofibroblasts.

NOX1/NOX2 IN HSCS

In addition to the TGFβ-NOX4 axis-mediated activation of HSC and expression of fibrogenic factors, other NOX isoforms, including NOX1, NOX2, and NOX2 regulatory subunit $p47^{phox}$, are also reported to orchestrate the progression of hepatic fibrosis (Aram et al., 2009; Jiang et al., 2010; Paik et al., 2011). $p47^{phox-/-}$ mouse was the first genetic model of NOX inhibition in the study of HSC function in liver fibrosis. After BDL-induced liver injury, $p47^{phox-/-}$ mice showed attenuated liver injury and fibrosis compared with WT mice. HSCs produce more type I collagen and TGFβ when treated with Angiotensin II (Ang II) (Yoshiji et al., 2001). Ang II also

stimulates ROS production, and activates intracellular signaling pathways involving PKC, PI3K-Akt, MAPKs, ERK, and c-Jun, which presumably promotes HSC migration and proliferation. Consistent with this notion, HSC isolated from $p47^{phox-/-}$ mice had reduced cell motility and expansion capacity, and displayed a reduced fibrogenic response to Ang II (Bataller et al., 2003). Although the detailed molecular mechanism underlying Ang II-induced NOX activation and ROS production is still unclear, studies have indicated that Ang II induces ROS production through two consecutive events in vascular smooth muscle cells: the first event, which occurs within 30 s after Ang II stimulation, is dependent on PKC-mediated phosphorylation of $p47^{phox}$. Phosphorylated $p47^{phox}$ then translocates to the membrane where it binds to and facilitates the activation of NOX1 and/or NOX2. The second event that leads to sustained NOX activation and the following ROS production induced by Ang II (peaked at 30 min) requires the activation of Rac GTPase. Ang II-mediated Rac activation is PI3K, EGFR, and c-Src dependent (Seshiah et al., 2002). In order to keep the prolonged signal induced by Ang II, the expression levels of NADPH catalytic subunits, as well as the regulatory subunits p47phox and p22phox are also upregualted during Ang II stimulation (Fukui et al., 1997; Lassègue et al., 2001; Touyz et al., 2002). Similarly in HSCs, the mRNA levels of both NOX1 and NOX4 are increased upon Ang II treatment (Aoyama et al., 2012).

Proliferation of HSCs is a prerequisite that mediates the proper function of HSC-derived myofibroblasts and fibrogenic response in general. It has been suggested that NOX1 is crucial for promoting HSC proliferation and ROS production in bile duct ligated mouse liver. The underlying molecular mechanism is proposed to involve oxidation and inactivation of phosphatase and tensin homolog (PTEN), leading to the activation of AKT/FOXO4/p27(kip) signaling pathway that promotes HSC proliferation and fibrogenesis following BDL-induced liver injury (Cui et al., 2011). Platelet-derived growth factor (PDGF) is considered the most potent mitogen that promotes HSC proliferation (Pinzani et al., 1989). It has been shown that NOXs play a crucial role in this process (Adachi et al., 2005). PDGF induces HSC proliferation through ROS production, and NOX inhibitor (DPI) or p38 MAPK inhibitor suppressed PDGF-induced ROS production and HSC proliferation (Adachi et al., 2005). Similarly, PDGF stimulates NOX-dependent proliferation of activated pancreatic stellate cells (PaSCs) in chronic alcoholic pancreatitis/fibrosis (Hu et al., 2007). Mechanistically, PDGF stimulation promotes NOX1 expression and ROS production. In line with this, NOX1 is critical in PDGF stimulated vascular hypertrophy through activation of PKCδ (Fan et al., 2005) and inducing transcription factor (ATF)-1 (Katsuyama et al., 2005). Additionally, it has been shown that the transcription factor AP1 binding site is critical for the promoter activity of NOX1 (Cevik et al., 2008). Recently, it has also been shown NOX1/NOX4 inhibitor suppressed PDGF mediated ROS production and proliferative gene expression in primary mouse HSCs (Lan et al., 2015).

NOXS IN HEPATOCYTES

Hepatocytes injury and death are important triggers of myofibroblasts activation. Dying hepatocyte can release DAMPs that induce the secretion of cytokines and chemokines from KCs/macrophage that eventually results in HSC activation and liver fibrosis. TGFβ1, secreted by active KCs/macrophage upon liver injury, can promote hepatocyte apoptosis (Oberhammer et al., 1992). The classical TGFβ mediated signaling pathway requires the binding of TGFβ1 to the TGFβ receptor (TGFβRI and II), leading to the phosphorylation and activation of Smad2/Smad3. Smad2/3 then interact with Smad4 to form an active Smad complex that enters the nucleus and binds to the promoter regions of TGFβ target genes to initiate their transcription. NOX4 is one of the TGFβ target genes. A key finding is that the expression of NOX4 is increased in NASH patients compared with healthy controls (Bettaieb et al., 2015). In hepatocytes, NOX4 expression is induced by TGFβ, and the activity of NOX4 is crucial for TGFβ mediated apoptosis of hepatocytes (Carmona-Cuenca et al., 2008). For instance, knocking down NOX4 in human hepatocytes cell lines (HepG2 and Hep3B) resulted in impaired NOX activity, caspase activation and cell death induced by TGFβ1 (Carmona-Cuenca et al., 2008). In rat fetal hepatocytes, TGFβ1 induces apoptosis through upregulating NOX4-mediated ROS production, followed by down-regulation of pro-survival protein Bcl-x_L, which ultimately results in the loss of mitochondrial membrane potential and initiation of cytochrome C release (Herrera et al., 2001). Additionally, TGFβ1-induced NOX4 activity also increases the levels of pro-apoptotic proteins BIM and BMF (Ramjaun et al., 2007; Caja et al., 2009), and thus further amplifies apoptotic signals. NOX4-derived ROS regulates the transcription of Bcl-x_L and Bmf, whereas its regulation of BIM occurs post-transcriptionally (Caja et al., 2009). Moreover, EGF blocks TGFβ-induced NOX4 expression and hepatocytes death in a MEK/ERK and PI3K/Akt dependent manner (Carmona-Cuenca et al., 2006). Interestingly, NOX4 not only contributes to TGFβ-mediated apoptosis, but also to death ligand (such as FasL or TNFα/actinomycin D)-induced hepatocyte apoptosis (Jiang et al., 2012). Hepatocyte-specific deletion of NOX4 reduced oxidative stress, lipid peroxidation and liver fibrosis in mice (Bettaieb et al., 2015). NOX4 was suggested to reduce the activity of the phosphatase PP1C, leading to prolonged activation of key stress signaling PKR/PERK pathway (Bettaieb et al., 2015). Therefore, NOX4 promotes myofibroblasts activation and hepatic fibrosis through at least two distinct mechanisms: (1) directly facilitating TGFβ-induced HSC activation and production of profibrogenic targets, (2) indirectly promoting TGFβ or death ligand-induced hepatocytes apoptosis, which contributes to the production of cytokines, chemokines, and microparticles that leads to HSC activation (Aoyama et al., 2012; Jiang et al., 2012).

Different from other NOX family proteins, the activity of NOX4 mainly depends on its expression levels, and not on agonist-induced assembly of a complex (Serrander et al.,

2007). NOX4 predominantly mediates H_2O_2 production instead of superoxide (Martyn et al., 2006; Serrander et al., 2007). Potentially, H_2O_2 generated by NOX4 may contribute to the activation of certain protein tyrosine kinases that play crucial roles in TGFβ downstream signaling pathways (Bae et al., 2011). Hepatocytes and sinusoidal endothelial cells also express all of the components for NOX1 and NOX2 H (Jiang and Török, 2014). Owever, the mechanisms underlying NOX1/2 enzyme activation in these cells and their roles in regulating fibrosis and myofibroblasts activation remain largely unknown.

TARGETING HEPATIC FIBROSIS BY INHIBITING NOXS

Fibrosis is an intrinsic wound healing response that helps to maintain organ integrity upon severe tissue damage. However, fibrosis may also become problematic when persistent injury and sustained inflammation occurs. Unresolved liver fibrosis leads to accumulation of excessive ECM proteins and scarring, which eventually progresses to cirrhosis and HCC. As the key cells that produce fibrotic ECM and other fibrogenic components, hepatic myofibroblasts, and their products are considered primary targets for antifibrotic therapies (Schuppan and Kim, 2013; Tsochatzis et al., 2014). However, there is still no FDA-approved drug for the treatment of liver fibrosis. Accumulating evidence have suggested the critical pathogenic effects of oxidative stress in the development of liver fibrosis, therapies that target ROS using antioxidants have therefore been applied in pre-clinical models of liver diseases. For example, a natural antioxidant Pyrroloquinoline-quinone (PQQ) found in human foods, suppresses oxidative stress, and liver fibrogenesis in mice with attenuated liver damage, hepatic inflammation and activation of HSCs (Jia et al., 2015). Similarly, Silybin, an extract of silymarin with antioxidant and anti-inflammatory properties, has been shown to be hepatoprotective in rat livers with secondary biliary cirrhosis (Serviddio et al., 2014). Additionally, a recent study suggested that blocking chloride channels prevented the increase of intracellular superoxide anion radicals, leading to attenuated activation of HSCs (den Hartog et al., 2014). However, it should also be noted that several antioxidants have failed in clinical trials to demonstrate their efficacy in antifibrotic response, such as polyenylphosphatidylcholine in alcoholic liver disease (Lieber et al., 2003), and Ursodeoxycholic acid (UDCA) and vitamin E in NASH (Lindor et al., 2004; Sanyal et al., 2010).

Given the vital role of NOX and NOX-derived ROS in hepatic fibrogenesis, the use of novel pharmacological NOX inhibitors to treat patients with chronic liver disease is being considered as the most promising antifibrotic therapeutics. However, historical NOX inhibitors, such as apocynin and diphenylene iodonium (DPI), do not specifically target NOX-derived ROS and are not isoform specialized. Until recently, GenKyoTex (Geneva, Switzerland) has developed a first-in-class small molecule NOX1/NOX4 dual inhibitor (GKT137831), with little affinity for Nox2 isoform (Laleu et al., 2010). Inhibition of NOX1/NOX4 using GKT137831 attenuated CCl4 or BDL-induced ROS production and hepatic fibrosis in mice (Aoyama et al., 2012; Jiang et al., 2012; Lan et al., 2015). Mechanistically, GKT137831 suppressed profibrotic gene expression and ROS production in HSCs (Aoyama et al., 2012; Lan et al., 2015), and also decreased hepatocyte apoptosis (Jiang et al., 2012). GenKyoTex is finalizing Phase II clinical study, and GKT137831 displayed an excellent safety profile and statistically significant reduction in both liver enzyme and inflammatory marker levels. Together with results from pre-clinical animal models of various fibrotic disorders, NOX inhibition shows strong potential as an effective treatment for hepatic fibrosis. However, chronic liver diseases of different etiologies may require specific and/or combination antifibrotic treatment approaches, based on the fact that the crosstalk between different cell types is critical for myofibroblasts activation. Future, studies on the components and functions of specific NOX isoforms in specific cell types and specific liver diseases will provide deeper insights for designing more specific and potent NOX inhibitors for the treatment of hepatic fibrosis.

CONCLUSIONS

Oxidative stress and inflammation are considered as the main cause of chronic liver diseases. Multiple lines of evidence indicate that NOX-generated ROS plays a pivotal role in the pathogenesis of liver fibrosis. A number of NOX isoforms, including NOX1, NOX2, and NOX4 are involved in the initiation of myofibroblasts activation and progression hepatic fibrosis. However, the intracellular pathways and molecular mechanisms involved in the role of NOX isoforms in specific cell types remain largely unknown. Targeting specific NOX isoforms with specific inhibitors, such as NOX1 and/or NOX4 to prevent HSC activation and protect hepatocyte injury may be promising to treat liver fibrosis, although future work is needed to fully confirm the clinical safety of these compounds. Moreover, the knowledge of molecular pathways involved in NOX-mediated myofibroblasts activation and fibrogenesis can provide new insights for developing novel anti-fibrotic treatments.

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REFERENCES

Adachi, T., Togashi, H., Suzuki, A., Kasai, S., Ito, J., Sugahara, K., et al. (2005). NAD(P)H oxidase plays a crucial role in PDGF-induced proliferation of hepatic stellate cells. *Hepatology* 41, 1272–1281. doi: 10.1002/hep.20719

Aliev, G., Priyadarshini, M., Reddy, V. P., Grieg, N. H., Kaminsky, Y., Cacabelos, R., et al. (2014). Oxidative stress mediated mitochondrial and vascular lesions as markers in the pathogenesis of Alzheimer disease. *Curr. Med. Chem.* 21, 2208–2217. doi: 10.2174/0929867321666131227161303

Ambasta, R. K., Kumar, P., Griendling, K. K., Schmidt, H. H., Busse, R., and Brandes, R. P. (2004). Direct interaction of the novel Nox proteins with p22phox is required for the formation of a functionally active NADPH oxidase. *J. Biol. Chem.* 279, 45935–45941. doi: 10.1074/jbc.M406486200

Aoyama, T., Paik, Y. H., and Seki, E. (2010). Toll-like receptor signaling and liver fibrosis. *Gastroenterol. Res. Pract.* 2010:192543. doi: 10.1155/2010/192543

Aoyama, T., Paik, Y. H., Watanabe, S., Laleu, B., Gaggini, F., Fioraso-Cartier, L., et al. (2012). Nicotinamide adenine dinucleotide phosphate oxidase in experimental liver fibrosis: GKT137831 as a novel potential therapeutic agent. *Hepatology* 56, 2316–2327. doi: 10.1002/hep.25938

Aram, G., Potter, J. J., Liu, X., Wang, L., Torbenson, M. S., and Mezey, E. (2009). Deficiency of nicotinamide adenine dinucleotide phosphate, reduced form oxidase enhances hepatocellular injury but attenuates fibrosis after chronic carbon tetrachloride administration. *Hepatology* 49, 911–919. doi: 10.1002/hep.22708

Bae, Y. S., Oh, H., Rhee, S. G., and Yoo, Y. D. (2011). Regulation of reactive oxygen species generation in cell signaling. *Mol. Cells* 32, 491–509. doi: 10.1007/s10059-011-0276-3

Bánfi, B., Clark, R. A., Steger, K., and Krause, K. H. (2003). Two novel proteins activate superoxide generation by the NADPH oxidase NOX1. *J. Biol. Chem.* 278, 3510–3513. doi: 10.1074/jbc.C200613200

Bataller, R., and Brenner, D. A. (2005). Liver fibrosis. *J. Clin. Invest.* 115, 209–218. doi: 10.1172/JCI24282

Bataller, R., Schwabe, R. F., Choi, Y. H., Yang, L., Paik, Y. H., Lindquist, J., et al. (2003). NADPH oxidase signal transduces angiotensin II in hepatic stellate cells and is critical in hepatic fibrosis. *J. Clin. Invest.* 112, 1383–1394. doi: 10.1172/JCI18212

Bettaieb, A., Jiang, J. X., Sasaki, Y., Chao, T. I., Kiss, Z., Chen, X., et al. (2015). Hepatocyte nicotinamide adenine dinucleotide phosphate reduced oxidase 4 regulates stress signaling, fibrosis, and insulin sensitivity during development of steatohepatitis in mice. *Gastroenterology* 149, 468–480.e410. doi: 10.1053/j.gastro.2015.04.009

Bondi, C. D., Manickam, N., Lee, D. Y., Block, K., Gorin, Y., Abboud, H. E., et al. (2010). NAD(P)H oxidase mediates TGF-beta1-induced activation of kidney myofibroblasts. *J. Am. Soc. Nephrol.* 21, 93–102. doi: 10.1681/ASN.2009020146

Boudreau, H. E., Casterline, B. W., Rada, B., Korzeniowska, A., and Leto, T. L. (2012). Nox4 involvement in TGF-beta and SMAD3-driven induction of the epithelial-to-mesenchymal transition and migration of breast epithelial cells. *Free Radic. Biol. Med.* 53, 1489–1499. doi: 10.1016/j.freeradbiomed.2012.06.016

Brenner, D. A., Kisseleva, T., Scholten, D., Paik, Y. H., Iwaisako, K., Inokuchi, S., et al. (2012). Origin of myofibroblasts in liver fibrosis. *Fibrog. Tiss. Repair* 5:S17. doi: 10.1186/1755-1536-5-S1-S17

Caja, L., Sancho, P., Bertran, E., Iglesias-Serret, D., Gil, J., and Fabregat, I. (2009). Overactivation of the MEK/ERK pathway in liver tumor cells confers resistance to TGF-{beta}-induced cell death through impairing up-regulation of the NADPH oxidase NOX4. *Cancer Res.* 69, 7595–7602. doi: 10.1158/0008-5472.CAN-09-1482

Carmona-Cuenca, I., Herrera, B., Ventura, J. J., Roncero, C., Fernandez, M., and Fabregat, I. (2006). EGF blocks NADPH oxidase activation by TGF-beta in fetal rat hepatocytes, impairing oxidative stress, and cell death. *J. Cell Physiol.* 207, 322–330. doi: 10.1002/jcp.20568

Carmona-Cuenca, I., Roncero, C., Sancho, P., Caja, L., Fausto, N., Fernandez, M., et al. (2008). Upregulation of the NADPH oxidase NOX4 by TGF-beta in hepatocytes is required for its pro-apoptotic activity. *J. Hepatol.* 49, 965–976. doi: 10.1016/j.jhep.2008.07.021

Carracedo, S., Lu, N., Popova, S. N., Jonsson, R., Eckes, B., and Gullberg, D. (2010). The fibroblast integrin alpha11beta1 is induced in a mechanosensitive manner involving activin A and regulates myofibroblast differentiation. *J. Biol. Chem.* 285, 10434–10445. doi: 10.1074/jbc.M109.078766

Cevik, M. O., Katsuyama, M., Kanda, S., Kaneko, T., Iwata, K., Ibi, M., et al. (2008). The AP-1 site is essential for the promoter activity of NOX1/NADPH oxidase, a vascular superoxide-producing enzyme: possible involvement of the ERK1/2-JunB pathway. *Biochem. Biophys. Res. Commun.* 374, 351–355. doi: 10.1016/j.bbrc.2008.07.027

Chan, E. C., Peshavariya, H. M., Liu, G. S., Jiang, F., Lim, S. Y., and Dusting, G. J. (2013). Nox4 modulates collagen production stimulated by transforming growth factor beta1 *in vivo* and *in vitro*. *Biochem. Biophys. Res. Commun.* 430, 918–925. doi: 10.1016/j.bbrc.2012.11.138

Cheng, G., and Lambeth, J. D. (2004). NOXO1, regulation of lipid binding, localization, and activation of Nox1 by the Phox homology (PX) domain. *J. Biol. Chem.* 279, 4737–4742. doi: 10.1074/jbc.M305968200

Christian, S., Winkler, R., Helfrich, I., Boos, A. M., Besemfelder, E., Schadendorf, D., et al. (2008). Endosialin (Tem1) is a marker of tumor-associated myofibroblasts and tumor vessel-associated mural cells. *Am. J. Pathol.* 172, 486–494. doi: 10.2353/ajpath.2008.070623

Cross, A. R., and Segal, A. W. (2004). The NADPH oxidase of professional phagocytes–prototype of the NOX electron transport chain systems. *Biochim. Biophys. Acta* 1657, 1–22. doi: 10.1016/j.bbabio.2004.03.008

Cucoranu, I., Clempus, R., Dikalova, A., Phelan, P. J., Ariyan, S., Dikalov, S., et al. (2005). NAD(P)H oxidase 4 mediates transforming growth factor-beta1-induced differentiation of cardiac fibroblasts into myofibroblasts. *Circ. Res.* 97, 900–907. doi: 10.1161/01.RES.0000187457.24338.3D

Cui, W., Matsuno, K., Iwata, K., Ibi, M., Matsumoto, M., Zhang, J., et al. (2011). NOX1/nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) oxidase promotes proliferation of stellate cells and aggravates liver fibrosis induced by bile duct ligation. *Hepatology* 54, 949–958. doi: 10.1002/hep.24465

De Deken, X., Corvilain, B., Dumont, J. E., and Miot, F. (2014). Roles of DUOX-mediated hydrogen peroxide in metabolism, host defense, and signaling. *Antioxid. Redox Signal.* 20, 2776–2793. doi: 10.1089/ars.2013.5602

De Minicis, S., Seki, E., Paik, Y. H., Osterreicher, C. H., Kodama, Y., Kluwe, J., et al. (2010). Role and cellular source of nicotinamide adenine dinucleotide phosphate oxidase in hepatic fibrosis. *Hepatology* 52, 1420–1430. doi: 10.1002/hep.23804

den Hartog, G. J., Qi, S., Van Tilburg, J. H., Koek, G. H., and Bast, A. (2014). Superoxide anion radicals activate hepatic stellate cells after entry through chloride channels: a new target in liver fibrosis. *Eur. J. Pharmacol.* 724, 140–144. doi: 10.1016/j.ejphar.2013.12.033

Desmoulière, A., Darby, I. A., and Gabbiani, G. (2003). Normal and pathologic soft tissue remodeling: role of the myofibroblast, with special emphasis on liver and kidney fibrosis. *Lab. Invest.* 83, 1689–1707. doi: 10.1097/01.LAB.0000101911.53973.90

Devasagayam, T. P., Tilak, J. C., Boloor, K. K., Sane, K. S., Ghaskadbi, S. S., and Lele, R. D. (2004). Free radicals and antioxidants in human health: current status and future prospects. *J. Assoc. Phys. India* 52, 794–804.

Diebold, B. A., and Bokoch, G. M. (2001). Molecular basis for Rac2 regulation of phagocyte NADPH oxidase. *Nat. Immunol.* 2, 211–215. doi: 10.1038/85259

Eklund, E. A., Jalava, A., and Kakar, R. (1998). PU.1, interferon regulatory factor 1, and interferon consensus sequence-binding protein cooperate to increase gp91(phox) expression. *J. Biol. Chem.* 273, 13957–13965. doi: 10.1074/jbc.273.22.13957

Fan, C. Y., Katsuyama, M., and Yabe-Nishimura, C. (2005). PKCdelta mediates up-regulation of NOX1, a catalytic subunit of NADPH oxidase, via transactivation of the EGF receptor: possible involvement of PKCdelta in vascular hypertrophy. *Biochem. J.* 390, 761–767. doi: 10.1042/BJ20050287

Friedman, S. L. (2000). Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. *J. Biol. Chem.* 275, 2247–2250. doi: 10.1074/jbc.275.4.2247

Friedman, S. L. (2008). Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. *Physiol. Rev.* 88, 125–172. doi: 10.1152/physrev.00013.2007

Fukui, T., Ishizaka, N., Rajagopalan, S., Laursen, J. B., Capers, Q. T., Taylor, W. R., et al. (1997). p22phox mRNA expression and NADPH oxidase activity are increased in aortas from hypertensive rats. *Circ. Res.* 80, 45–51. doi: 10.1161/01.RES.80.1.45

Geiszt, M., Kopp, J. B., Várnai, P., and Leto, T. L. (2000). Identification of renox, an NAD(P)H oxidase in kidney. *Proc. Natl. Acad. Sci. U.S.A.* 97, 8010–8014. doi: 10.1073/pnas.130135897

Grande, M. T., Sánchez-Laorden, B., López-Blau, C., De Frutos, C. A., Boutet, A., Arévalo, M., et al. (2015). Snail1-induced partial epithelial-to-mesenchymal transition drives renal fibrosis in mice and can be targeted to reverse established disease. *Nat. Med.* 21, 989–997. doi: 10.1038/nm.3901

Gressner, A. M., Weiskirchen, R., Breitkopf, K., and Dooley, S. (2002). Roles of TGF-beta in hepatic fibrosis. *Front. Biosci.* 7, d793–d807. doi: 10.2741/gressner

Groemping, Y., and Rittinger, K. (2005). Activation and assembly of the NADPH oxidase: a structural perspective. *Biochem. J.* 386, 401–416. doi: 10.1042/BJ20041835

Hardie, W. D., Glasser, S. W., and Hagood, J. S. (2009). Emerging concepts in the pathogenesis of lung fibrosis. *Am. J. Pathol.* 175, 3–16. doi: 10.2353/ajpath.2009.081170

Hecker, L., Vittal, R., Jones, T., Jagirdar, R., Luckhardt, T. R., Horowitz, J. C., et al. (2009). NADPH oxidase-4 mediates myofibroblast activation and fibrogenic responses to lung injury. *Nat. Med.* 15, 1077–1081. doi: 10.1038/nm.2005

Herrera, B., Fernández, M., Alvarez, A. M., Roncero, C., Benito, M., Gil, J., et al. (2001). Activation of caspases occurs downstream from radical oxygen species production, Bcl-xL down-regulation, and early cytochrome C release in apoptosis induced by transforming growth factor beta in rat fetal hepatocytes. *Hepatology* 34, 548–556. doi: 10.1053/jhep.2001.27447

Higashiyama, R., Moro, T., Nakao, S., Mikami, K., Fukumitsu, H., Ueda, Y., et al. (2009). Negligible contribution of bone marrow-derived cells to collagen production during hepatic fibrogenesis in mice. *Gastroenterology* 137, 1459–1466.e1451. doi: 10.1053/j.gastro.2009.07.006

Hilenski, L. L., Clempus, R. E., Quinn, M. T., Lambeth, J. D., and Griendling, K. K. (2004). Distinct subcellular localizations of Nox1 and Nox4 in vascular smooth muscle cells. *Arterioscler. Thromb. Vasc. Biol.* 24, 677–683. doi: 10.1161/01.ATV.0000112024.13727.2c

Hinz, B. (2010). The myofibroblast: paradigm for a mechanically active cell. *J. Biomech.* 43, 146–155. doi: 10.1016/j.jbiomech.2009.09.020

Hinz, B., Phan, S. H., Thannickal, V. J., Prunotto, M., Desmoulière, A., Varga, J., et al. (2012). Recent developments in myofibroblast biology: paradigms for connective tissue remodeling. *Am. J. Pathol.* 180, 1340–1355. doi: 10.1016/j.ajpath.2012.02.004

Hu, R., Wang, Y. L., Edderkaoui, M., Lugea, A., Apte, M. V., and Pandol, S. J. (2007). Ethanol augments PDGF-induced NADPH oxidase activity and proliferation in rat pancreatic stellate cells. *Pancreatology* 7, 332–340. doi: 10.1159/000105499

Humphreys, B. D., Lin, S. L., Kobayashi, A., Hudson, T. E., Nowlin, B. T., Bonventre, J. V., et al. (2010). Fate tracing reveals the pericyte and not epithelial origin of myofibroblasts in kidney fibrosis. *Am. J. Pathol.* 176, 85–97. doi: 10.2353/ajpath.2010.090517

Jaeschke, H. (2011). Reactive oxygen and mechanisms of inflammatory liver injury: present concepts. *J. Gastroenterol. Hepatol.* 26 (Suppl. 1), 173–179. doi: 10.1111/j.1440-1746.2010.06592.x

Jia, D., Duan, F., Peng, P., Sun, L., Ruan, Y., and Gu, J. (2015). Pyrroloquinoline-quinone suppresses liver fibrogenesis in mice. *PLoS ONE* 10:e0121939. doi: 10.1371/journal.pone.0121939

Jiang, J. X., Chen, X., Serizawa, N., Szyndralewiez, C., Page, P., Schröder, K., et al. (2012). Liver fibrosis and hepatocyte apoptosis are attenuated by GKT137831, a novel NOX4/NOX1 inhibitor *in vivo*. *Free Radic. Biol. Med.* 53, 289–296. doi: 10.1016/j.freeradbiomed.2012.05.007

Jiang, J. X., and Török, N. J. (2014). NADPH oxidases in chronic liver Diseases. *Adv. Hepatol.* 2014:742931. doi: 10.1155/2014/742931

Jiang, J. X., Venugopal, S., Serizawa, N., Chen, X., Scott, F., Li, Y., et al. (2010). Reduced nicotinamide adenine dinucleotide phosphate oxidase 2 plays a key role in stellate cell activation and liver fibrogenesis *in vivo*. *Gastroenterology* 139, 1375–1384. doi: 10.1053/j.gastro.2010.05.074

Katsuyama, M., Fan, C., Arakawa, N., Nishinaka, T., Miyagishi, M., Taira, K., et al. (2005). Essential role of ATF-1 in induction of NOX1, a catalytic subunit of NADPH oxidase: involvement of mitochondrial respiratory chain. *Biochem. J.* 386, 255–261. doi: 10.1042/BJ20041180

Kent, G., Gay, S., Inouye, T., Bahu, R., Minick, O. T., and Popper, H. (1976). Vitamin A-containing lipocytes and formation of type III collagen in liver injury. *Proc. Natl. Acad. Sci. U.S.A.* 73, 3719–3722. doi: 10.1073/pnas.73.10.3719

Kinnman, N., Francoz, C., Barbu, V., Wendum, D., Rey, C., Hultcrantz, R., et al. (2003). The myofibroblastic conversion of peribiliary fibrogenic cells distinct from hepatic stellate cells is stimulated by platelet-derived growth factor during liver fibrogenesis. *Lab. Invest.* 83, 163–173. doi: 10.1097/01.LAB.0000054178.01162.E4

Koga, H., Terasawa, H., Nunoi, H., Takeshige, K., Inagaki, F., and Sumimoto, H. (1999). Tetratricopeptide repeat (TPR) motifs of p67(phox) participate in interaction with the small GTPase Rac and activation of the phagocyte NADPH oxidase. *J. Biol. Chem.* 274, 25051–25060. doi: 10.1074/jbc.274.35.25051

Kuwahara, F., Kai, H., Tokuda, K., Kai, M., Takeshita, A., Egashira, K., et al. (2002). Transforming growth factor-beta function blocking prevents myocardial fibrosis and diastolic dysfunction in pressure-overloaded rats. *Circulation* 106, 130–135. doi: 10.1161/01.CIR.0000020689.12472.E0

Laleu, B., Gaggini, F., Orchard, M., Fioraso-Cartier, L., Cagnon, L., Houngninou-Molango, S., et al. (2010). First in class, potent, and orally bioavailable NADPH oxidase isoform 4 (Nox4) inhibitors for the treatment of idiopathic pulmonary fibrosis. *J. Med. Chem.* 53, 7715–7730. doi: 10.1021/jm100773e

Lan, T., Kisseleva, T., and Brenner, D. A. (2015). Deficiency of NOX1 or NOX4 prevents liver inflammation and fibrosis in mice through inhibition of hepatic stellate cell activation. *PLoS ONE* 10:e0129743. doi: 10.1371/journal.pone.0129743

Lassègue, B., Sorescu, D., Szöcs, K., Yin, Q., Akers, M., Zhang, Y., et al. (2001). Novel gp91(phox) homologues in vascular smooth muscle cells: nox1 mediates angiotensin II-induced superoxide formation and redox-sensitive signaling pathways. *Circ. Res.* 88, 888–894. doi: 10.1161/hh0901.090299

Lieber, C. S., Weiss, D. G., Groszmann, R., Paronetto, F., Schenker, S., and Veterans Affairs Cooperative Study 391 Group (2003). II. Veterans Affairs Cooperative Study of polyenylphosphatidylcholine in alcoholic liver disease. *Alcohol Clin. Exp. Res.* 27, 1765–1772. doi: 10.1097/01.ALC.0000093743.03049.80

Lindor, K. D., Kowdley, K. V., Heathcote, E. J., Harrison, M. E., Jorgensen, R., Angulo, P., et al. (2004). Ursodeoxycholic acid for treatment of nonalcoholic steatohepatitis: results of a randomized trial. *Hepatology* 39, 770–778. doi: 10.1002/hep.20092

Lovisa, S., Lebleu, V. S., Tampe, B., Sugimoto, H., Vadnagara, K., Carstens, J. L., et al. (2015). Epithelial-to-mesenchymal transition induces cell cycle arrest and parenchymal damage in renal fibrosis. *Nat. Med.* 21, 998–1009. doi: 10.1038/nm.3902

Lyle, A. N., Deshpande, N. N., Taniyama, Y., Seidel-Rogol, B., Pounkova, L., Du, P., et al. (2009). Poldip2, a novel regulator of Nox4 and cytoskeletal integrity in vascular smooth muscle cells. *Circ. Res.* 105, 249–259. doi: 10.1161/CIRCRESAHA.109.193722

Martinon, F., Chen, X., Lee, A. H., and Glimcher, L. H. (2010). TLR activation of the transcription factor XBP1 regulates innate immune responses in macrophages. *Nat. Immunol.* 11, 411–418. doi: 10.1038/ni.1857

Martyn, K. D., Frederick, L. M., von Loehneysen, K., Dinauer, M. C., and Knaus, U. G. (2006). Functional analysis of Nox4 reveals unique characteristics compared to other NADPH oxidases. *Cell Signal.* 18, 69–82. doi: 10.1016/j.cellsig.2005.03.023

Novo, E., Marra, F., Zamara, E., Valfrè di Bonzo, L., Caligiuri, A., Cannito, S., et al. (2006). Dose dependent and divergent effects of superoxide anion on cell death, proliferation, and migration of activated human hepatic stellate cells. *Gut* 55, 90–97. doi: 10.1136/gut.2005.069633

Oberhammer, F. A., Pavelka, M., Sharma, S., Tiefenbacher, R., Purchio, A. F., Bursch, W., et al. (1992). Induction of apoptosis in cultured hepatocytes and in regressing liver by transforming growth factor beta 1. *Proc. Natl. Acad. Sci. U.S.A.* 89, 5408–5412. doi: 10.1073/pnas.89.12.5408

Ozbek, E. (2012). Induction of oxidative stress in kidney. *Int. J. Nephrol.* 2012:465897. doi: 10.1155/2012/465897

Paik, Y. H., Iwaisako, K., Seki, E., Inokuchi, S., Schnabl, B., Osterreicher, C. H., et al. (2011). The nicotinamide adenine dinucleotide phosphate oxidase (NOX) homologues NOX1 and NOX2/gp91(phox) mediate hepatic fibrosis in mice. *Hepatology* 53, 1730–1741. doi: 10.1002/hep.24281

Paik, Y. H., Kim, J., Aoyama, T., De Minicis, S., Bataller, R., and Brenner, D. A. (2014). Role of NADPH oxidases in liver fibrosis. *Antioxid. Redox Signal.* 20, 2854–2872. doi: 10.1089/ars.2013.5619

Paik, Y. H., Lee, K. S., Lee, H. J., Yang, K. M., Lee, S. J., Lee, D. K., et al. (2006). Hepatic stellate cells primed with cytokines upregulate inflammation in response to peptidoglycan or lipoteichoic acid. *Lab. Invest.* 86, 676–686. doi: 10.1038/labinvest.3700422

Park, H. S., Chun, J. N., Jung, H. Y., Choi, C., and Bae, Y. S. (2006). Role of NADPH oxidase 4 in lipopolysaccharide-induced proinflammatory responses by human aortic endothelial cells. *Cardiovasc. Res.* 72, 447–455. doi: 10.1016/j.cardiores.2006.09.012

Park, H. S., Jung, H. Y., Park, E. Y., Kim, J., Lee, W. J., and Bae, Y. S. (2004). Cutting edge: direct interaction of TLR4 with NAD(P)H oxidase 4 isozyme is essential for lipopolysaccharide-induced production of reactive oxygen species and activation of NF-kappa B. *J. Immunol.* 173, 3589–3593. doi: 10.4049/jimmunol.173.6.3589

Pinzani, M., Gesualdo, L., Sabbah, G. M., and Abboud, H. E. (1989). Effects of platelet-derived growth factor and other polypeptide mitogens on DNA synthesis and growth of cultured rat liver fat-storing cells. *J. Clin. Invest.* 84, 1786–1793. doi: 10.1172/JCI114363

Proell, V., Carmona-Cuenca, I., Murillo, M. M., Huber, H., Fabregat, I., and Mikulits, W. (2007). TGF-beta dependent regulation of oxygen radicals during transdifferentiation of activated hepatic stellate cells to myofibroblastoid cells. *Comp. Hepatol.* 6:1. doi: 10.1186/1476-5926-6-1

Ramjaun, A. R., Tomlinson, S., Eddaoudi, A., and Downward, J. (2007). Upregulation of two BH3-only proteins, Bmf and Bim, during TGF beta-induced apoptosis. *Oncogene* 26, 970–981. doi: 10.1038/sj.onc.1209852

Reth, M. (2002). Hydrogen peroxide as second messenger in lymphocyte activation. *Nat. Immunol.* 3, 1129–1134. doi: 10.1038/ni1202-1129

Robert, A. M., and Robert, L. (2014). Xanthine oxido-reductase, free radicals and cardiovascular disease. A critical review. *Pathol. Oncol. Res.* 20, 1–10. doi: 10.1007/s12253-013-9698-x

Russo, F. P., Alison, M. R., Bigger, B. W., Amofah, E., Florou, A., Amin, F., et al. (2006). The bone marrow functionally contributes to liver fibrosis. *Gastroenterology* 130, 1807–1821. doi: 10.1053/j.gastro.2006.01.036

Sampson, N., Koziel, R., Zenzmaier, C., Bubendorf, L., Plas, E., Jansen-Durr, P., et al. (2011). ROS signaling by NOX4 drives fibroblast-to-myofibroblast differentiation in the diseased prostatic stroma. *Mol. Endocrinol.* 25, 503–515. doi: 10.1210/me.2010-0340

Sánchez-Valle, V., Chávez-Tapia, N. C., Uribe, M., and Méndez-Sánchez, N. (2012). Role of oxidative stress and molecular changes in liver fibrosis: a review. *Curr. Med. Chem.* 19, 4850–4860. doi: 10.2174/092986712803341520

Sancho, P., Mainez, J., Crosas-Molist, E., Roncero, C., Fernández-Rodriguez, C. M., Pinedo, F., et al. (2012). NADPH oxidase NOX4 mediates stellate cell activation and hepatocyte cell death during liver fibrosis development. *PLoS ONE* 7:e45285. doi: 10.1371/journal.pone.0045285

Sanyal, A. J., Chalasani, N., Kowdley, K. V., Mccullough, A., Diehl, A. M., Bass, N. M., et al. (2010). Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. *N. Engl. J. Med.* 362, 1675–1685. doi: 10.1056/NEJMoa0907929

Scholten, D., Osterreicher, C. H., Scholten, A., Iwaisako, K., Gu, G., Brenner, D. A., et al. (2010). Genetic labeling does not detect epithelial-to-mesenchymal transition of cholangiocytes in liver fibrosis in mice. *Gastroenterology* 139, 987–998. doi: 10.1053/j.gastro.2010.05.005

Schuppan, D., and Kim, Y. O. (2013). Evolving therapies for liver fibrosis. *J. Clin. Invest.* 123, 1887–1901. doi: 10.1172/JCI66028

Seki, E., and Brenner, D. A. (2008). Toll-like receptors and adaptor molecules in liver disease: update. *Hepatology* 48, 322–335. doi: 10.1002/hep.22306

Seki, E., De Minicis, S., Osterreicher, C. H., Kluwe, J., Osawa, Y., Brenner, D. A., et al. (2007). TLR4 enhances TGF-beta signaling and hepatic fibrosis. *Nat. Med.* 13, 1324–1332. doi: 10.1038/nm1663

Serrander, L., Cartier, L., Bedard, K., Banfi, B., Lardy, B., Plastre, O., et al. (2007). NOX4 activity is determined by mRNA levels and reveals a unique pattern of ROS generation. *Biochem. J.* 406, 105–114. doi: 10.1042/BJ20061903

Serviddio, G., Bellanti, F., Stanca, E., Lunetti, P., Blonda, M., Tamborra, R., et al. (2014). Silybin exerts antioxidant effects and induces mitochondrial biogenesis in liver of rat with secondary biliary cirrhosis. *Free Radic. Biol. Med.* 73, 117–126. doi: 10.1016/j.freeradbiomed.2014.05.002

Seshiah, P. N., Weber, D. S., Rocic, P., Valppu, L., Taniyama, Y., and Griendling, K. K. (2002). Angiotensin II stimulation of NAD(P)H oxidase activity: upstream mediators. *Circ. Res.* 91, 406–413. doi: 10.1161/01.RES.0000033523.08033.16

Smeyne, M., and Smeyne, R. J. (2013). Glutathione metabolism and Parkinson's disease. *Free Radic. Biol. Med.* 62, 13–25. doi: 10.1016/j.freeradbiomed.2013.05.001

Sturrock, A., Cahill, B., Norman, K., Huecksteadt, T. P., Hill, K., Sanders, K., et al. (2006). Transforming growth factor-beta1 induces Nox4 NAD(P)H oxidase and reactive oxygen species-dependent proliferation in human pulmonary artery smooth muscle cells. *Am. J. Physiol. Lung. Cell. Mol. Physiol.* 290, L661–L673. doi: 10.1152/ajplung.00269.2005

Suh, Y. A., Arnold, R. S., Lassegue, B., Shi, J., Xu, X., Sorescu, D., et al. (1999). Cell transformation by the superoxide-generating oxidase Mox1. *Nature* 401, 79–82. doi: 10.1038/43459

Sumimoto, H., Miyano, K., and Takeya, R. (2005). Molecular composition and regulation of the Nox family NAD(P)H oxidases. *Biochem. Biophys. Res. Commun.* 338, 677–686. doi: 10.1016/j.bbrc.2005.08.210

Tan, J., Peng, X., Luo, G., Ma, B., Cao, C., He, W., et al. (2010). Investigating the role of P311 in the hypertrophic scar. *PLoS ONE* 5:e9995. doi: 10.1371/journal.pone.0009995

Taura, K., Miura, K., Iwaisako, K., Osterreicher, C. H., Kodama, Y., Penz-Osterreicher, M., et al. (2010). Hepatocytes do not undergo epithelial-mesenchymal transition in liver fibrosis in mice. *Hepatology* 51, 1027–1036. doi: 10.1002/hep.23368

Touyz, R. M., Chen, X., Tabet, F., Yao, G., He, G., Quinn, M. T., et al. (2002). Expression of a functionally active gp91phox-containing neutrophil-type NAD(P)H oxidase in smooth muscle cells from human resistance arteries: regulation by angiotensin II. *Circ. Res.* 90, 1205–1213. doi: 10.1161/01.RES.0000020404.01971.2F

Tsochatzis, E. A., Bosch, J., and Burroughs, A. K. (2014). Liver cirrhosis. *Lancet* 383, 1749–1761. doi: 10.1016/S0140-6736(14)60121-5

Tuchweber, B., Desmouliere, A., Bochaton-Piallat, M. L., Rubbia-Brandt, L., and Gabbiani, G. (1996). Proliferation and phenotypic modulation of portal fibroblasts in the early stages of cholestatic fibrosis in the rat. *Lab. Invest.* 74, 265–278.

Wang, B., Trippler, M., Pei, R., Lu, M., Broering, R., Gerken, G., et al. (2009). Toll-like receptor activated human and murine hepatic stellate cells are potent regulators of hepatitis C virus replication. *J. Hepatol.* 51, 1037–1045. doi: 10.1016/j.jhep.2009.06.020

Watsky, M. A., Weber, K. T., Sun, Y., and Postlethwaite, A. (2010). New insights into the mechanism of fibroblast to myofibroblast transformation and associated pathologies. *Int. Rev. Cell. Mol. Biol.* 282, 165–192. doi: 10.1016/S1937-6448(10)82004-0

Wong, C. M., Bansal, G., Pavlickova, L., Marcocci, L., and Suzuki, Y. J. (2013). Reactive oxygen species and antioxidants in pulmonary hypertension. *Antioxid. Redox Signal.* 18, 1789–1796. doi: 10.1089/ars.2012.4568

Yang, L., and Seki, E. (2012). Toll-like receptors in liver fibrosis: cellular crosstalk and mechanisms. *Front. Physiol.* 3:138. doi: 10.3389/fphys.2012.00138

Yoshiji, H., Kuriyama, S., Yoshii, J., Ikenaka, Y., Noguchi, R., Nakatani, T., et al. (2001). Angiotensin-II type 1 receptor interaction is a major regulator for liver fibrosis development in rats. *Hepatology* 34, 745–750. doi: 10.1053/jhep.2001.28231

Zhan, S. S., Jiang, J. X., Wu, J., Halsted, C., Friedman, S. L., Zern, M. A., et al. (2006). Phagocytosis of apoptotic bodies by hepatic stellate cells induces NADPH oxidase and is associated with liver fibrosis *in vivo*. *Hepatology* 43, 435–443. doi: 10.1002/hep.21093

Zuo, L., Ushio-Fukai, M., Ikeda, S., Hilenski, L., Patrushev, N., and Alexander, R. W. (2005). Caveolin-1 is essential for activation of Rac1 and NAD(P)H oxidase after angiotensin II type 1 receptor stimulation in vascular smooth muscle cells: role in redox signaling and vascular hypertrophy. *Arterioscler. Thromb. Vasc. Biol.* 25, 1824–1830. doi: 10.1161/01.ATV.0000175295.09607.18

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Interplay of Matrix Stiffness and c-SRC in Hepatic Fibrosis

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Introduction: In liver fibrosis activation of hepatic stellate cells (HSC) comprises phenotypical change into profibrotic and myofibroplastic cells with increased contraction and secretion of extracellular matrix (ECM) proteins. The small GTPase RhoA orchestrates cytoskeleton formation, migration, and mobility via non-receptor tyrosine-protein kinase c-SRC (cellular sarcoma) in different cells. Furthermore, RhoA and its downstream effector Rho-kinase also play a crucial role in hepatic stellate cells and hepatic fibrogenesis. Matrix stiffness promotes HSC activation via cytoskeleton modulation. This study investigated the interaction of c-SRC and RhoA under different matrix stiffness conditions.

Methods: Liver fibrosis was induced in rats using bile duct ligation (BDL), thioacetamide (TAA) or carbon tetrachloride (CCl_4) models. mRNA levels of *albumin, PDGF-R, RHOA, COL1A1,* and *αSMA* were analyzed via qRT-PCR. Western Blots using phospho-specific antibodies against p-c-SRC418 and p-c-SRC530 analyzed the levels of activating and inactivating c-SRC, respectively. LX2 cells and hepatocytes were cultured on acrylamide gels of 1 and 12 kPa or on plastic to mimic non-fibrotic, fibrotic, or cirrhotic environments then exposed to SRC-inhibitor PP2. Overexpression of RhoA was performed by transfection using RhoA-plasmids. Additionally, samples from cirrhotic patients and controls were collected at liver transplantations and tumor resections were analyzed for RhoA and c-SRC protein expression by Western Blot.

Results: Transcription of albumin and RhoA was decreased, whereas transcription and activation of c-SRC was increased in hepatocytes cultured on 12 kPa compared to 1 kPa gels. LX2 cells cultured on 12 kPa gels showed upregulation of *RHOA*, *COL1A1,* and *αSMA* mRNA levels. Inhibition of c-SRC by PP2 in LX2 cells led to an increase in *COL1A1* and *αSMA* most prominently in 12 kPa gels. In LX2 cells with RhoA overexpression, c-SRC inhibition by PP2 failed to improve fibrosis. RhoA expression was significantly elevated in human and experimental liver fibrosis, while c-SRC was inactivated.

Conclusions: This study shows that c-SRC is inactive in activated myofibroblast-like HSC in liver cirrhosis. Inactivation of c-SRC is mediated by a crosstalk with RhoA upon hepatic stellate cell activation and fibrosis progression.

Keywords: RhoA, SRC, liver fibrosis, matrix stiffness, hepatic stellate cells, PP2

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INTRODUCTION

End-stage liver disease is characterized by fibrosis and loss of organ function, and is one of the leading causes of death worldwide (World Health Organisation, 2002; National Institute on Alcohol Abuse and Alcoholism, 2014). In chronic liver injury, hepatic stellate cells (HSC) get activated, proliferate, and migrate within liver tissue (Rockey, 1997). Moreover, activated HSCs are the major contributors to liver fibrogenesis by extracellular matrix (ECM) synthesis (Mederacke et al., 2013). The small GTPase RhoA is a master regulator protein and mediates HSC activity and motility by signaling downstream to effectors Rho-kinase (ROCK) or mDia1 (Thumkeo et al., 2013). In liver fibrosis, upregulation of the RhoA/ROCK axis leads to increased vascular contractility and portal pressure (Hennenberg et al., 2006; Trebicka et al., 2007). The RhoA/ROCK signaling exerts these effects via activated HSCs (Trebicka et al., 2010). Inhibition of RhoA/ROCK activity in liver fibrosis reduces portal pressure and attenuates hepatic fibrosis by induction of HSC senescence (Trebicka et al., 2010; Klein et al., 2012a,b). Besides the RhoA/ROCK axis, RhoA also interacts with the cytosolic tyrosine kinase c-SRC via mDia1 (Yamana et al., 2006). c-SRC is a transmembraneous regulator, which plays a role in focal adhesion complexes and cytoskeletal dynamics and mediates proliferatition via the platelet-derived growth factor receptor (PDGF-R) (Brown and Cooper, 1996; Yamana et al., 2006; Huveneers and Danen, 2009). Furthermore, pro-oncogenic properties make c-SRC an important target in cancer research (Musumeci et al., 2012; Gargalionis et al., 2014; Varkaris et al., 2014).

The regulation of the downstream signaling of RhoA via either ROCK or mDia1/c-SRC plays an important role in the dynamics and contractility of intracellular stress fibers. While stimulation of the RhoA/mDia1/c-SRC-axis leads to actin polymerization, the stimulation of the RhoA/ROCK-axis leads to actomyosin contractility and inhibits actin depolymerization (Takai et al., 2001; Quack et al., 2009). In migration and contraction of activated HSCs the signaling via RhoA/ROCK is well-investigated. However, little is known about the role of c-SRC and the interplay with RhoA in liver fibrosis in general and in activated HSCs in particular.

In this work, we show for the first time the changes exerted by liver fibrosis in the expression of activating and inactivating phosphorylation sites of c-SRC in both human and rat liver samples. Furthermore, we demonstrate that c-SRC plays a decisive role in RhoA and cytoskeletal protein activity by inhibition of c-SRC in cultivated human hepatic stellate cells.

MATERIALS AND METHODS

Animals

All animal testing was carried out using wild type rats. Our studies were approved by the committee responsible for animal studies in North Rhine-Westphalia (LANUV reference number 84-02.04.2014.A137).

Cholestatic Model of Fibrosis

Bile duct ligation (BDL) was performed in rats with an initial body weight between 180 and 200 g as previously described (Heller et al., 2003). Sham-operated rats served as controls. Experiments were performed after a 4 week interval to allow development of liver fibrosis.

Toxic Model of Fibrosis

Rats with an initial body weight between 80 and 100 g were administered carbon tetrachloride (CCl_4) via inhalation for 14–16 weeks as described previously (Granzow et al., 2014). Age-matched rats who did not receive CCl_4 served as controls. Additionally, rats with an initial body weight between 200 and 250 g were orally administered thioacetamide (TAA) weekly for 18 weeks as described previously (Verbeke et al., 2014).

Tissue Collection

After induction of liver fibrosis, the rats were anesthetized and laparotomy was performed for tissue collection. The livers were cut into fragments and stored at −80°C until they were used for qRT-PCR and western blot analysis as described previously (Trebicka et al., 2008, 2010).

Human Liver Samples

Human liver samples were taken during liver transplantation from patients with alcohol-induced cirrhosis. Liver samples from non-cirrhotic patients who underwent liver resection served as controls. No patient or donor received catecholamines, angiotensin receptor antagonists or ACE inhibitors prior to transplantation. All samples were snap frozen after excision. The use of human liver samples was approved by the Human Ethics Committee of the University of Bonn (reference number 029/13). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

Isolation of Primary Hepatocytes and Hepatic Stellate Cells

Primary rat hepatocytes and hepatic stellate cells were isolated and cultured as described previously (Herman et al., 1988; Wojtalla et al., 2012; Granzow et al., 2014). Viability and purity were routinely more than 95%. For early activation of HSCs, cells were harvested at day 10 and for advanced activation with differentiation to myofibroblast-like phenotype, cells were harvested at third passage.

Cell Culture

Snap-frozen LX2 cells and primary rat hepatic stellate cells were incubated with cell culture medium (DMEM + 20% FBS + Penicillin/Streptomycin) in 250 ml plastic flasks at 37°C. After reaching 80% confluency, cells were passaged with a 1:3 split ratio. Detachment was achieved by incubating the cells with 0.05% Trypsin/EDTA solution (solved in PBS) for 5 min at 37°C. Before transfection, LX2 cells were incubated with transfection media (DMEM with 10% FBS) for 24 h. Plasmids with wildtype RhoA, constitutively active RhoA and dominant negative RhoA were kindly provided by Prof. Dr. Fürst (Institute for Cell Biology, University of Bonn, Germany). Fifteen microliter of

the respective plasmid and 37.5 µl of lipofectamine were then incubated for 20 min with 3.6 ml transfection media. Cell media was then aspirated and the cells were incubated with the plasmid/lipofectamine mix, which was carefully added dropwise. After 4 h, cells were again incubated in transfection media and harvested 3 days later. Efficacy of transfection was tested using qRT-PCR.

Inhibition of c-SRC

For inhibition of c-SRC 4-Amino-3-(4-chlorophenyl)-1-(t-butyl)-1H-pyrazolo[3,4-d]pyrimidine (PP2; Sigma-Aldrich, Munich, Germany) has been used to blunt c-SRC mediated effects (Hanke et al., 1996; Yoshizumi et al., 2000). Ten micrometer of PP2 were added to the cell culture medium and cells were incubated for 2 days before harvesting.

Polyacrylamide Gels

Either snap-frozen LX2 cells or isolated rat hepatocytes were seeded on fibrinogen-coated polyacrylamide (PAA) gels of variable stiffness. Matrices were prepared on 12 mm cover slips in 6-well cell culture plates as previously described (Olsen et al., 2011). Briefly, gels were cross-linked using a Spectroline Microprocessor controlled UV Cross-linker (Thermo Scientific, Waltham, USA). Prepared gels were then coated with fibronectin (Sigma-Aldrich, Munich, Germany). Healthy parenchymatous conditions were simulated by soft matrices with 1 kPa shear modulus, while matrices used to simulate stiff conditions had a shear modulus of 12 kPa. Cell culture was then performed on these gels as described above.

qRT-PCR

Liver homogenates from either fibrotic or non-fibrotic rats were prepared using previously described methods (Trebicka et al., 2010). RNA was isolated from samples using the Qiazol reagent as instructed by the manufacturer (Qiagen, Hilden, Germany) (Trebicka et al., 2013; Anadol et al., 2015). The following assays provided by Applied Biosystems (Foster City, USA) were used: *ACTA2* (αSMA, Hs00426835_g1), *COL1A1* (Hs00164004_m1), *Src* (Rn01418228_m1) *PDGFRB* (Hs01019589_m1), *RHOA* (for human; Hs01051295-m1), and *RhoA* (for rat; Rn04219609_m1). *Albumin* (Rn-Alb_1_SG) was provided by Qiagen (Hilden, Germany). Samples were normalized to 18s rRNA.

Western Blotting

Snap-frozen cells and liver samples were processed as previously described using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and nitrocellulose membranes (Kwiecinski et al., 2011). Equal protein loading was assured using Ponceau-S staining. GAPDH served as endogenous control of protein expression. Membranes were incubated with rabbit-anti-p-c-SRC (Tyr418) from Invitrogen (Darmstadt, Germany), rabbit-anti-c-SRC, rabbit-anti-p-c-SRC (Tyr530), mouse-anti-RhoA, and rabbit-anti-GAPDH primary antibodies and corresponding peroxidase-coupled secondary antibodies from Santa Cruz Biotechnology (Heidelberg, Germany). Results were analyzed using Chemi-Smart digital detection (PeqLab, Biotechnologies, Erlangen, Germany) after enhanced chemiluminescence (ECL, Amersham, UK).

Statistical Analysis

Group size was at least $n = 5$ for each group. Graphs are presented as means ± standard deviation and $p < 0.05$ were considered statistically significant. Western blots were measured using digital densitometry software (Bio-1D v.15.02, Vilber Lourmat, Marne-la-Vallée, France) and the respective density of each band was calculated. The fibrosis groups were tested for significance to their corresponding controls using Mann-Whitney *U* test. In qPCR experiments, 2^{-ddCT} was calculated and normalized to the respective control group. Plotting of diagrams and statistic analysis were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, La Jolla, California, USA).

RESULTS

RhoA and c-SRC Crosstalk in Hepatocytes

Rat hepatocytes, which were cultivated on PAA gels with a shear modulus of 12 kPa, simulating stiff liver tissue, showed reduced function marked by a significant decrease in transcription levels of albumin compared to hepatocytes cultivated on 1 kPa gels (**Figure 1A**). Reduced hepatocyte function further led to a significant downregulation of *RhoA* transcription under stiff conditions, while mRNA levels of *c-Src* were increased in these cells (**Figure 1A**).

Besides transcription of c-Src, also the activation of the c-SRC protein was altered in hepatocytes cultivated on PAA gels with 12 kPa. Phosphorylation at tyrosine 488 (p-c-Src418), the c-SRC activating phosphorylation site, was significantly increased and phosphorylation at tyrosine 530, the c-SRC inactivating phosphorylation site, showed a trend to be decreased under stiff conditions (**Figure 1B**).

RhoA and c-SRC Crosstalk in Human Derived HSC Cell Line LX2 under Stiff Conditions

In contrast to hepatocytes, cultivation on PAA gels with shear modulus of 12 kPa led to stimulation and activation of hepatic stellate cells (HSC). Both, proliferation as shown by transcriptional levels of *Pdgf-r*, as well as activation and collagen secretion as shown by the surrogate marker *αSma* and *collagen 1* mRNA levels, were increased when cells were cultivated on PAA gels with stiffness of 12 kPa compared to cells cultivated on PAA gels with stiffness of 1 kPa (**Figure 2A**). Furthermore, transcription of *RhoA* was increased significantly, but less pronounced, in LX2 cells under stiff conditions (**Figure 2A**).

Incubation with PP2, a selective inhibitor of the c-SRC tyrosine kinases, decreased transcriptional levels of *RhoA* and of the HSC activation markers *collagen 1* and *αSma* even under soft conditions when LX2 cells were cultivated on PAA gels with shear modulus of 1 kPa (**Figure 2B**). On PAA gels with 12 kPa stiffness incubation with PP2 revealed similar results compared to the experiments performed on 1 kPa gels. However, the effect of c-SRC inhibition by PP2 on reduction of *RhoA*, *collagen 1*, and *αSma* mRNA levels was stronger in LX2 cells cultivated on 12 kPa PAA gels (**Figure 2C**).

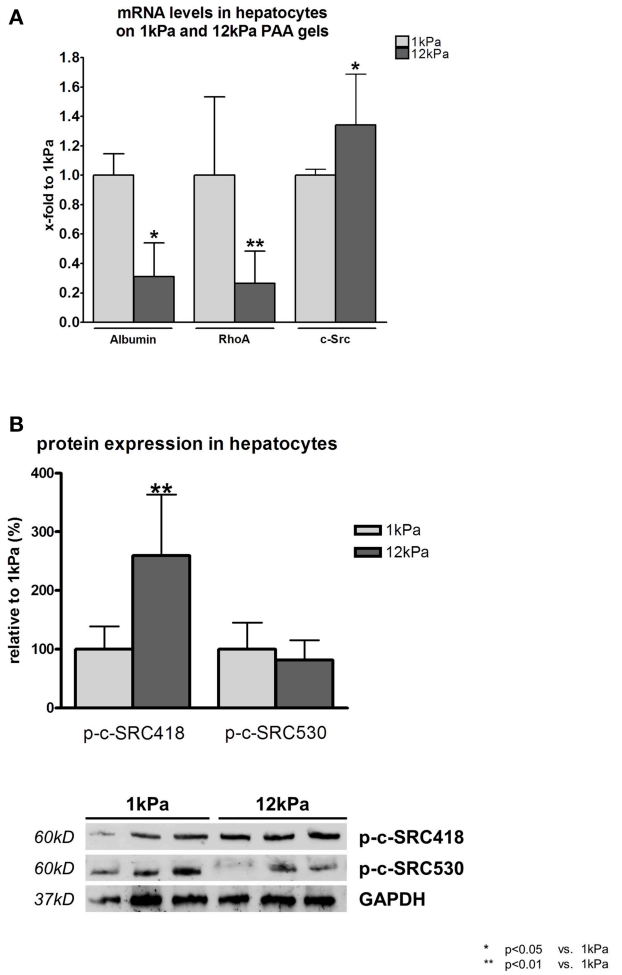

FIGURE 1 | RhoA and c-SRC crosstalk in hepatocytes. (A) Hepatocytes were incubated on gels with a shear modulus of 1 kPa, simulating healthy liver tissue, and with a shear modulus of 12 kPa, simulating fibrotic tissue. Hepatocyte function was reduced under stiff conditions as shown by *albumin* transcription levels. Transcription of *RhoA* was decreased, while *c-Src* mRNA was increased under stiff conditions. **(B)** Activating phosphorylation (p-c-SRC418) of c-SRC was increased, while inactivating (p-c-SRC530) was decreased under stiff conditions in hepatocytes.

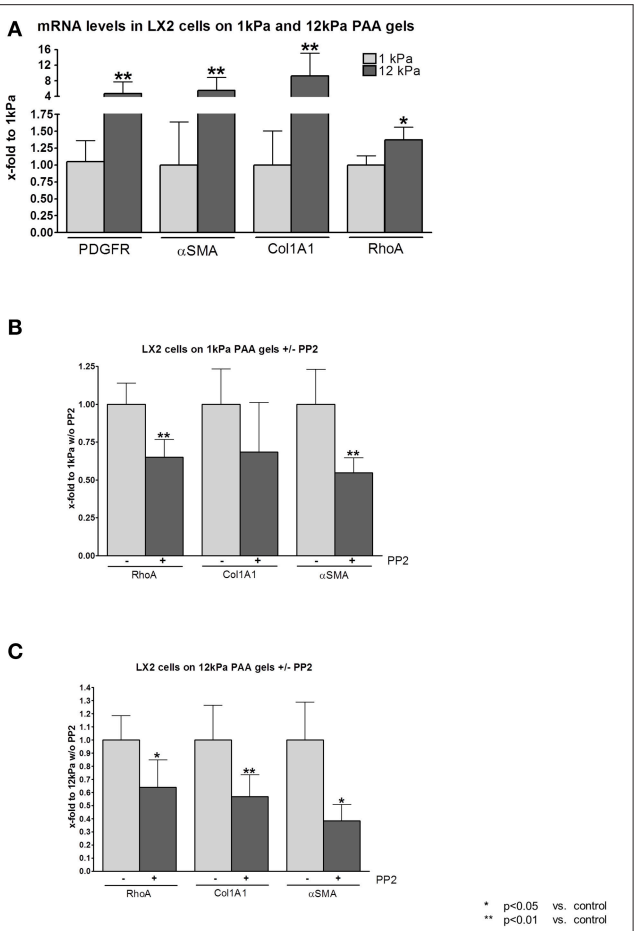

FIGURE 2 | RhoA and c-SRC crosstalk in human derived HSC cell line LX2 under fibrotic conditions. (A) Human derived hepatic stellate cell line (HSC) LX2 were incubated on gels with shear modulus of 1 kPa, simulating healthy liver tissue, and with shear modulus of 12 kPa, simulating fibrotic tissue. Transcription of proliferative (PDGF-R) and activation (αSMA) markers was increased under stiff conditions. Stiff conditions stimulated LX2 cells to increase collagen production. RhoA was slightly increased by mRNA levels under stiff conditions. **(B)** PP2 administration reduced RhoA, Collagen 1 and αSMA mRNA levels under soft conditions. **(C)** Similarly, in LX2 cell incubated on 12 kPa gels, PP2 administration led to decreased transcription of RhoA, Collagen 1 and αSMA. However, the effect of PP2 in LX2 cells on 12 kPa gels was more pronounced than in LX2 cells on 1 kPa.

RhoA and c-SRC Crosstalk in Hepatic Stellate Cells under Extremely Stiff Conditions

The high matrix stiffness prevailing in plastic cell culture flasks simulates the extremely stiff matrix conditions in liver cirrhosis. Under these conditions the c-SRC activation in primary rat HSCs drops with progressive HSC activation as shown by a significant increase in p-c-SRC530 and a decrease in p-c-SRC418 (**Figure 3A**). Total protein levels of c-SRC remained unchanged upon progressive HSC activation under extremely stiff conditions (**Figure 3A**).

Inhibition of c-SRC by PP2 under extremely stiff conditions led to a significant upregulation of *RhoA* mRNA (**Figure 3B**). Furthermore, PP2 significantly increased HSC activation as demonstrated by *αSma* mRNA levels. Additionally, also collagen production showed a trend toward an increase in transcriptional level (**Figure 3B**).

RhoA and c-SRC Crosstalk in Experimental and Human Liver Cirrhosis

In bile duct ligated (BDL) rats, a model for cholestatic liver cirrhosis, the inactivating phosphorylation at tyrosine 530 of c-SRC was significantly increased compared to sham operated rats (**Figures 4A,B**). As a consequence, phosphorylation at tyrosine 418 of c-SRC was significantly decreased in these animals, while total protein levels of c-SRC remained unchanged upon BDL (**Figures 4A,B**). In addition, RhoA was highly upregulated in BDL rats (**Figures 4A,B**).

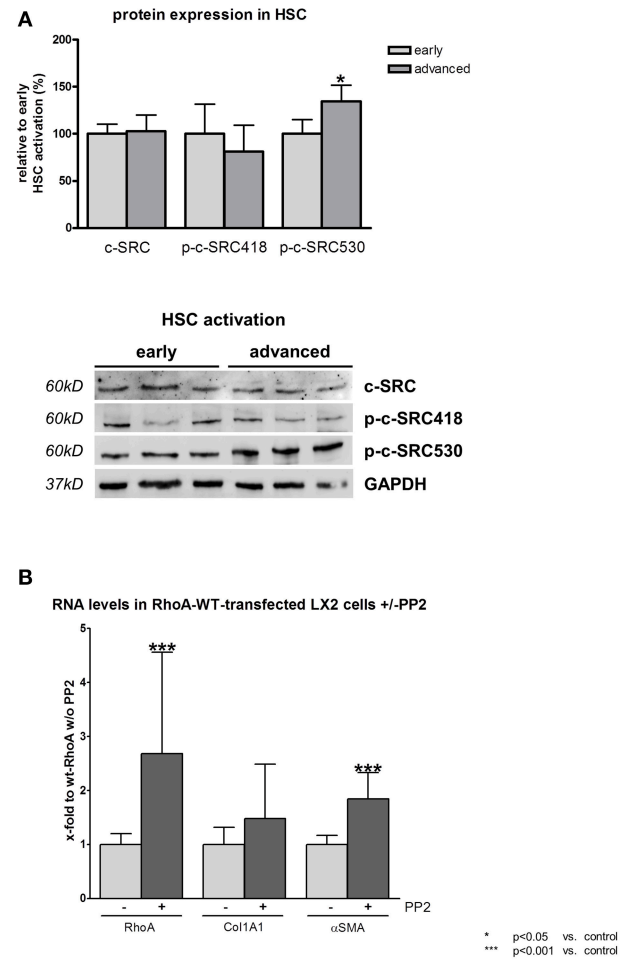

FIGURE 3 | RhoA and c-SRC crosstalk in hepatic stellate cells under cirrhotic conditions. (A) Primary rat hepatic stellate cells (HSC) were cultivated on plastic, simulating extremely stiff conditions. For early activation of HSCs, cells were harvested at day 10 and for advanced activation to myofibroblast-like phenotype, cells were harvested at third passage. Inactivating phosphorylation at tyrosine 530 of c-SRC was increased in activated myofibroblast-like HSCs, while activating phosphorylation at tyrosine 418 of c-SRC showed a trend toward a decrease. Overall c-SRC protein did not change upon HSC activation progress. **(B)** Inhibition of c-SRC by PP2 led to increased mRNA levels of RhoA, Collagen 1 and αSMA in activated myofibroblast-like HSCs.

Carbon tetrachloride (CCl_4) and thioacetomide (TAA) intoxication are both models for toxic liver cirrhosis. Parallel to the results observed in BDL rats, phosphorylation at tyrosine 530 of c-SRC was significantly increased, while phosphorylation at tyrosine 418 of c-SRC was significantly decreased in CCl_4 (**Figures 4C,D**) and TAA (**Figures 4E,F**) intoxicated rats compared to untreated control rats. Also in experimental models of toxic liver cirrhosis levels of total c-SRC remained unchanged, while RhoA was significantly increased (**Figures 4C–F**).

Changes in c-SRC activation in models of experimental liver damage mirrored the situation in human liver cirrhosis. In liver samples of cirrhotic patients c-SRC activating phosphorylation at tyrosine 418 was significantly downregulated compared to non-cirrhotic control liver samples (**Figures 4G,H**). As a consequence c-SRC inactivating phosphorylation at tyrosine 530 was increased in liver samples of cirrhotic patients. As shown for experimental liver cirrhosis, also in human liver cirrhosis RhoA expression is highly upregulated compared to the non-cirrhotic control liver samples (**Figures 4G,H**).

These data suggest a marked counterplay of RhoA and c-SRC expression in human and experimental liver cirrhosis (**Figure 5A**).

DISCUSSION

In the present study, we demonstrate for the first time that the activity of c-SRC decreases with progressive liver fibrogenesis and hepatic stellate cell (HSC) activation (**Figure 5B**). We could show that this effect on c-SRC is regulated by a counterplay with RhoA, which, in contrast, is upregulated with progressing HSC activation (**Figure 5A**). Thereby, c-SRC inactivation was reached by increased phosphorylation at tyrosine 530 and decreased phosphorylation at tyrosine 418, while total protein levels of SRC in HSCs were unchanged upon progressing liver damage. Since this crosstalk of RhoA and c-SRC could be observed in cholestatic and toxic models of liver cirrhosis, as well as in alcohol-induced human liver cirrhosis, our data suggest that the mechanisms are independent of the etiology.

SRC consists of four SRC homology (SH) domains and phosphorylation at tyrosine 530 on SH2 leads to inactivation of SRC. Dephosphorylation at tyrosine 530 by different kinases, such as SH2-containing phosphatases, allows autophosphorylation at tyrosine 418/419 on SH1, which includes the kinase domain, and leads to activation of SRC (reviewed in Yeatman, 2004). Members of the SRC family kinases have been broadly investigated in cancer due to their pro-oncogenic characteristics (Musumeci et al., 2012; Gargalionis et al., 2014; Varkaris et al., 2014). While first results for c-SRC targeting have been reported in the treatment of idiopathic pulmonary fibrosis, systemic sclerosis and glioblastoma (Beyer and Distler, 2013; Ceccherini et al., 2015), its role in liver fibrosis progression is not yet understood.

To simulate either healthy or fibrotic environments, we chose to incubate hepatocytes and hepatic stellate cells on polyacrylamide matrices of a defined shear modulus, since it has been shown that a stiff environment is required for differentiation of HSCs to a myofibroblastic phenotype (Olsen et al., 2011). Plastic cell culture flasks are extremely stiff and out of the physiologic range, but provide a useful model system to mimic cells receiving maximal stimulation from a stiff matrix. Hepatic stellate cells that grow in these containers can often only be observed in a highly activated and fully transdifferentiated myofibroblastic state. Results obtained from cells incubated on plastic are therefore restricted to describe only end-stage liver disease. Polyacrylamide matrices allow a better analysis of cells, since different stages of fibrosis progression can be simulated under more physiologically relevant conditions (Olsen et al., 2011).

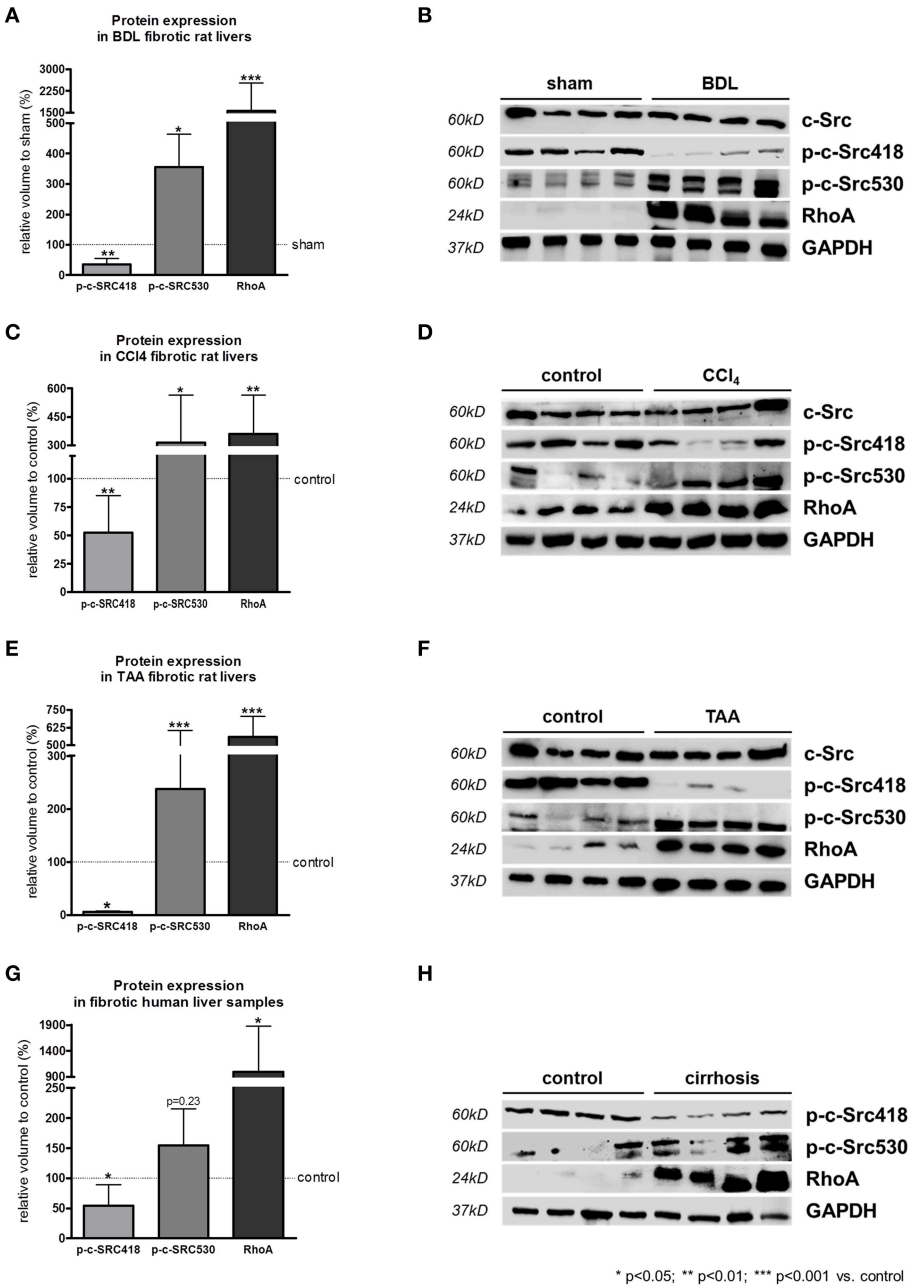

FIGURE 4 | RhoA and c-SRC crosstalk in experimental and human liver cirrhosis. Protein expression of phosphorylated c-SRC and RhoA in cholestatic BDL **(A,B)**, toxic CCl_4 **(C,D)**, or TAA **(E,F)** intoxication liver cirrhosis models, as well as in samples of human liver tissue **(G,H)** showed increased inactivation of c-SRC (p-c-SRC530) in liver cirrhosis. In contrast, activating phosphorylation (p-c-SRC418) was decreased. RhoA was highly upregulated in experimental and human liver cirrhosis. Controls were sham operated rats for the BDL model, untreated rats for CCl_4 and TAA model an liver biopsies from non-cirrhotic patients.

Inhibition of c-SRC by PP2 as a therapeutic approach may be promising only in early fibrotic stages, when HSCs are migrating. In this stage c-SRC is still active and drives cytoskeletal dynamics and cell motility by stimulation of actin polymerization (Takai et al., 2001; Quack et al., 2009). Furthermore, c-SRC is increased in monocytes and macrophages and mediates secretion of pro-inflammatory cytokines (Sarang et al., 2011; Yokoi et al., 2011), which may contribute to HSC activation upon liver damage.

In contrast, in progressive liver disease activated HSCs develop a myofibroblast-like phenotype. Activated myofibroblast-like HSCs are contractile and drive hepatic fibrogenesis (Friedman, 2003; Mederacke et al., 2013). These effects are mediated by upregulated RhoA/Rho-kinase signaling

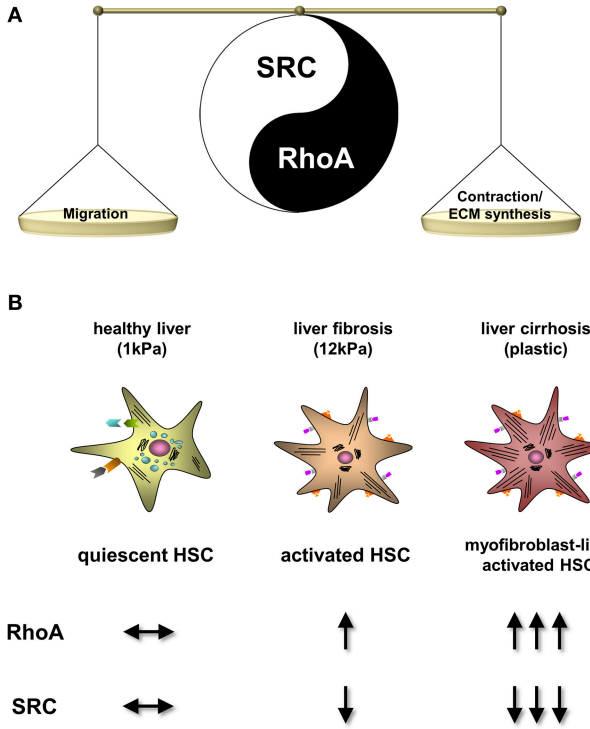

FIGURE 5 | Regulation of RhoA and c-SRC activity upon progression of liver fibrosis. (A) Scheme of the RhoA and c-SRC crosstalk in hepatic stellate cells (HSC). c-SRC activation primary stimulates migration of HSCs and activation of RhoA leads to contraction and collagen production. Therefore, c-SRC seems to play a important role in early stages of fibrosis, when HSCs are proliferating and migrating. In contrast, RhoA plays a pivotal role in progressed fibrosis, when HSC phenotype changes into a myofibroblast-like phenotype. **(B)** Progression of HSC activation into myofibroblast-like cells and the corresponding cell culture conditions mimicking fibrosis progression. Upon HSC activation RhoA becomes highly upregulated, while c-SRC activation is suppressed.

upon liver damage (Trebicka et al., 2007, 2010). Our group has demonstrated several times that the signaling cascade via JAK2, RhoA, and Rho-kinase signaling is upregulated in liver fibrosis and located mainly in myofibroblast-like activated HSC (Zhou et al., 2006; Granzow et al., 2014; Klein et al., 2015). With the current study we show that increased RhoA activity leads to decreased c-SRC activity with progressive HSC activation. At this stage, inhibition of c-SRC by PP2 failed to improve transcription of fibrogenic markers, probably since c-SRC activity is already low under these conditions, due to the increased phosphorylation at tyrosine 530. This data suggests that the phenotype change of HSC is mediated by a crosstalk of RhoA and c-SRC.

ECM components are responsible for increased matrix stiffness in liver fibrosis and may influence expression of c-SRC via growth factor or hyaluronan receptors (e.g., CD44) or focal adhesion complexes (Nikitovic et al., 2013). Furthermore, ECM components may have direct paracrine and endocrine effects on HSC function and intracellular signaling in liver fibrosis (reviewed in Wells, 2013; Karsdal et al., 2015). Besides increased matrix stiffness, which is only one factor of liver fibrosis, the crosstalk of RhoA, and c-SRC may be triggered by other factors such as proinflammatory or profibrotic cytokines upon liver disease progression. Also regulatory miRNAs in response to inflammatory stimuli could play a role. However, the molecular mechanisms which regulate the crosstalk of RhoA and c-SRC remain unclear and should be investigated in future studies.

Besides inhibition of c-SRC, PP2 has been described to have a weak affinity to inhibit other kinases like JAK2 (Hanke et al., 1996), which is an upstream regulator of RhoA activity (Granzow et al., 2014; Klein et al., 2015). However, the doses of PP2 used in our *in vitro* experiments was very low and much higher doses would be of need to inhibit the JAK2/RhoA axis (Hanke et al., 1996).

In conclusion, this study provides insight into the role of matrix stiffness on c-SRC activity and the crosstalk of RhoA and c-SRC upon progressing liver damage. Furthermore, the usage of PAA gels of different elasticities, simulating different stages of fibrosis progression, proves to be useful for *in vitro* experiments to investigate molecular and pathomechanistic changes triggered by liver fibrosis progression.

AUTHOR CONTRIBUTIONS

JG, RS designed the original study, wrote the first draft of the article and acquired, analyzed, and interpreted the data. JB, SK, FU acquired, analyzed, and interpreted the data. PV, DF, WL, JP provided substantial material and methods and interpreted data. CS provided administrative support and interpreted data. JT designed the original study, interpreted the data wrote the first draft of the article, provided administrative support and supervised the study. All authors commented on the drafts of the article and approved the final article.

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REFERENCES

Anadol, E., Schierwagen, R., Elfimova, N., Tack, K., Schwarze-Zander, C., Eischeid, H., et al. (2015). Circulating miRNAs as marker for liver injury in HIV patients. *Hepatology* 61, 46–55. doi: 10.1002/hep.27369

Beyer, C., and Distler, J. H. W. (2013). Tyrosine kinase signaling in fibrotic disorders: translation of basic research to human disease. *Biochim. Biophys. Acta* 1832, 897–904. doi: 10.1016/j.bbadis.2012.06.008

Brown, M. T., and Cooper, J. A. (1996). Regulation, substrates and functions of src. *Biochim. Biophys. Acta* 1287, 121–149.

Ceccherini, E., Indovina, P., Zamperini, C., Dreassi, E., Casini, N., Cutaia, O., et al. (2015). SRC family kinase inhibition through a new pyrazolo[3,4-d]pyrimidine derivative as a feasible approach for glioblastoma treatment. *J. Cell. Biochem.* 116, 856–863. doi: 10.1002/jcb.25042

Friedman, S. L. (2003). Liver fibrosis – from bench to bedside. *J. Hepatol.* 38(Suppl. 1), 38–53. doi: 10.1016/S0168-8278(02)00429-4

Gargalionis, A. N., Karamouzis, M. V., and Papavassiliou, A. G. (2014). The molecular rationale of Src inhibition in colorectal carcinomas. *Int. J. Cancer* 134, 2019–2029. doi: 10.1002/ijc.28299

Granzow, M., Schierwagen, R., Klein, S., Kowallick, B., Huss, S., Linhart, M., et al. (2014). Angiotensin-II type 1 receptor-mediated Janus kinase 2 activation induces liver fibrosis. *Hepatology* 60, 334–348. doi: 10.1002/hep.27117

Hanke, J. H., Gardner, J. P., Dow, R. L., Changelian, P. S., Brissette, W. H., Weringer, E. J., et al. (1996). Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. *J. Biol. Chem.* 271, 695–701.

Heller, J., Shiozawa, T., Trebicka, J., Hennenberg, M., Schepke, M., Neef, M., et al. (2003). Acute haemodynamic effects of losartan in anaesthetized cirrhotic rats. *Eur. J. Clin. Invest.* 33, 1006–1012. doi: 10.1046/j.1365-2362.2003.01251.x

Hennenberg, M., Biecker, E., Trebicka, J., Jochem, K., Zhou, Q., Schmidt, M., et al. (2006). Defective RhoA/Rho-kinase signaling contributes to vascular hypocontractility and vasodilation in cirrhotic rats. *Gastroenterology* 130, 838–854. doi: 10.1053/j.gastro.2005.11.029

Herman, B., Nieminen, A. L., Gores, G. J., and Lemasters, J. J. (1988). Irreversible injury in anoxic hepatocytes precipitated by an abrupt increase in plasma membrane permeability. *FASEB J.* 2, 146–151.

Huveneers, S., and Danen, E. H. J. (2009). Adhesion signaling—crosstalk between integrins, Src and Rho. *J. Cell Sci.* 122, 1059–1069. doi: 10.1242/jcs.039446

Karsdal, M. A., Manon-Jensen, T., Genovese, F., Kristensen, J. H., Nielsen, M. J., Sand, J. M. B., et al. (2015). Novel insights into the function and dynamics of extracellular matrix in liver fibrosis. *Am. J. Physiol. Gastrointest. Liver Physiol.* 308, G807–G830. doi: 10.1152/ajpgi.00447.2014

Klein, S., Klösel, J., Schierwagen, R., Körner, C., Granzow, M., Huss, S., et al. (2012a). Atorvastatin inhibits proliferation and apoptosis, but induces senescence in hepatic myofibroblasts and thereby attenuates hepatic fibrosis in rats. *Lab. Invest.* 92, 1440–1450. doi: 10.1038/labinvest.2012.106

Klein, S., Rick, J., Lehmann, J., Schierwagen, R., Schierwagen, I. G., Verbeke, L., et al. (2015). Janus-kinase-2 relates directly to portal hypertension and to complications in rodent and human cirrhosis. *Gut* 10:e0138732. doi: 10.1136/gutjnl-2015-309600

Klein, S., van Beuge, M. M., Granzow, M., Beljaars, L., Schierwagen, R., Kilic, S., et al. (2012b). HSC-specific inhibition of Rho-kinase reduces portal pressure in cirrhotic rats without major systemic effects. *J. Hepatol.* 57, 1220–1227. doi: 10.1016/j.jhep.2012.07.033

Kwiecinski, M., Noetel, A., Elfimova, N., Trebicka, J., Schievenbusch, S., Strack, I., et al. (2011). Hepatocyte growth factor (HGF) inhibits collagen I and IV synthesis in hepatic stellate cells by miRNA-29 induction. *PLoS ONE* 6:e24568. doi: 10.1371/journal.pone.0024568

Mederacke, I., Hsu, C. C., Troeger, J. S., Huebener, P., Mu, X., Dapito, D. H., et al. (2013). Fate tracing reveals hepatic stellate cells as dominant contributors to liver fibrosis independent of its aetiology. *Nat. Commun.* 4, 2823. doi: 10.1038/ncomms3823

Musumeci, F., Schenone, S., Brullo, C., and Botta, M. (2012). An update on dual Src/Abl inhibitors. *Future Med. Chem.* 4, 799–822. doi: 10.4155/fmc.12.29

National Institute on Alcohol Abuse and Alcoholism (2014). *Liver Cirrhosis Mortality in the United States: National, State, and Regional Trends, 2000–2011.* Arlington, VA: CSR, Incorporated.

Nikitovic, D., Kouvidi, K., Karamanos, N. K., Tzanakakis, G. N., Nikitovic, D., Kouvidi, K., et al. (2013). The roles of hyaluronan/RHAMM/CD44 and their respective interactions along the insidious pathways of fibrosarcoma progression, the roles of hyaluronan/RHAMM/CD44 and their respective interactions along the insidious pathways of fibrosarcoma progression. *BioMed Res. Int.* 2013:e929531. doi: 10.1155/2013/929531

Olsen, A. L., Bloomer, S. A., Chan, E. P., Gaça, M. D. A., Georges, P. C., Sackey, B., et al. (2011). Hepatic stellate cells require a stiff environment for myofibroblastic differentiation. *Am. J. Physiol. Gastrointest. Liver Physiol.* 301, G110–G118. doi: 10.1152/ajpgi.00412.2010

Quack, T., Knobloch, J., Beckmann, S., Vicogne, J., Dissous, C., and Grevelding, C. G. (2009). The formin-homology protein SmDia interacts with the Src kinase SmTK and the GTPase SmRho1 in the gonads of *Schistosoma mansoni*. *PLoS ONE* 4:e6998. doi: 10.1371/journal.pone.0006998

Rockey, D. (1997). The cellular pathogenesis of portal hypertension: stellate cell contractility, endothelin, and nitric oxide. *Hepatology* 25, 2–5. doi: 10.1053/jhep.1997.v25.ajhep0250002

Sarang, Z., Köröskényi, K., Pallai, A., Duró, E., Melino, G., Griffin, M., et al. (2011). Transglutaminase 2 null macrophages respond to lipopolysaccharide stimulation by elevated proinflammatory cytokine production due to an enhanced αvβ3 integrin-induced Src tyrosine kinase signaling. *Immunol. Lett.* 138, 71–78. doi: 10.1016/j.imlet.2011.03.004

Takai, Y., Sasaki, T., and Matozaki, T. (2001). Small GTP-binding proteins. *Physiol. Rev.* 81, 153–208. doi: 10.1016/S0074-7696(08)61861-6

Thumkeo, D., Watanabe, S., and Narumiya, S. (2013). Physiological roles of Rho and Rho effectors in mammals. *Eur. J. Cell Biol.* 92, 303–315. doi: 10.1016/j.ejcb.2013.09.002

Trebicka, J., Anadol, E., Elfimova, N., Strack, I., Roggendorf, M., Viazov, S., et al. (2013). Hepatic and serum levels of miR-122 after chronic HCV-induced fibrosis. *J. Hepatol.* 58, 234–239. doi: 10.1016/j.jhep.2012.10.015

Trebicka, J., Hennenberg, M., Laleman, W., Shelest, N., Biecker, E., Schepke, M., et al. (2007). Atorvastatin lowers portal pressure in cirrhotic rats by inhibition of RhoA/Rho-kinase and activation of endothelial nitric oxide synthase. *Hepatology* 46, 242–253. doi: 10.1002/hep.21673

Trebicka, J., Hennenberg, M., Odenthal, M., Shir, K., Klein, S., Granzow, M., et al. (2010). Atorvastatin attenuates hepatic fibrosis in rats after bile duct ligation via decreased turnover of hepatic stellate cells. *J. Hepatol.* 53, 702–712. doi: 10.1016/j.jhep.2010.04.025

Trebicka, J., Leifeld, L., Hennenberg, M., Biecker, E., Eckhardt, A., Fischer, N., et al. (2008). Hemodynamic effects of urotensin II and its specific receptor antagonist palosuran in cirrhotic rats. *Hepatology* 47, 1264–1276. doi: 10.1002/hep.22170

Varkaris, A., Katsiampoura, A. D., Araujo, J. C., Gallick, G. E., and Corn, P. G. (2014). Src signaling pathways in prostate cancer. *Cancer Metastasis Rev.* 33, 595–606. doi: 10.1007/s10555-013-9481-1

Verbeke, L., Farre, R., Trebicka, J., Komuta, M., Roskams, T., Klein, S., et al. (2014). Obeticholic acid, a farnesoid X receptor agonist, improves portal hypertension by two distinct pathways in cirrhotic rats. *Hepatology* 59, 2286–2298. doi: 10.1002/hep.26939

Wells, R. G. (2013). Tissue mechanics and fibrosis. *Biochim. Biophys. Acta* 1832, 884–890. doi: 10.1016/j.bbadis.2013.02.007

Wojtalla, A., Herweck, F., Granzow, M., Klein, S., Trebicka, J., Huss, S., et al. (2012). The endocannabinoid N-arachidonoyl dopamine (NADA) selectively induces oxidative stress-mediated cell death in hepatic stellate cells but not in hepatocytes. *Am. J. Physiol. Gastrointest. Liver Physiol.* 302, G873–G887. doi: 10.1152/ajpgi.00241.2011

World Health Organisation (2002). *Reducing Risks, Promoting Healthy Life.* Geneva: World Health Report.

Yamana, N., Arakawa, Y., Nishino, T., Kurokawa, K., Tanji, M., Itoh, R. E., et al. (2006). The Rho-mDia1 pathway regulates cell polarity and focal adhesion turnover in migrating cells through mobilizing Apc and c-Src. *Mol. Cell. Biol.* 26, 6844–6858. doi: 10.1128/MCB.00283-06

Yeatman, T. J. (2004). A renaissance for SRC. *Nat. Rev. Cancer* 4, 470–480. doi: 10.1038/nrc1366

Yokoi, K., Hawke, D., Oborn, C. J., Jang, J.-Y., Nishioka, Y., Fan, D., et al. (2011). Identification and validation of SRC and phospho-SRC family proteins

in circulating mononuclear cells as novel biomarkers for pancreatic cancer. *Transl. Oncol.* 4, 83–91. doi: 10.1593/tlo.10202

Yoshizumi, M., Abe, J., Haendeler, J., Huang, Q., and Berk, B. C. (2000). Src and Cas mediate JNK activation but not ERK1/2 and p38 kinases by reactive oxygen species. *J. Biol. Chem.* 275, 11706–11712. doi: 10.1074/jbc.275.16.11706

Zhou, Q., Hennenberg, M., Trebicka, J., Jochem, K., Leifeld, L., Biecker, E., et al. (2006). Intrahepatic upregulation of RhoA and Rho-kinase signalling contributes to increased hepatic vascular resistance in rats with secondary biliary cirrhosis. *Gut* 55, 1296–1305. doi: 10.1136/gut.2005.081059

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Promising Therapy Candidates for Liver Fibrosis

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Liver fibrosis is a wound-healing process in response to repeated and chronic injury to hepatocytes and/or cholangiocytes. Ongoing hepatocyte apoptosis or necrosis lead to increase in ROS production and decrease in antioxidant activity, which recruits inflammatory cells from the blood and activate hepatic stellate cells (HSCs) changing to myofibroblasts. Injury to cholangiocytes also recruits inflammatory cells to the liver and activates portal fibroblasts in the portal area, which release molecules to activate and amplify cholangiocytes. No matter what origin of myofibroblasts, either HSCs or portal fibroblasts, they share similar characteristics, including being positive for α-smooth muscle actin and producing extracellular matrix. Based on the extensive pathogenesis knowledge of liver fibrosis, therapeutic strategies have been designed to target each step of this process, including hepatocyte apoptosis, cholangiocyte proliferation, inflammation, and activation of myofibroblasts to deposit extracellular matrix, yet the current therapies are still in early-phase clinical development. There is an urgent need to translate the molecular mechanism of liver fibrosis to effective and potent reagents or therapies in human.

Keywords: liver fibrosis, hepatocytes, cholangiocyte, inflammation, myofibroblasts, early-phase clinical trial

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INTRODUCTION

Liver fibrosis is a wound-healing process of the liver in response to repeated and chronic liver injury with distinct etiologies, such as infectious diseases (e.g., viral hepatitis), metabolic derangements (non-alcoholic steatohepatitis), exposure to chemicals (e.g., alcohol liver diseases), or autoimmune diseases (e.g., primary biliary cirrhosis, primary sclerosing cholangitis, and autoimmune hepatitis). The identical morphology characteristics of liver fibrosis are the quantitative and qualitative deposition of extracellular matrix which is produced by myofibroblasts. Myofibroblasts are absent from the healthy liver, accumulate in the injured liver and serve as the principle effector cells of fibrogenesis. Although control and clearance of the underlying causative etiology (e.g., virus eradication or alcohol absence) can slow down fibrosis progression and lead to fibrosis regression, the extensive knowledge on the mechanism leading to liver fibrosis through hepatocyte injury, chaolangiocyte proliferation, inflammation, and activation of myofibroblasts to deposit extracellular matrix has not been translated into effective and potent reagents or therapies in human so far (Hauff et al., 2015). In this review we would like to summarize the current knowledge of targeting each step of the pathologenesis process to relieve liver fibrosis.

INJURY-INDUCED HEPATOCYTE APOPTOSIS

Chronic liver injury, including viral hepatitis and nonalcoholic steatohepatitis (NASH), is typically associated with apoptosis of hepatocytes, which recruit inflammatory cells and promote liver fibrosis. Inactivation of Caspase 1, Caspase 2, or Caspase 3 protects hepatocytes against apoptosis, reduces cytokines involved in inflammatory signaling, and ameliorates fibrogenesis in diet-induced NASH models (Dixon et al., 2012; Machado et al., 2014; Thapaliya et al., 2014). The pan-caspase inhibitor, VX-166 could reduce oxidative stress, inflammation, HSC activation, and extracellular matrix deposition in non-alcoholic steatohepatis mouse (Witek et al., 2009; Anstee et al., 2010). Another pan-caspase inhibitor, IDN-6556, also attenuates hepatic inflammation, hepatic stellate cells (HSCs) activation, and liver fibrosis in murine bile duct ligation (BDL) model (Canbay et al., 2004) and steatohepatitis models (Barreyro et al., 2015). In a clinical study, oral administration of IDN-6556 can reduce aminotransferase activity in chronic hepatitis C patients (Pockros et al., 2007). Now, IDN-6556 has been completed the investigation in randomized, double-blind phase II studies for the treatment of Non-alcoholic fatty liver disease (NAFLD), hepatitis C virus (HCV), and cirrhosis (**Table 1**). Interestingly, co-administration of an antioxidant (lithospermate B) and caspase inhibitor (nivocasan) can suppress oxidative stress and hepatocyte apoptosis, resulting in a combined effect on reversal of liver fibrosis in rats (Kim do et al., 2013), which provides a better basis for effective therapy to liver fibrosis.

INJURY-INDUCED CHOLANGIOCYTE PROLIFERATION

Injury to cholangiocytes recruits inflammatory cells from the blood and activates portal fibroblasts in the portal area, which release molecules to activate and amplify the proliferation biliary progenitor cells (activated cholangiocytes). In BDL cholestatic injury model, bile ductular cells and fibroblastic-appearing cells in the portal area express Hedgehog (Hh) ligands, receptor, and/or target genes (Omenetti et al., 2007). Furthermore, myofibroblasts release soluble Hh ligands that stimulate cholangiocytes to produce Cxcl16 and recruit NKT cells (Omenetti et al., 2009). Hh signaling antagonist GDC-0449 could inhibit liver myofibroblasts and progenitors, relieve liver fibrosis, and even promotes regression of HCC in phospholipid flippase (Mdr2) knockout mice (Philips et al., 2011). Integrin αvβ6, which is another molecular absent in normal liver, promotes proliferation of cholangiocytes and plays functional roles in activating latent TGF-β1. Cholangiocytes exhibit marked increased expression of αvβ6 integrin in thioacetamide (TAA)- and BDL-induced fibrosis, and in human HCV fibrosis and end-stage cirrhosis (Wang et al., 2007; Popov et al., 2008). Inhibition of αvβ6 could attenuate collagen deposition, improve liver function, and retard progression of biliary fibrosis in mouse orthotopic liver transplantation model (Chen et al., 2013). Impressively, a single dose of integrin αvβ6 inhibitor exhibits antifibrogenic and profibrolytic effects (Popov et al., 2008), which might serve as a target for anti-fibrosis therapy.

TABLE 1 | Liver injury animal model findings with potential antifibrotic effects.

Targets	Mechanism	Intervention	Animal model
Injured hepatocytes	Pan-caspase inhibitor	VX-166	NAFLD
	Pan-caspase inhibitor	IDN-6556	BDL; NAFLD
Cholangiocyte	Hh signaling antagonist	GDC-0449	Mdr2-/-
Proliferation	Integrin αvβ6 antagonist	Integrin αvβ6 antibody	Ischemia-related biliary fibrosis after orthotopic liver transplantation
	Integrin αvβ6 antagonist	EMD527040	Mdr2-/-
Recruitment of inflammatory cells	P2X7 antagonist	A438079	CCl4; acetaminophen
	CXCR2-FPR1	BOC-1 and DF2156a	acetaminophen
	HMGB1-mediated inflammatory signaling	E5564	ischemia and reperfusion
	TLR9	COV08-0064	acetaminophen
	IL17A	IL17A antibody	S. japonicum; NASH
Nox/ROS signaling	NOX1/NOX4 inhibitor	GKT137831	CCl4; BDL; Fast-food Diet
	antioxidant	Anthocyanin	Acetaminophen; BDL; Alcohol
	antioxidant	β-Lapachone	alcoholic fatty liver disease
Hh signaling	Progenitor response		CCl4; MCD; DDC
Myofibroblasts Activation	TGF-β antibody	1D11	TAA
	CTGF siRNA	NA	CCl4
	Hh signaling antagonist	GDC-0449	BDL; NASH
	Hh signaling antagonist	CYA	BDL
	S1P antagonist	suramin	CCl4; BDL; TAA
	LOXL2 antibody	AB0023	CCl4

MCD, methionine-choline deficient diet; DDC, 3,5,-diethoxycarbonyl-1,4-dihydrocollidine diet; TAA, thioacetamide.

DAMAGED PARENCHYMAL CELLS RECRUIT INFLAMMATORY CELLS TO THE LIVER

Endogenous damage-associated molecular patterns (DAMPS) released or leaked from dying hepatocytes act as danger signals recruiting immune cells to the site of injury and initiating inflammatory response (Kubes and Mehal, 2012).

A large variety DAMPS have been identified, including nucleic acids, proteins [for example, high mobility group box-1 (HMGB1)], and cellular components [for example, adenosine triphosphate, (ATP)]. It has been shown that ATP released from necrotic hepatocytes generates an inflammatory microenvironment attracting neutrophils to the liver sinusoids via P2X7R, a sensor monitoring the release of ATP at inflammation sites, in a murine hepatic necrosis model by localized thermal injury (McDonald et al., 2010). Neutrophil recruitment is significantly reduced in response to tissue injury either by blocking P2X7 receptor or by genetic deficiency of *P2x7r*$^{-/-}$ in mice (McDonald et al., 2010). The specific P2X7 antagonist, A438079, could markedly reduce acetaminophen-induced necrosis (Hoque et al., 2012) and alive CCl4-induced liver inflammation and injury (Huang et al., 2014). Although ATP signals initiate neutrophil adhesion to liver sinusoid, it is the chemokines and mitochondrial contain DAMPs, including formyl-peptide receptor 1 (FPR1), that guide and direct neutrophils migration through health tissue toward injured foci (McDonald et al., 2010). CXCR2-FPR1 antagonism can block neutrophil infiltration and hepatotoxicity in acetaminophen-induced liver injury (Marques et al., 2012). These findings suggest blocking P2X7R and/or CXCR2-FPR1 could be a promising therapeutic approach to control liver inflammation.

The HMGB1 is a nonhistone nuclear protein that facilitates regulatory proteins binding to DNA. HGMB1 is constitutively expressed by most of the cells and will be released under injury and death. HGMB1 is highly induced during liver injury (Ge et al., 2014) and contributes to inflammation through binding to TLR4 (McDonald et al., 2015). It is proposed that HMGB1 may not have a direct proinflammatory effect but acts with other proinflammatory mediators such as lipopolysaccharide (LPS; Bianchi, 2009). LPS, a cell-wall component of Gram-negative bacteria, is among the strongest known inducers of inflammation. It has been shown that patients suffered from chronic liver disease had altered gut microbiota and intestinal permeability resulting in releasing bacterial endotoxins, including LPS, to the circulation (Cesaro et al., 2011; Hartmann et al., 2013; Brenner et al., 2015). The leaky gut further induces and stimulates the progression of liver inflammation in addition to the direct injury. As the receptor of HMGB1 and LPS, TLR4 increases TGF-β1 production by Kupffer cells and activates HSCs promoting liver fibrosis (Federico et al., 2015). An inhibitor of TLR4, Eritoran tetrasodium (E5564), can prevent the gut barrier permeability and reduce liver damage by lower IL-6 level and less NF-κB activation in hemorrhagic shock mice with resuscitation (Korff et al., 2013). Pharmacological inhibition of TLR4 by Eritoran tetrasodium ameliorates liver injury through blocking HMGB1-mediated inflammatory signaling in ischemia and reperfusion mice (McDonald et al., 2015) In addition, both bacterial DNA and mitochondrial DNA and nuclear DNA released by damaged hepatocyte can activate TLR9 and induce a number of cellular immune responses (Kubes and Mehal, 2012). A specific antagonist of TLR9, COV08-0064, could limit the sterile inflammation in rat and mouse models of acute liver injury and acute pancratitis (Hoque et al., 2013). So, the antagonists of TLRs provide us new options of combination drug therapy for controlling liver inflammation.

Th17 cells are derived from Naive CD4^{+} T cells, which are rare in normal liver, but accumulate in the portal areas around bile ducts in chronic liver diseases (Wang et al., 2011; Oo et al., 2012). Th17 cells express CCL20, a ligand for CCR6. Using cytokine-treated human cholangiocytes, CCL20 could induce CCR6-dependent migration of Th17 cells (Oo et al., 2012), which might be one of the mechanisms for inflammatory cells recruiting to the liver. IL17 expressed by Th17 cells activates Kupffer cells to produce proinflammatory and profibrotic cytokines for fibrogenesis, and directly increase the expression of collagen, α-SMA, TGF-β in HSCs (Meng et al., 2012). IL-17 monoclonal antibody ameliorates hepatic granulomatous inflammation through downregulation of inflammatory cytokines and recruitment of neutrophils in mouse infected with *Schistosoma japonicum* (*S. japonicum*) larvae (Zhang et al., 2012). And neutralization of IL-17 in NASH mice ameliorates LPS-induced inflammatory cell infiltrating into the liver (Tang et al., 2011). Antibodies against IL17A has been used in phase II-III clinical trials to establish their therapeutic effects in autoimmune diseases (Schuppan and Kim, 2013), yet no information is available for clinical trials on liver fibrosis now.

APOPTOTIC HEPATOCYTES ACTIVATE HSCs THROUGH Nox/ROS SIGNALING

Ongoing hepatocyte apoptosis or necrosis leads to increase in ROS production and facilitates HSCs activation and migration, which is one of the characteristics of chronic liver disease that triggers liver fibrogenesis. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) produces reactive oxygen species (ROS) via transferring electron from nicotinamide adenine dinucleotide phosphate to molecular oxygen, which is different from other redox enzymes that produce superoxide as a byproduct. The mammalian NOX family is composed of seven isoforms: NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1, and DUOX2, which are distinctively expressed in specific cell types in the liver. HSCs express three Nox isoforms, Nox1, Nox2, Nox 3 (Paik et al., 2014). HSCs from p47^{phox}-deficient mice (without a regulatory component of NOX) fail to generate ROS in response to angiotensin II, platelet derived growth factor (PDGF), leptin, or apoptotic bodies, and p47^{phox}-deficient mice demonstrate reduced liver fibrosis after BDL or the hepatotoxin CCl4. (De Minicis and Brenner, 2007; De Minicis et al., 2010). Since NOX1 and NOX4 are expressed in α-SMA positive activated HSCs, GKT137831, a potent dual NOX1/NOX4 inhibitor, attenuates ROS production and inhibits activation of HSCs (Paik et al., 2014). *In vivo* experiments show that GKT137831 could suppress ROS production, liver inflammation and ameliorate CCl4-, BDL-, and Fast-Food Diet-induced liver fibrosis (Aoyama et al., 2012; Jiang et al., 2012; Bettaieb et al., 2015).

Although hepatocyts express many isoforms of Nox, the development of steatosis by a high-fat, methionine and

choline-deficient (MCD) diet is independent from Nox activation in hepatocytes. In steatosis, the majority of ROS production may derive from hepatocellular lipid deposition and subsequent peroxidation. Anthocyanin, a plant-derived antioxidant, could reduce oxidative stress, relieve hepatic inflammation, and protect hepatocytes against injury, indicating its potential antifibrotic effects (Choi et al., 2009; Hou et al., 2010; Donepudi et al., 2012). Administration of beta-Lapachone (3,4-dihydro-2,2-dimethyl-2H-naphthol[1,2-b]pyran-5,6-dione), a natural compound extracted from the bark of the lapacho tree (Tabebuia avellanedae), upregulates apoB100 synthesis and lipid mobilization via modulation of NAD(+)/NADH ratio to activate AMPK signaling (Shin et al., 2014). Although vitamin E may reduce the liver oxidative stress and the fibrosis development, administration vitamin E supplementation does not consistently result in protection from liver injury. Multicenter, long-term clinical trials are still needed to evaluate the role of antioxidants in NASH.

DYING HEPATOCYTES ACTIVATE HEPATIC PROGENITORS THROUGH Hh SIGNALING

Hh signal released by dying hepatocyte could activate the compensatory outgrowth of hepatic progenitors, which are involved in liver regeneration (Jung et al., 2010). As a Hh target, osteopontin is highly expressed in fibrotic liver tissue and influences the function of hepatic progenitors (Coombes et al., 2015). And neutralization of osteopontin could suppress progenitor cell response and attenuate liver fibrosis in CCl4, methionine-choline deficient diet (MCD) and 3,5,-diethoxycarbonyl-1,4-dihydrocollidine diet (DDC) mice (Coombes et al., 2015).

TREATING LIVER FIBROSIS BY TARGETING MYOFIBROBLAST ACTIVATION

Chronic inflammation is linked to liver fibrosis through activating the fibrogenetic effector cells, HSCs, portal fibroblasts, bone marrow-derived fibrocytes, and mesenchymal stem cells. HSCs are the major source of hepatic myofibroblasts during development of liver fibrosis under different etiologies (Mederacke et al., 2013). Portal fibroblasts play a less role in the pathogenesis of liver fibrosis than HSCs because they were implicated to pathogenesis of cholestatic liver injury (Wells, 2014). Fibrocytes with dual characteristics of fibroblasts and hematopoietic cells migrate to the injured liver in response to BDL and CCl4-damaged liver and comprise about 5% of the collagen type I expressing myofibroblasts (Kisseleva et al., 2006; Scholten et al., 2011). Bone marrow-derived mesenchymal stem cells could also be recruited to the injured liver and facilitate fibrogenesis (Russo et al., 2006; Li et al., 2009). Although epithelial-mesenchymal transition of hepatocytes and cholangiocytes has been reported to be another origin of myofibroblasts (Omenetti et al., 2007; Zeisberg et al., 2007; Nitta et al., 2008; Syn et al., 2009), recent studies using cell fate mapping detect only minimal or no contribution of EMT by hepatocytes, cholangiocytes, or hepatic progenitors to myofibroblasts (Scholten et al., 2010; Taura et al., 2010; Chu et al., 2011). And, although endothelial cell injury and neovascularization play a critical role in liver fibrosis, the transition of endothelial cells to mesenchymal cells (EndMT) giving rise myofibroblasts is still not definitively resolved. So, anti-fibrotic therapy targeting the myofibroblast activation process of HSCs, portal fibroblasts, fibrocytes, and mesenchymal stem cells might be more useful than blocking EMT or EndMT.

After engulfment of apoptotic bodies, Kupffer cells are stimulated to produce TGF-β1 (Szondy et al., 2003), which is a potent cytokine to activate HSCs, fibrocytes, and mesenchymal stem cells into myofibroblasts (Kisseleva et al., 2006; Li et al., 2009; Meindl-Beinker et al., 2012). Although TGF-β is one of the most potent stimuli of extracellular matrix synthesis, suppressing its expression remains a major challenge of antifibrotic therapy, since systemic blocking of TGF-β1 can provoke inflammation and increase the risk of neoplasia. Neutralization TGF-β in animal models inhibits liver fibrosis and reduces the risk in developing cholangiocarcinoma (Fan et al., 2013; Ling et al., 2013). Fresolimumab (GC1008) is a human anti-TGF-β1 monoclonal antibody that neutralizes all isoform of TGF-β. In patients with advanced malignant melanoma and renal cell carcinoma, fresolimumab demonstrated acceptable safety and preliminary evidence of antitumor activity (Trachtman et al., 2011; Morris et al., 2014; Lacouture et al., 2015). Using radio-labeled (89)Zr-conjugated fresolimumab for PET to analyze TGF-β expression, GC1008 accumulated in primary tumors and metastases in a manner similar to IgG, and (89) Zr-fresolimumab uptake is seen in sites of tumor ulceration and in scar tissue, where TGF-β is highly active (Oude Munnink et al., 2011). Although there is phase II clinical trial ongoing of fresolimumab, optimal strategies are still needed to restrict it to the fibrotic milieu. TGF-β transduces its signal to target genes through the ALK5 ser/thr kinase receptor. GW6604 (2-phenyl-4-(3-pyridin-2-yl-1H-pyrazol-4-yl)-pyridine), an ALK5 inhibitor, inhibits the transcription and deposition of extracellular matrix and improves the deterioration of liver function in mice (de Gouville et al., 2005). Yet, considering of the pleiotropic effects of TGF-β, treatment with an ALK5 inhibitor should be carefully examined to avoid the unwanted effects (de Gouville and Huet, 2006).

As a TGF-β target gene, CTGF is considered as a central mediator which is specific for promoting fibrogenensis. It has been reported that inhibition of CTGF expression by siRNA prevents CCl4-induced liver fibrosis and can induce regression of liver fibrosis (Hao et al., 2014). A human monoclonal antibody to CTGF, FG-3019, has been investigated in various animal models (e.g., liver fibrosis, diabetes, pulmonary fibrosis) and demonstrated a reduction and regression of fibrogenesis. FG-3019 binds to the second domain of human CTGF and is currently under phase II drug investigation for the treatment of liver fibrosis (Lipson et al., 2012; Hauff et al., 2015).

Platelet-derived growth factor (PDGF) is the most potent mitogen for HSC. Imatinib mesylate, a clinically used PDGF receptor tyrpsine kinase inhibitor, attenuates liver fibrosis at

early stages, but does not prevent advanced liver fibrosis in animal experiments (Yoshiji et al., 2005; Neef et al., 2006). PTPRO (protein tyrosine phosphatase, receptor type O) shRNA significantly neutralized PDGF-BB-induced HSC proliferation and myofibroblast marker expression through downregulated phosphorylation of extracellular signal-regulated kinase (ERK) and AKT. PTPRO knockout mice [PTPRO(-/-)] have attenuated liver injury, release of inflammatory factors, tissue remodeling, and liver fibrosis in fibrogenesis induced by BDL or carbon tetrachloride (CCl4) administration (Zhang et al., 2015).

Not only does Hh signaling participate in cholangiocyte-chemokine secretion and progenitor response, but also it is involved in liver injury and inflammation after ischemia reperfusion and BDL (Pratap et al., 2010, 2011). Pharmacological inhibition of Hedgehog signaling by vismodegib (GDC-0449) and CYA could attenuate BDL-induced liver fibrosis (Pratap et al., 2012). And vismodegib relieves hepatic inflammation and liver fibrosis in a mouse nutrient excess model of NASH (Hirsova et al., 2013).

Sphingosine 1-phosphate (S1P) is a multifunctional mediator with increased synthesis in the liver following acute and chronic liver injury (Li et al., 2009, 2011), while its concentration remains a relative low level in the bone marrow. This S1P gradient between liver and bone marrow drives the recruitment of bone marrow-derived mesenchymal stem cells into the circulation and into the liver afterwards. Selective S1P receptor antagonist, suramin, has anti-fibrotic effects in CCl4 and BDL liver fibrosis (Li et al., 2009) and hepatoprotective and antitumor activities in TAA-induced liver injury (Tayel et al., 2014).

Inhibitors of receptor tyrosine kinase and Ser/Thr kinase also demonstrate some anti-fibrosis effects. Multitargeted receptor tyrosine kinase inhibitor Sorafenib, which has been approved for the treatment of advanced renal cell carcinoma and hepatocellular carcinoma (HCC), and Sunitinib, can improve

TABLE 2 | Ongoing clinical studies of antifibrotic drugs.

Targets	Mechanism	Intervention	NCT no.	Disease condition	Phase	Status
Injured hepatocytes	Pan-caspase inhibitor	IDN 6556	NCT02230683	Liver cirrhosis with portal hypertension	Phase 2	Complete
	Pan-caspase inhibitor	IDN 6556	NCT02077374	NAFLD	Phase 2	Complete
	Pan-caspase inhibitor	IDN 6556	NCT00088140	Chronic HCV	Phase 2	Complete
	Pan-caspase inhibitor	IDN 6556	NCT02230670	Liver cirrhosis	Phase 2	Active, not recruiting
	Pan-caspase inhibitor	IDN 6556	NCT01912404	Severe AH	Phase 2	Terminated
Cholangiocyte proliferation	hedgehog inhibitor	LDE225	NCT02151864	HCC and liver cirrhosis	Phase 1	Recruiting
Nox/ROS	Anti-oxidant	Vitamin E	NCT01792115	NAFLD	Phase 2	Recruiting
Signaling	Anti-oxidant	Anthocyanin	NCT01940263	NAFLD	Phase 0	Complete
Myofibroblasts	CTGF antibody	FG-3019	NCT01217632	Liver Fibrosis Due to HBV	Phase 2	Active, not recruiting
	LOXL antibody	Simtuzumab	NCT01452308	Liver Fibrosis	Phase 2	Complete
	LOXL antibody	Simtuzumab	NCT01672879	NASH	Phase 2	Active, not recruiting
	LOXL antibody	Simtuzumab	NCT01672853	PSC	Phase 2	Active, not recruiting
Mesenchymal stem cells	Umbilical cord MSCs	Transplantation	NCT01724398	Liver failure	Phase 1 Phase 2	Unknown
	Umbilical cord MSCs	Transplantation	NCT01218464	Liver failure	Phase 1 Phase 2	Unknown
	Autologous MSCs	Transplantation	NCT01741090	Alcoholic Liver Cirrhosis	Phase 2	Unknown
	Umbilical cord MSCs Bone marrow MSCs	Transplantation	NCT01844063	Liver failure	Phase 1 Phase 2	recruiting
	Autologous MSCs	Transplantation	NCT00420134	Liver failure, cirrhosis	Phase 1 Phase 2	Complete
	Autologous MSCs	Transplantation	NCT00956891	Liver failure	Phase 1 Phase 2	Complete

experimental hepatic fibrosis, inflammation, and angiogenesis (Tugues et al., 2007; Mejias et al., 2009). SiRNA of transient receptor potential melastatin 7 (TRPM7), a non-selective cation channel with protein serine/threonine kinase activity, attenuates TGF-β1-induced expression of myofibroblast markers, increases the ratio of MMPs/TIMPs, and decrease the phosphorylation of Smad2 and Smad3 associated collagen production (Fang et al., 2013, 2014). Hepatic nuclear factor kappa B (NF-κB)-inducing kinase (NIK), a Ser/Thr kinase, which is increased in injured livers in both mice and humans, induces hepatocyte injury, activates bone marrow-derived macrophages, and leads to liver fibrosis, and this might serve as a candidate for liver fibrosis therapy (Shen et al., 2014).

No matter what the origin of myofibroblasts is, the common feature of them is expressing extracellular matrix. In liver fibrosis, type I collagen is the most prominent increased components of extracellular matrix. The cross-linking of type I collagen is also increased, which is modulated by the matrix enzyme lysyl oxidase-like-2 (LOXL2). Although blocking collagens have unwanted off-target effects, inhibition of LOXL2 by a monoclonal antibody (AB0023) reduces the production of cytokines, attenuates TGF-β signaling, and inhibits the activate fibroblasts (Barry-Hamilton et al., 2010). Similar to AB0023, another humanized monoclonal LOXL2 antibody (GS-6624) is in randomized, double blind, phase II clinical trials to treat NASH and PSC (Schuppan and Kim, 2013).

TRANSPLANTATION OF MESENCHYMAL STEM CELLS TO RELIEVE LIVER FIBROSIS

Mesenchymal stem cells, which reside in various tissue, such as bone marrow, umbilical cord blood, adipose tissue and others, can differentiate into multiple cell lineages *in vitro*, including hepatocyte (Ye et al., 2015). Yet, few engraft human cells could be found in the liver after transplantation of human mesenchymal stem cells into the sublethally irradiated NOD/SCID mice exposed to acute or chronic CCl4 injury (di Bonzo et al., 2008). Although endogenous mesenchymal stem cells contribute to liver fibrogenesis during liver injury as described previously, recent studies have demonstrated that exogenous transplantation of mesenchymal stem cells have immunomodulation, inflammation suppression and antifibrogenic effects (Lin et al., 2011; Wang et al., 2014; Christ et al., 2015). Many clinical investigations on the safety and efficacy of mesenchymal stem cells for treatment of liver failure and liver cirrhosis are under phase I and phase II studies (**Table 2**).

CONCLUSION

Currently, there are many potential antifibrotic targets obtained from animal experiments (**Table 1**), yet most of the therapies are still in preclinical evaluation stages (**Table 2**). Since both animal experiments and clinical studies have revealed that liver fibrosis, even early cirrhosis, is reversible, treating patients by combined therapies on underline etiology and fibrosis simultaneously might expedite the regression of liver fibrosis and promote liver regeneration. In the circumstance that the underline etiology of liver fibrosis could not be eradicated, therapies on liver fibrosis might help restrict the disease progression to cirrhosis and reduce the risk of cirrhosis related complication. In the future, the elucidation of the molecular steps of regression of liver fibrosis might provide new preventive and therapeutic strategies for fibrosis even cirrhosis patients.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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REFERENCES

Anstee, Q. M., Concas, D., Kudo, H., Levene, A., Pollard, J., Charlton, P., et al. (2010). Impact of pan-caspase inhibition in animal models of established steatosis and non-alcoholic steatohepatitis. *J. Hepatol.* 53, 542–550. doi: 10.1016/j.jhep.2010.03.016

Aoyama, T., Paik, Y. H., Watanabe, S., Laleu, B., Gaggini, F., Fioraso-Cartier, L., et al. (2012). Nicotinamide adenine dinucleotide phosphate oxidase in experimental liver fibrosis: GKT137831 as a novel potential therapeutic agent. *Hepatology* 56, 2316–2327. doi: 10.1002/hep.25938

Barreyro, F. J., Holod, S., Finocchietto, P. V., Camino, A. M., Aquino, J. B., Avagnina, A., et al. (2015). The pan-caspase inhibitor Emricasan (IDN-6556) decreases liver injury and fibrosis in a murine model of non-alcoholic steatohepatitis. *Liver Int.* 35, 953–966. doi: 10.1111/liv.12570

Barry-Hamilton, V., Spangler, R., Marshall, D., McCauley, S., Rodriguez, H. M., Oyasu, M., et al. (2010). Allosteric inhibition of lysyl oxidase-like-2 impedes the development of a pathologic microenvironment. *Nat. Med.* 16, 1009–1017. doi: 10.1038/nm.2208

Bettaieb, A., Jiang, J. X., Sasaki, Y., Chao, T. I., Kiss, Z., Chen, X., et al. (2015). Hepatocyte nicotinamide adenine dinucleotide phosphate reduced oxidase 4 regulates stress signaling, fibrosis, and insulin sensitivity during development of Steatohepatitis in mice. *Gastroenterology* 149, 468.e10–480.e10. doi: 10.1053/j.gastro.2015.04.009

Bianchi, M. E. (2009). HMGB1 loves company. *J. Leukoc. Biol.* 86, 573–576. doi: 10.1189/jlb.1008585

Brenner, D. A., Paik, Y. H., and Schnabl, B. (2015). Role of gut microbiota in liver disease. *J. Clin. Gastroenterol.* 49(Suppl. 1), S25–S27. doi: 10.1097/MCG.0000000000000391

Canbay, A., Feldstein, A., Baskin-Bey, E., Bronk, S. F., and Gores, G. J. (2004). The caspase inhibitor IDN-6556 attenuates hepatic injury and fibrosis in the bile duct ligated mouse. *J. Pharmacol. Exp. Ther.* 308, 1191–1196. doi: 10.1124/jpet.103.060129

Cesaro, C., Tiso, A., Del Prete, A., Cariello, R., Tuccillo, C., Cotticelli, G., et al. (2011). Gut microbiota and probiotics in chronic liver diseases. *Dig. Liver Dis.* 43, 431–438. doi: 10.1016/j.dld.2010.10.015

Chen, G., Zhang, L., Chen, L., Wang, H., Zhang, Y., and Bie, P. (2013). Role of integrin αvβ6 in the pathogenesis of ischemia-related biliary fibrosis after liver transplantation. *Transplantation* 95, 1092–1099. doi: 10.1097/TP.0b013e3182884866

Choi, J. H., Choi, C. Y., Lee, K. J., Hwang, Y. P., Chung, Y. C., and Jeong, H. G. (2009). Hepatoprotective effects of an anthocyanin fraction from purple-fleshed sweet potato against acetaminophen-induced liver damage in mice. *J. Med. Food.* 12, 320–326. doi: 10.1089/jmf.2007.0691

Christ, B., Brückner, S., and Winkler, S. (2015). The Therapeutic Promise of mesenchymal stem cells for liver restoration. *Trends Mol. Med.* 21, 673–686. doi: 10.1016/j.molmed.2015.09.004

Chu, A. S., Diaz, R., Hui, J. J., Yanger, K., Zong, Y., Alpini, G., et al. (2011). Lineage tracing demonstrates no evidence of cholangiocyte epithelial-to-mesenchymal transition in murine models of hepatic fibrosis. *Hepatology* 53, 1685–1695. doi: 10.1002/hep.24206

Coombes, J. D., Swiderska-Syn, M., Dollé, L., Reid, D., Eksteen, B., Claridge, L., et al. (2015). Osteopontin neutralisation abrogates the liver progenitor cell response and fibrogenesis in mice. *Gut* 64, 1120–1131. doi: 10.1136/gutjnl-2013-306484

de Gouville, A. C., Boullay, V., Krysa, G., Pilot, J., Brusq, J. M., Loriolle, F., et al. (2005). Inhibition of TGF-beta signaling by an ALK5 inhibitor protects rats from dimethylnitrosamine-induced liver fibrosis. *Br. J. Pharmacol.* 145, 166–177. doi: 10.1038/sj.bjp.0706172

de Gouville, A. C., and Huet, S. (2006). Inhibition of ALK5 as a new approach to treat liver fibrotic diseases. *Drug News Perspect.* 19, 85–90. doi: 10.1358/dnp.2006.19.2.977444

De Minicis, S., and Brenner, D. A. (2007). NOX in liver fibrosis. *Arch. Biochem. Biophys.* 462, 266–272. doi: 10.1016/j.abb.2007.04.016

De Minicis, S., Seki, E., Paik, Y. H., Osterreicher, C. H., Kodama, Y., Kluwe, J., et al. (2010). Role and cellular source of nicotinamide adenine dinucleotide phosphate oxidase in hepatic fibrosis. *Hepatology* 52, 1420–1430. doi: 10.1002/hep.23804

di Bonzo, L. V., Ferrero, I., Cravanzola, C., Mareschi, K., Rustichell, D., Novo, E., et al. (2008). Human mesenchymal stem cells as a two-edged sword in hepatic regenerative medicine: engraftment and hepatocyte differentiation versus profibrogenic potential. *Gut* 57, 223–231. doi: 10.1136/gut.2006.111617

Dixon, L. J., Berk, M., Thapaliya, S., Papouchado, B. G., and Feldstein, A. E. (2012). Caspase-1-mediated regulation of fibrogenesis in diet-induced steatohepatitis. *Lab. Invest.* 92, 713–723. doi: 10.1038/labinvest.2012.45

Donepudi, A. C., Aleksunes, L. M., Driscoll, M. V., Seeram, N. P., and Slitt, A. L. (2012). The traditional ayurvedic medicine, Eugenia jambolana (Jamun fruit), decreases liver inflammation, injury and fibrosis during cholestasis. *Liver Int.* 32, 560–573. doi: 10.1111/j.1478-3231.2011.02724.x

Fan, X., Zhang, Q., Li, S., Lv, Y., Su, H., Jiang, H., et al. (2013). Attenuation of CCl4-induced hepatic fibrosis in mice by vaccinating against TGF-β1. *PLoS ONE* 8:e82190. doi: 10.1371/journal.pone.0082190

Fang, L., Huang, C., Meng, X., Wu, B., Ma, T., Liu, X., et al. (2014). TGF-β1-elevated TRPM7 channel regulates collagen expression in hepatic stellate cells via TGF-β1/Smad pathway. *Toxicol. Appl. Pharmacol.* 280, 335–344. doi: 10.1016/j.taap.2014.08.006

Fang, L., Zhan, S., Huang, C., Cheng, X., Lv, X., Si, H., et al. (2013). TRPM7 channel regulates PDGF-BB-induced proliferation of hepatic stellate cells via PI3K and ERK pathways. *Toxicol. Appl. Pharmacol.* 272, 713–725. doi: 10.1016/j.taap.2013.08.009

Federico, A., Dallio, M., Godos, J., Loguercio, C., and Salomone, F. (2015). Targeting gut-liver axis for the treatment of nonalcoholic steatohepatitis: translational and clinical evidence. *Transl. Res.* 167, 116–124. doi: 10.1016/j.trsl.2015.08.002

Ge, X., Antoine, D. J., Lu, Y., Arriazu, E., Leung, T. M., Klepper, A. L., et al. (2014). High mobility group box-1 (HMGB1) participates in the pathogenesis of alcoholic liver disease (ALD). *J. Biol. Chem.* 289, 22672–22691. doi: 10.1074/jbc.M114.552141

Hao, C., Xie, Y., Peng, M., Ma, L., Zhou, Y., Zhang, Y., et al. (2014). Inhibition of connective tissue growth factor suppresses hepatic stellate cell activation *in vitro* and prevents liver fibrosis *in vivo*. *Clin. Exp. Med.* 14, 141–150. doi: 10.1007/s10238-013-0229-6

Hartmann, P., Chen, P., Wang, H. J., Wang, L., McCole, D. F., Brandl, K., et al. (2013). Deficiency of intestinal mucin-2 ameliorates experimental alcoholic liver disease in mice. *Hepatology* 58, 108–119. doi: 10.1002/hep.26321

Hauff, P., Gottwald, U., and Ocker, M. (2015). Early to Phase II drugs currently under investigation for the treatment of liver fibrosis. *Expert Opin. Investig. Drugs* 24, 309–327. doi: 10.1517/13543784.2015.997874

Hirsova, P., Ibrahim, S. H., Bronk, S. F., Yagita, H., and Gores, G. J. (2013). Vismodegib suppresses TRAIL-mediated liver injury in a mouse model of nonalcoholic steatohepatitis. *PLoS ONE* 8:e70599. doi: 10.1371/journal.pone.0070599

Hoque, R., Farooq, A., Malik, A., Trawick, B. N., Berberich, D. W., McClurg, J. P., et al. (2013). A novel small-molecule enantiomeric analogue of traditional (-)-morphinans has specific TLR9 antagonist properties and reduces sterile inflammation-induced organ damage. *J. Immunol.* 190, 4297–4304. doi: 10.4049/jimmunol.1202184

Hoque, R., Sohail, M. A., Salhanick, S., Malik, A. F., Ghani, A., Robson, S. C., et al. (2012). P2X7 receptor-mediated purinergic signaling promotes liver injury in acetaminophen hepatotoxicity in mice. *Am. J. Physiol. Gastrointest. Liver Physiol.* 302, G1171–G1179. doi: 10.1152/ajpgi.00352.2011

Hou, Z., Qin, P., and Ren, G. (2010). Effect of anthocyanin-rich extract from black rice (*Oryza sativa* L. Japonica) on chronically alcohol-induced liver damage in rats. *J Agric. Food Chem.* 58, 3191–3196. doi: 10.1021/jf904407x

Huang, C., Yu, W., Cui, H., Wang, Y., Zhang, L., Han, F., et al. (2014). P2X7 blockade attenuates mouse liver fibrosis. *Mol. Med. Rep.* 9, 57–62. doi: 10.3892/mmr.2013.1807

Jiang, J. X., Chen, X., Serizawa, N., Szyndralewiez, C., Page, P., Schröder, K., et al. (2012). Liver fibrosis and hepatocyte apoptosis are attenuated by GKT137831, a novel NOX4/NOX1 inhibitor *in vivo*. *Free Radic. Biol. Med.* 53, 289–296. doi: 10.1016/j.freeradbiomed.2012.05.007

Jung, Y., Witek, R. P., Syn, W. K., Choi, S. S., Omenetti, A., Premont, R., et al. (2010). Signals from dying hepatocytes trigger growth of liver progenitors. *Gut* 59, 655–665. doi: 10.1136/gut.2009.204354

Kim do, Y., Chung, S. I., Ro, S. W., Paik, Y. H., Lee, J. I., Jung, M. K., et al. (2013). Combined effects of an antioxidant and caspase inhibitor on the reversal of hepatic fibrosis in rats. *Apoptosis* 18, 1481–1491. doi: 10.1007/s10495-013-0896-5

Kisseleva, T., Uchinami, H., Feirt, N., Quintana-Bustamante, O., Segovia, J. C., Schwabe, R. F., et al. (2006). Bone marrow-derived fibrocytes participate in pathogenesis of liver fibrosis. *J. Hepatol.* 45, 429–438. doi: 10.1016/j.jhep.2006.04.014

Korff, S., Loughran, P., Cai, C., Lee, Y. S., Scott, M., and Billiar, T. R. (2013). Eritoran attenuates tissue damage and inflammation in hemorrhagic shock/trauma. *J. Surg. Res.* 184, e17–e25. doi: 10.1016/j.jss.2013.03.023

Kubes, P., and Mehal, W. Z. (2012). Sterile inflammation in the liver. *Gastroenterology* 143, 1158–1172. doi: 10.1053/j.gastro.2012.09.008

Lacouture, M. E., Morris, J. C., Lawrence, D. P., Tan, A. R., Olencki, T. E., Shapiro, G. I., et al. (2015). Cutaneous keratoacanthomas/squamous cell carcinomas associated with neutralization of transforming growth factor β by the monoclonal antibody fresolimumab (GC1008). *Cancer Immunol. Immunother.* 64, 437–446. doi: 10.1007/s00262-015-1653-0

Li, C., Kong, Y., Wang, H., Wang, S., Yu, H., Liu, X., et al. (2009). Homing of bone marrow mesenchymal stem cells mediated by sphingosine 1-phosphate contributes to liver fibrosis. *J. Hepatol.* 50, 1174–1183. doi: 10.1016/j.jhep.2009.01.028

Li, C., Zheng, S., You, H., Liu, X., Lin, M., Yang, L., et al. (2011). Sphingosine 1-phosphate (S1P)/S1P receptors are involved in human liver fibrosis by action on hepatic myofibroblasts motility. *J. Hepatol.* 54, 1205–1213. doi: 10.1016/j.jhep.2010.08.028

Lin, H., Xu, R., Zhang, Z., Chen, L., Shi, M., and Wang, F. S. (2011). Implications of the immunoregulatory functions of mesenchymal stem cells in the treatment of human liver diseases. *Cell Mol. Immunol.* 8, 19–22. doi: 10.1038/cmi.2010.57

Ling, H., Roux, E., Hempel, D., Tao, J., Smith, M., Lonning, S., et al. (2013). Transforming growth factor β neutralization ameliorates pre-existing hepatic fibrosis and reduces cholangiocarcinoma in thioacetamide-treated rats. *PLoS ONE* 8:e54499. doi: 10.1371/journal.pone.0054499

Lipson, K. E., Wong, C., Teng, Y., and Spong, S. (2012). CTGF is a central mediator of tissue remodeling and fibrosis and its inhibition can reverse the process of fibrosis. *Fibrogenesis Tissue Repair* 5:S24. doi: 10.1186/1755-1536-5-S1-S24

Machado, M. V., Michelotti, G. A., Pereira, T. D., Boursier, J., Kruger, L., Swiderska-Syn, M., et al. (2014). Reduced lipoapoptosis, hedgehog pathway activation and fibrosis in caspase-2 deficient mice with non-alcoholic steatohepatitis. *Gut* 67, 1148–1157. doi: 10.1136/gutjnl-2014-307362

Marques, P. E., Amaral, S. S., Pires, D. A., Nogueira, L. L., Soriani, F. M., Lima, B. H., et al. (2012). Chemokines and mitochondrial products activate neutrophils to amplify organ injury during mouse acute liver failure. *Hepatology* 56, 1971–1882. doi: 10.1002/hep.25801

McDonald, B., Pittman, K., Menezes, G. B., Hirota, S. A., Slaba, I., Waterhouse, C. C., et al. (2010). Intravascular danger signals guide neutrophils to sites of sterile inflammation. *Science* 330, 362–366. doi: 10.1126/science.1195491

McDonald, K. A., Huang, H., Tohme, S., Loughran, P., Ferrero, K., Billiar, T., et al. (2015). Toll-like receptor 4 (TLR4) antagonist eritoran tetrasodium attenuates liver ischemia and reperfusion injury through inhibition of high-mobility group box protein B1 (HMGB1) Signaling. *Mol. Med.* 20, 639–648. doi: 10.2119/molmed.2014.00076

Mederacke, I., Hsu, C. C., Troeger, J. S., Huebener, P., Mu, X., Dapito, D. H., et al. (2013). Fate tracing reveals hepatic stellate cells as dominant contributors to liver fibrosis independent of its aetiology. *Nat. Commun.* 4, 2823. doi: 10.1038/ncomms3823

Meindl-Beinker, N. M., Matsuzaki, K., and Dooley, S. (2012). TGF-β signaling in onset and progression of hepatocellular carcinoma. *Dig. Dis.* 30, 514–523. doi: 10.1159/000341704

Mejias, M., Garcia-Pras, E., Tiani, C., Miquel, R., Bosch, J., and Fernandez, M. (2009). Beneficial effects of sorafenib on splanchnic, intrahepatic, and portocollateral circulations in portal hypertensive and cirrhotic rats. *Hepatology* 49, 1245–1256. doi: 10.1002/hep.22758

Meng, F., Wang, K., Aoyama, T., Grivennikov, S. I., Paik, Y., Scholten, D., et al. (2012). Interleukin-17 signaling in inflammatory, Kupffer cells, and hepatic stellate cells exacerbates liver fibrosis in mice. *Gastroenterology* 143, 765–776. doi: 10.1053/j.gastro.2012.05.049

Morris, J. C., Tan, A. R., Olencki, T. E., Shapiro, G. I., Dezube, B. J., Reiss, M., et al. (2014). Phase I study of GC1008 (fresolimumab): a human anti-transforming growth factor-beta (TGFβ) monoclonal antibody in patients with advanced malignant melanoma or renal cell carcinoma. *PLoS ONE* 9:e90353. doi: 10.1371/journal.pone.0090353

Neef, M., Ledermann, M., Saegesser, H., Schneider, V., Widmer, N., Decosterd, L. A., et al. (2006). Oral imatinib treatment reduces early fibrogenesis but does not prevent progression in the long term. *J. Hepatol.* 44, 167–175. doi: 10.1016/j.jhep.2005.06.015

Nitta, T., Kim, J. S., Mohuczy, D., and Behrns, K. E. (2008). Murine cirrhosis induces hepatocyte epithelial mesenchymal transition and alterations in survival signaling pathways. *Hepatology* 48, 909–919. doi: 10.1002/hep.22397

Omenetti, A., Syn, W. K., Jung, Y., Francis, H., Porrello, A., Witek, R. P., et al. (2009). Repair-related activation of hedgehog signaling promotes cholangiocyte chemokine production. *Hepatology* 50, 518–527. doi: 10.1002/hep.23019

Omenetti, A., Yang, L., Li, Y. X., McCall, S. J., Jung, Y., Sicklick, J. K., et al. (2007). Hedgehog-mediated mesenchymal-epithelial interactions modulate hepatic response to bile duct ligation. *Lab. Invest.* 87, 499–514. doi: 10.1038/labinvest.3700537

Oo, Y. H., Banz, V., Kavanagh, D., Liaskou, E., Withers, D. R., Humphreys, E., et al. (2012). CXCR3-dependent recruitment and CCR6-mediated positioning of Th-17 cells in the inflamed liver. *J. Hepatol* 57, 1044–1051. doi: 10.1016/j.jhep.2012.07.008

Oude Munnink, T. H., Arjaans, M. E., Timmer-Bosscha, H., Schröder, C. P., Hesselink, J. W., Vedelaar, S. R., et al. (2011). PET with the 89Zr-labeled transforming growth factor-β antibody fresolimumab in tumor models. *J. Nucl. Med.* 52, 2001–2008. doi: 10.2967/jnumed.111.092809

Paik, Y. H., Kim, J., Aoyama, T., De Minicis, S., Bataller, R., and Brenner, D. A. (2014). Role of NADPH oxidases in liver fibrosis. *Antioxid. Redox Signal.* 20, 2854–2872. doi: 10.1089/ars.2013.5619

Philips, G. M., Chan, I. S., Swiderska, M., Schroder, V. T., Guy, C., Karaca, G. F., et al. (2011). Hedgehog signaling antagonist promotes regression of both liver fibrosis and hepatocellular carcinoma in a murine model of primary liver cancer. *PLoS ONE* 6:e23943. doi: 10.1371/journal.pone.0023943

Pockros, P. J., Schiff, E. R., Shiffman, M. L., McHutchison, J. G., Gish, R. G., Afdhal, N. H., et al. (2007). Oral IDN-6556, an antiapoptotic caspase inhibitor, may lower aminotransferase activity in patients with chronic hepatitis C. *Hepatology* 46, 324–329. doi: 10.1002/hep.21664

Popov, Y., Patsenker, E., Stickel, F., Zaks, J., Bhaskar, K. R., Niedobitek, G., et al. (2008). Integrin alphavbeta6 is a marker of the progression of biliary and portal liver fibrosis and a novel target for antifibrotic therapies. *J. Hepatol.* 48, 453–464. doi: 10.1016/j.jhep.2007.11.021

Pratap, A., Panakanti, R., Yang, N., Eason, J. D., and Mahato, R. I. (2010). Inhibition of endogenous hedgehog signaling protects against acute liver injury after ischemia reperfusion. *Pharm. Res.* 27, 2492–2504. doi: 10.1007/s11095-010-0246-z

Pratap, A., Panakanti, R., Yang, N., Lakshmi, R., Modanlou, K. A., Eason, J. D., et al. (2011). Cyclopamine attenuates acute warm ischemia reperfusion injury in cholestatic rat liver: hope for marginal livers. *Mol. Pharm.* 8, 958–968. doi: 10.1021/mp200115v

Pratap, A., Singh, S., Mundra, V., Yang, N., Panakanti, R., Eason, J. D., et al. (2012). Attenuation of early liver fibrosis by pharmacological inhibition of smoothened receptor signaling. *J. Drug Target* 20, 770–782. doi: 10.3109/1061186X.2012.719900

Russo, F. P., Alison, M. R., Bigger, B. W., Amofah, E., Florou, A., Amin, F., et al. (2006). The bone marrow functionally contributes to liver fibrosis. *Gastroenterology*. 130, 1807–1821. doi: 10.1053/j.gastro.2006.01.036

Scholten, D., Osterreicher, C. H., Scholten, A., Iwaisako, K., Gu, G., Brenner, D. A., et al. (2010). Genetic labeling does not detect epithelial-to-mesenchymal transition of cholangiocytes in liver fibrosis in mice. *Gastroenterology* 139, 987–998. doi: 10.1053/j.gastro.2010.05.005

Scholten, D., Reichart, D., Paik, Y. H., Lindert, J., Bhattacharya, J., Glass, C. K., et al. (2011). Migration of fibrocytes in fibrogenic liver injury. *Am. J. Pathol.* 179, 189–198. doi: 10.1016/j.ajpath.2011.03.049

Schuppan, D., and Kim, Y. O. (2013). Evolving therapies for liver fibrosis. *J. Clin. Invest.* 123, 1887–1901. doi: 10.1172/JCI66028

Shen, H., Sheng, L., Chen, Z., Jiang, L., Su, H., Yin, L., et al. (2014). Mouse hepatocyte overexpression of NF-κB-inducing kinase (NIK) triggers fatal macrophage-dependent liver injury and fibrosis. *Hepatology* 60, 2065–2076. doi: 10.1002/hep.27348

Shin, S., Park, J., Li, Y., Min, K. N., Kong, G., Hur, G. M., et al. (2014). β-Lapachone alleviates alcoholic fatty liver disease in rats. *Cell. Signal.* 26, 295–305. doi: 10.1016/j.cellsig.2013.11.020

Syn, W. K., Jung, Y., Omenetti, A., Abdelmalek, M., Guy, C. D., Yang, L., et al. (2009). Hedgehog-mediated epithelial-to-mesenchymal transition and fibrogenic repair in nonalcoholic fatty liver disease. *Gastroenterology* 137, 1478.e8–1488.e8. doi: 10.1053/j.gastro.2009.06.051

Szondy, Z., Sarang, Z., Molnar, P., Nemeth, T., Piacentini, M., Mastroberardino, P. G., et al. (2003). Transglutaminase 2-/- mice reveal a phagocytosis-associated crosstalk between macrophages and apoptotic cells. *Proc. Natl. Acad. Sci. U.S.A.* 100, 7812–7817. doi: 10.1073/pnas.0832466100

Tang, Y., Bian, Z., Zhao, L., Liu, Y., Liang, S., Wang, Q., et al. (2011). Interleukin-17 exacerbates hepatic steatosis and inflammation in non-alcoholic fatty liver disease. *Clin. Exp. Immunol.* 166, 281–290. doi: 10.1111/j.1365-2249.2011.04471.x

Taura, K., Miura, K., Iwaisako, K., Osterreicher, C. H., Kodama, Y., Penz-Osterreicher, M., et al. (2010). Hepatocytes do not undergo epithelial-mesenchymal transition in liver fibrosis in mice. *Hepatology* 51, 1027–1036. doi: 10.1002/hep.23368

Tayel, A., Abd El Galil, K. H., Ebrahim, M. A., Ibrahim, A. S., El-Gayar, A. M., and Al-Gayyar, M. M. (2014). Suramin inhibits hepatic tissue damage in hepatocellular carcinoma through deactivation of heparanase enzyme. *Eur. J. Pharmacol.* 728, 151–160. doi: 10.1016/j.ejphar.2014.02.001

Thapaliya, S., Wree, A., Povero, D., Inzaugarat, M. E., Berk, M., Dixon, L., et al. (2014). Caspase 3 inactivation protects against hepatic cell death and ameliorates fibrogenesis in a diet-induced NASH model. *Dig. Dis. Sci.* 59, 1197–1206. doi: 10.1007/s10620-014-3167-6

Trachtman, H., Fervenza, F. C., Gipson, D. S., Heering, P., Jayne, D. R., Peters, H., et al. (2011). A phase 1, single-dose study of fresolimumab, an anti-TGF-β antibody, in treatment-resistant primary focal segmental glomerulosclerosis. *Kidney Int.* 79, 1236–1243. doi: 10.1038/ki.2011.33

Tugues, S., Fernandez-Varo, G., Muñoz-Luque, J., Ros, J., Arroyo, V., Rodés, J., et al. (2007). Antiangiogenic treatment with sunitinib ameliorates inflammatory infiltrate, fibrosis, and portal pressure in cirrhotic rats. *Hepatology* 46, 1919–1926. doi: 10.1002/hep.21921

Wang, B., Dolinski, B. M., Kikuchi, N., Leone, D. R., Peters, M. G., Weinreb, P. H., et al. (2007). Role of alphavbeta6 integrin in acute biliary fibrosis. *Hepatology* 46, 1404–1412. doi: 10.1002/hep.21849

Wang, L., Chen, S., and Xu, K. (2011). IL-17 expression is correlated with hepatitis B-related liver diseases and fibrosis. *Int. J. Mol. Med.* 27, 385–392. doi: 10.3892/ijmm.2011.594

Wang, Y., Chen, X., Cao, W., and Shi, Y. (2014). Plasticity of mesenchymal stem cells in immunomodulation: pathological and therapeutic implications. *Nat. Immunol.* 15, 1009–1016. doi: 10.1038/ni.3002

Wells, R. G. (2014). The portal fibroblast: not just a poor man's stellate cell. *Gastroenterology* 147, 41–47. doi: 10.1053/j.gastro.2014.05.001

Witek, R. P., Stone, W. C., Karaca, F. G., Syn, W. K., Pereira, T. A., Agboola, K. M., et al. (2009). Pan-caspase inhibitor VX-166 reduces fibrosis in an animal model of nonalcoholic steatohepatitis. *Hepatology* 50, 1421–1430. doi: 10.1002/hep.23167

Ye, J. S., Su, X. S., Stoltz, J. F., de Isla, N., and Zhang, L. (2015). Signalling pathways involved in the process of mesenchymal stem cells differentiating into hepatocytes. *Cell Prolif.* 48, 157–165. doi: 10.1111/cpr.12165

Yoshiji, H., Noguchi, R., Kuriyama, S., Ikenaka, Y., Yoshii, J., Yanase, K., et al. (2005). Imatinib mesylate (STI-571) attenuates liver fibrosis development in rats. *Am. J. Physiol. Gastrointest. Liver Physiol.* 288, G907–G913. doi: 10.1152/ajpgi.00420.2004

Zeisberg, M., Yang, C., Martino, M., Duncan, M. B., Rieder, F., Tanjore, H., et al. (2007). Fibroblasts derive from hepatocytes in liver fibrosis via epithelial to mesenchymal transition. *J. Biol. Chem.* 282, 23337–23347. doi: 10.1074/jbc.M700194200

Zhang, X., Tan, Z., Wang, Y., Tang, J., Jiang, R., Hou, J., et al. (2015). PTPRO-associated hepatic stellate cell activation plays a critical role in liver fibrosis. *Cell. Physiol. Biochem.* 35, 885–898. doi: 10.1159/000369746

Zhang, Y., Chen, L., Gao, W., Hou, X., Gu, Y., Gui, L., et al. (2012). IL-17 neutralization significantly ameliorates hepatic granulomatous inflammation and liver damage in *Schistosoma japonicum* infected mice. *Eur. J. Immunol.* 42, 1523–1535. doi: 10.1002/eji.201141933

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REVIEW
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The hop constituent xanthohumol exhibits hepatoprotective effects and inhibits the activation of hepatic stellate cells at different levels

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Xanthohumol is the principal prenylated flavonoid of the female inflorescences of the hop plant. In recent years, various beneficial xanthohumol effects including anti-inflammatory, antioxidant, hypoglycemic activities, and anticancer effects have been revealed. This review summarizes present studies indicating that xanthohumol also inhibits several critical pathophysiological steps during the development and course of chronic liver disease, including the activation and pro-fibrogenic genotype of hepatic stellate cells. Also the various mechanism of action and molecular targets of the beneficial xanthohumol effects will be described. Furthermore, the potential use of xanthohumol or a xanthohumol-enriched hop extract as therapeutic agent to combat the progression of chronic liver disease will be discussed. It is notable that in addition to its hepatoprotective effects, xanthohumol also holds promise as a therapeutic agent for treating obesity, dysregulation of glucose metabolism and other components of the metabolic syndrome including hepatic steatosis. Thus, therapeutic xanthohumol application appears as a promising strategy, particularly in obese patients, to inhibit the development as well as the progression of non-alcoholic fatty liver disease.

Keywords: xanthohumol, hops, fibrosis, hepatic stellate cells, liver disease

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Introduction

Hop (*Humulus lupulus* L.) has been used since ancient times as a medicinal plant. Traditional medicinal indications included the treatment of anxiety and insomnia, mild pain reduction or combating dyspepsia (Zanoli and Zavatti, 2008). Today, hops are used in the manufacturing of beer and female infertile plants are cultivated on high trolleys especially for brewing (**Figure 1A**). Biologically active substances, which are also important for brewing, are concentrated inside hop cones (**Figure 1B**) in lupulin glands (**Figure 1C**) which contain hop resins, bitter acids, essential oils and prenylated flavonoids. These lupulin glands are tiny yellow sacs that are located at the base of the petals of the hop cone (**Figure 1D**) that are found in female plants, while cones from the male hop plant contain relatively few lupulin glands.

Xanthohumol (XN; 3′-[3,3-dimethyl allyl]-2′,4′,4-trihydroxy-6′-methoxychalcone) is the principal prenylated chalcone of the hop plant (**Figure 1E**). The yellow compound (Greek:

Abbreviations: BW, body weight; ECM, extracellular matrix; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HSC, hepatic stellate cells; IFN, interferon; MS, metabolic syndrome; NF-κB, nuclear factor-κB; XN, xanthohumol.

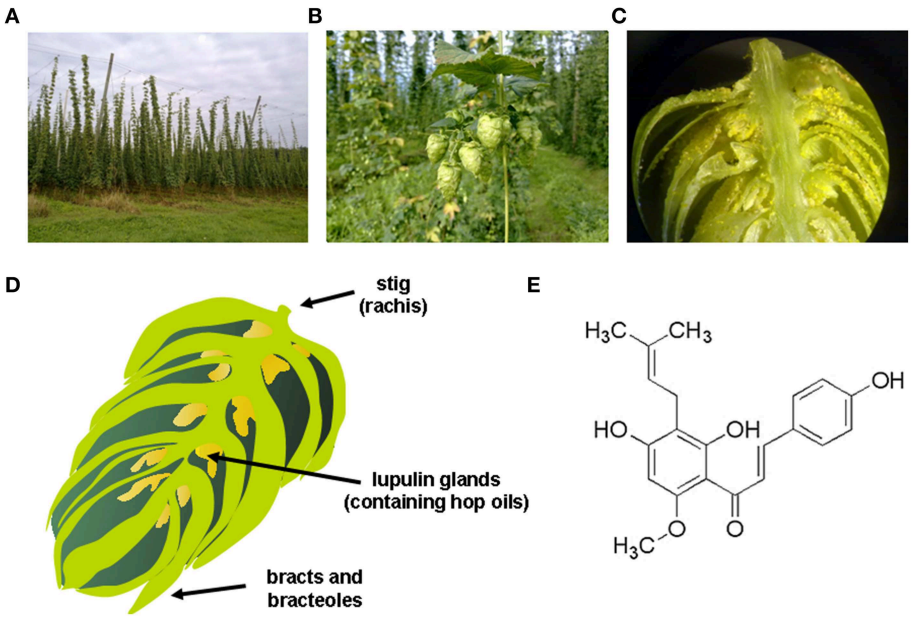

FIGURE 1 | Hop plant and xanthohumol. (A) Hop plant (*Humulus lupulus*) field in Bavaria, showing the typical form of cultivation on high trolleys. **(B)** Female hop flowers (hop cones), where the biologically active substances, which are also important for brewing, are concentrated inside in the lupulin glands; **(C)** lupulin glands, which contain hop resins, bitter acids, essential oils and prenylated flavonoids. **(D)** Schematic drawing of a female hop cone that is composed of a central spine (i.e., the strig), bracts (i.e., pear-shaped petal that does not contain lupulin glands), bracteoles (i.e., pear-shaped petal of the hop cone that shelters the lupulin glands and any seeds that may be present), and the characteristic lupulin glands that are tiny yellow sacs containing the hop oils. **(E)** Chemical structure of xanthohumol (3′-[3,3-dimethyl allyl]-2′,4′,4-trihydroxy-6′-methoxychalcone), the major prenylated chalcone of the hop plant.

xantho = yellow) is found in high quantities in the lupulin glands. Since the 1990s, interest in health-promoting activities of XN increased constantly, scientific investigations were initialized worldwide and papers and patents on this topic have increased steadily (Gerhauser and Frank, 2005).

Many studies identified XN as a broad-spectrum cancer chemopreventive agent acting by multiple mechanisms relevant for cancer development and progression (Gerhauser et al., 2002). XN is able to scavenge reactive oxygen species and it modulates many enzymes involved in carcinogen metabolism and detoxification (Gerhauser et al., 2002). Furthermore, XN inhibits cyclooxygenase 2 (COX-2) expression and the activity of both Cox-1 and Cox-2 in lipopolysaccharide-mediated iNOS induction in the macrophages (Stevens and Page, 2004). XN also has been shown to decrease prostaglandin-E2 (PGE2) expression (Jongthawin et al., 2012). These anti-inflammatory properties may contribute to the inhibition of tumor promotion by the inhibition of nuclear factor-κB (NF-κB) signaling and subsequent down-regulation of pro-inflammatory factors (Albini et al., 2006; Colgate et al., 2007). Moreover, estrogen-mediated tumor promotion may be prevented by XN, which suppresses estrogen-signaling through the inhibition of the interaction between the oncoprotein brefeldin A-inhibited guanine nucleotide-exchange protein 3 (BIG3) and tumor suppressor prohibitin 2 (PHB2) (Yoshimaru et al., 2014). XN also inhibits the enzyme aromatase (CYP19), which plays a crucial role in the conversion of testosterone to estrogen (Monteiro et al., 2006). Furthermore, XN inhibits tumor cell growth by different mechanism such as decrease of DNA polymerase alpha activity and inhibition of DNA synthesis (Gerhauser et al., 2002). Moreover, XN induces apoptosis by poly(ADP-ribose)polymerase (PARP) cleavage, activation of caspases or down-regulation of Bcl-2 protein expression (Pan et al., 2005). XN has further been shown to modulate drug metabolism *in vitro* by inhibition of various Cyp enzymes and by induction of quinone reductase activity (Henderson et al., 2000; Miranda et al., 2000a), which has been considered as a biomarker for cancer chemoprevention (Cuendet et al., 2006). In addition to the molecular mechanism by which XN affects cancer cells, it has been shown to exhibit several further biological effects (**Figure 2**) which are also playing an important role during the course of chronic liver disease. Hepatic fibrosis is the peril that determines morbidity and mortality in patients with liver disease. Cirrhosis, as the end stage of hepatic fibrosis, is a major clinical issue for its high prevalence in the world and its tight relationship with hepatocellular carcinoma (HCC) incidence (Gines et al., 2004; Villanueva et al., 2007; Minguez et al., 2009). The activation of hepatic stellate cells (HSC) is the key event of hepatic fibrosis (Lang and Brenner, 1999; Kisseleva and Brenner, 2007; Elpek, 2014). Activated HSC/myofibroblasts are the cellular source of the excessive extracellular matrix (ECM) deposition (Lang and Brenner, 1999; Kisseleva and Brenner, 2007). Furthermore, activated HSC/myofibroblasts form and infiltrate the tumor stroma and promote HCC progression (Amann et al., 2009).

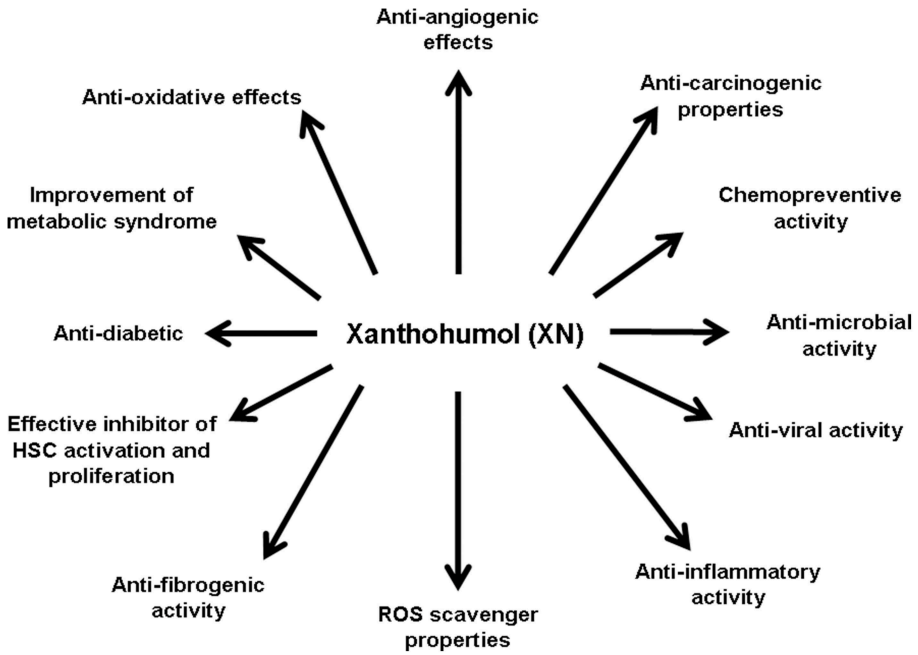

FIGURE 2 | Biological effects of xanthohumol. Xanthohumol (XN) has been shown to have wide spectrum of biological effects, by which it may also affect different pathophysiological mechanisms involved in the development and progression of chronic liver disease. Studies have shown that XN acts anti-angiogenic (Albini et al., 2006; Shamoto et al., 2013), anti-carcinogenic (Dorn et al., 2010a,b,c; Araujo et al., 2011), chemopreventive (Miranda et al., 2000c; Gerhauser et al., 2002; Dorn et al., 2010a), anti-microbial (Gerhauser and Frank, 2005; Rozalski et al., 2013; Kramer et al., 2015), anti-viral (Buckwold et al., 2004; Zhang et al., 2009, 2010; Lou et al., 2014), anti-inflammatoric (Dorn et al., 2010a, 2013; Jongthawin et al., 2012), as a ROS scavenger (Gerhauser et al., 2002), anti-diabetic (Legette et al., 2013), and anti-oxidative (Gerhauser et al., 2002). In regard to liver fibrogenesis, it was shown that XN has anti-fibrogenic potential (Dorn et al., 2010a, 2013; Yang et al., 2013) and inhibits HSC activation and proliferation (Dorn et al., 2010a). Moreover, XN improves the metabolic syndrome (Legette et al., 2013).

Therefore, these cells are a critical target for therapy during the whole course of chronic liver disease. However, up to date, no effective therapy is available to block the activation of HSC or to inhibit the pro-inflammatory and pro-fibrogenic activity of the activated HSC. In the following, we provide a summary of present studies indicating the potential of this hop constituent as a therapeutic agent to beneficially affect hepatic fibrosis as well as various further pathological mechanisms during the course of chronic liver disease.

Effects of Xanthohumol on Hepatic Stellate Cells *in vitro*

As already mentioned, the activation of HSC plays a critical pathophysiological role in the progression of chronic liver disease and the activation of these cells in response to liver injury is considered as the key event of hepatic fibrosis (Bataller and Brenner, 2001). Interestingly, XN has been shown to inhibit the activation of primary human HSC *in vitro* in concentrations as low as 5 μM XN (Dorn et al., 2010a). Furthermore, XN induced apoptosis in activated HSC *in vitro* in a dose-dependent manner (0–20 μM). Moreover, XN reduced expression of the pro-inflammatory factors such as the monocyte chemoattractant protein-1 (MCP-1) or pro-fibrogenic genes such as type I collagen in HSC. MCP-1 is regulated by NF-κB and increased levels are associated with fibrosis progression in chronic liver disease (Jarrar et al., 2008; Wouters et al., 2008). Further, NF-κB activation is a central pathophysiological mechanism during HSC activation (Hellerbrand et al., 1998a,b; Elsharkawy et al., 2005). Importantly, XN inhibited NF-κB activity in activated HSC *in vitro* (Dorn et al., 2010a). In summary, *in vitro* studies revealed that XN inhibits several key pathological factors of HSC activation and their contribution to fibrosis progression in chronic liver disease (**Figure 3**). In addition, XN has been shown to inhibit HSC-activation and hepatic fibrosis in experimental models of liver injury (please see below). Future research, may aim at the identification of further molecular pathways which may contribute to the anti-fibrogenic effect of XN in HSC.

Anti-viral and Antimicrobial Activities of XN

HSC activation occurs in response to hepatocellular injury, and hepatitis C virus (HCV) infection is a one of the major causes of liver infectious diseases. *In vitro* studies using virus that causes bovine diarrhea (bovine viral diarrhea virus - BVDV E2), which shows considerable similarities with the human HCV, showed that XN inhibits BVDV replication and enhanced the anti-viral activity of interferon (IFN)-α (Buckwold et al., 2004; Zhang et al., 2009, 2010). Antiviral activity of XN in combination with IFNα-2b, was also demonstrated against

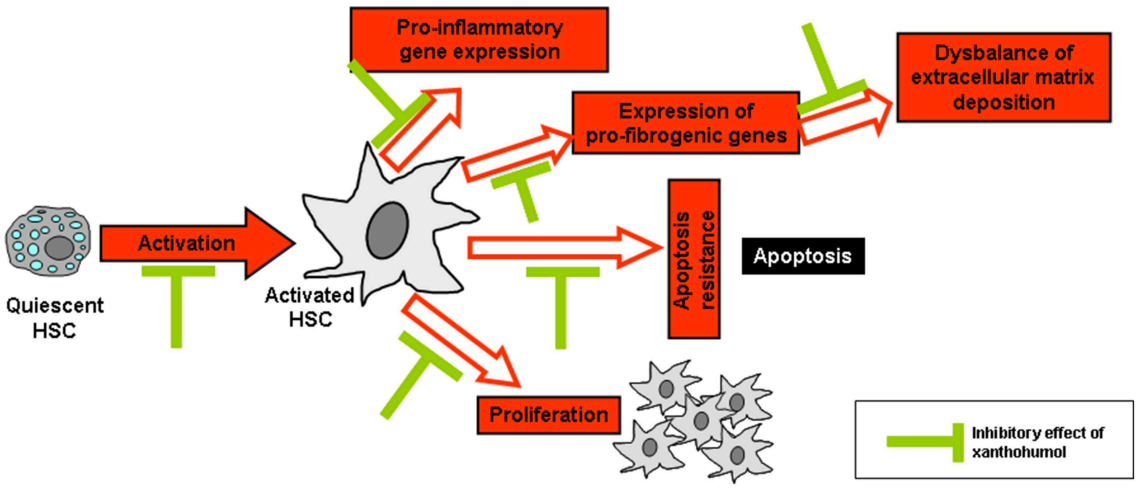

FIGURE 3 | Reported *in vitro* effects of xanthohumol in hepatic stellate cells. Xanthohumol (XN) inhibits the activation of hepatic stellate cells (HSC) as well as several key pathological factors of activated HSC including pro-inflammatory and -fibrogenic gene expression, proliferation, apoptosis resistance, and composition of extracellular matrix. In particular, it is known that XN inhibits pro-inflammatory and pro-fibrogenic (Dorn et al., 2010a, 2013) gene expression and HSC activation (Yang et al., 2013) thereby preventing the excessive formation and deposition of extracellular matrix as well as the activation and proliferation of HSC. In addition, XN was shown to interfere with HSC apoptosis (Dorn et al., 2010a).

herpes simplex virus and cytomegalovirus (Buckwold et al., 2004). More recently, XN was examined for its ability to inhibit HCV virus replication in a cell culture system carrying replicating HCV RNA replicon and it was shown that XN has similar inhibitory effects as IFNα-2b (Lou et al., 2014). Bacteria and bacterial translocation from the intestine have been shown to promote the progression of chronic liver disease, including HSC-activation and fibrogenesis (Seki et al., 2007). More recently, it has been shown that antibiotics improved the intestinal permeability and attenuated liver fibrosis development associated with Nonalcoholic Steatohepatitis (NASH) *via* the inhibition of HSC activation (Douhara et al., 2015). With regards to this, it is important, that the broad spectrum of antimicrobial activity of XN is well known (Gerhauser, 2005). More recently, the inhibitory effect of a hop extract containing XN was demonstrated against different gram-positive bacteria including *Listeria monocytogenes* and *Staphylococcus aureus* (Kramer et al., 2015).

In summary, there is a high rationale that XN may exhibit beneficial effects in liver disease also *via* inhibiting bacterial translocation or the growth of (gram-positive) bacteria. Still experimental proof of this hypothesis is missing, which may be the subject of future research in appropriate (*in vivo*) models or clinical studies.

XN Effects on Hepatocellular Cancer Cells and Primary Hepatocytes

As already mentioned, a number of studies indicated the potential of XN to prevent and treat different types of cancers (Araujo et al., 2011). In the pathogenesis of HCC, XN exhibited anti-tumorous effects and induced the apoptosis in two different human HCC cell lines (HepG2 and Huh7) *in vitro* at concentration of 25 μM (Dorn et al., 2010b). Furthermore, XN repressed proliferation and migration in both cell lines even at lower concentrations (Dorn et al., 2010a). In a study with HepG2 cells, anti-mutagenic effects of XN were demonstrated even at concentrations of 0.01–0.1 μM (Plazar et al., 2008; Viegas et al., 2012). Chemopreventative effects can also occur by detoxifying carcinogens through the action of specific enzymes. One of these enzymes is NAD(P)H: quinone reductase that catalyzes the reduction of quinone to hydroquinones, which are more suitable substrates for subsequent conjugation. It was found that XN increased by several fold the activity of quinone reductase in murine liver cells (Hepa-1c1c7) (Miranda et al., 2000b) at concentrations above 1 μM. Importantly, even at XN concentrations as high as 100 μM, XN did not affect the viability of primary human hepatocytes *in vitro* (Dorn et al., 2010a).

It should be noted that in cellular experiments performed with XN the reproducibility of all these findings might be affected by the low solubility of XN in cell culture medium, the composition of media used, the tendency of XN to absorb to various plastic materials routinely used in the cell culture, and the reduction of the effective XN dose by conversion to isoxanthohumol (Motyl et al., 2012). Likewise, the bioavailability of XN and its prenylated flavanone (i.e., Isoxanthohumol) is marked by inter-individual variability that is induced by variations in the intestinal microbial community and their degradation pathways that have direct impact on biotransformation of respective compounds (Possemiers et al., 2005).

Therefore, studies that investigate pharmacological effects of XN or its natural or synthetic derivatives should punctiliously describe all details of the chosen experimental setting, not only including sources and concentrations of employed prenylflavonoid but also specification of media and buffer

composition, identity and origin of cell lines and animals, details about culture conditions, animal accommodation, supplier of plastic ware, and many others.

Furthermore, future research may focus on the identification of (downstream) signaling pathways responsible for xanthohumol effects in hepatocytes. Recently, it has been shown that the activation of Nrf2 pathway and subsequently phase II enzymes in concert with p53 induction may account for the molecular mechanism of the chemopreventive activity of XN in hepatocytes (Krajka-Kuzniak et al., 2013). On the other hand its cytotoxicity toward HCC cells indicates that it may also be considered as potentially chemotherapeutic (Dorn et al., 2010a). Moreover, the antimutagenic effects of XN were demonstrated against various procarcinogens, which are activated by cytochrome P450 enzymes (Plazar et al., 2007; Ferk et al., 2010). The mechanisms are possibly related to the inhibition of the metabolic activation by human cytochrome P450 1A2 (CYP1A2) and inhibition of the binding of the metabolites to DNA and proteins (Miranda et al., 2000c). Moreover, XN interferes with several stages in the angiogenic process, including inhibition of endothelial cell invasion and migration, growth, and formation of tubular-like structures (Gerhauser et al., 2002; Albini et al., 2006). The mechanisms for its inhibition of angiogenesis are related to the blockage of both the nuclear factor-κB (NFκB) and Akt pathways in endothelial cells (Albini et al., 2006). Furthermore, in addition to the direct effect of XN on the vascular cells, XN inhibits the production of angiogenic factors, e.g., vascular endothelial growth factor (VEGF) and interleukin 8 (IL-8) via the inhibition of NF-κB (Shamoto et al., 2013). Still, according analyses in primary hepatocytes are missing.

XN Effects in Models of Acute Liver Injury

There is already a multitude of reports describing beneficial effects of XN on liver injury *in vitro* and in *vivo* (**Table 1**). Hepatic ischemia/reperfusion (I/R) injury occurs in a variety of clinical scenarios, including transplantation, liver resection, trauma, and hypovolemic shock. The process of hepatic I/R-injury can be divided into two phases; an acute phase (the first 6 h after reperfusion) and the following sub-acute phase (Fan et al., 1999). The acute phase is characterized by generation of reactive oxygen species (ROS) subsequent to reoxygenation of the liver leading to marked hepatocellular damage (Parks and Granger, 1988; Rauen et al., 1994). Noteworthy, pretreatment of mice with XN (1,000 mg/kg body weight for 5 days) significantly ameliorated I/R-induced oxidative stress 6 h after reperfusion (Dorn et al., 2013). Although hepatocellular damage was not modulated at this early phase, the I/R-induced NF-κB activation and pro-inflammatory gene expression was almost completely blunted (Dorn et al., 2013). These factors play a crucial role in the later course of hepatic I/R-injury *via* recruitment and activation of pro-inflammatory cells (Jaeschke et al., 1992; Jaeschke, 1996). Also in an *ex vivo*-model of cold hepatic I/R XN revealed an antioxidant and inhibitory effect on NF-κB activity (Hartkorn et al., 2009).

The liver is also frequently exposed to various insults, including toxic chemicals (Zimmerman and Lewis, 1995; Grunhage et al., 2003). Liver damage caused by hepatotoxic chemicals induces liver necrosis due to direct damage of hepatocytes and subsequent inflammation (Mehendale et al., 1994). Carbon tetrachloride (CCl_4), an industrial solvent, is a hepatotoxic agent and its administration is widely used as an animal model of toxin-induced liver injury that allows the evaluation of both necrosis and subsequent inflammation (Huh et al., 2004) as well as fibrosis (Iredale, 2007). In this model, oral application of XN (500 mg/kg BW) was shown to significantly inhibit the pro-inflammatory and pro-fibrogenic hepatic gene expression (Dorn et al., 2012). Noteworthy, these effects occurred despite the fact that hepatocellular injury as reflected by serum levels of transaminases or histomorphological analysis was comparable between control mice and XN-fed mice. These findings suggest that the suppressive effect of XN against the progress of acute CCl_4-induced hepatic fibrosis involved direct mechanisms related to its ability to block both hepatic inflammation and the activation of HSC.

In summary, these findings indicate the potential of XN to exhibit a beneficial effect in acute liver injury or failure. Still, it has to be mentioned that in the studies cited above XN has be applied before the onset of liver injury, i.e., prevented acute liver injury in experimental models. Further studies are warranted to analyzed the therapeutic potential of XN, i.e., after the onset of liver injury. A further potential clinical application of XN might be the prevention of oxidative stress in conservation solutions during the organ transplantation process. However, also here, future studies are warranted.

Effect of XN in Models of Chronic Liver Injury

Chronic HCV infection is one of the most frequent liver diseases worldwide (Yang et al., 2013). In an elegant study, Yang et al. analyzed the hepatoprotective effect of XN in an *in vivo* model of HCV infected *Tupaias*. XN was applied by gavage at a dose of 100 mg/kg BW which led to a significant reduction of hepatic inflammation and fibrosis compared to control animals. Interestingly, XN also inhibited the hepatic steatosis in this model, which was found to be related to an inhibitory effect on microsomal triglyceride transfer protein activity and inhibition of hematopoietic stem cells (Yang et al., 2013).

Besides alcohol abuse, non-alcoholic fatty liver disease (NAFLD) has emerged as the most frequent liver disease in Western countries (Clark et al., 2002; Cobbold et al., 2010; Vernon et al., 2011). Today, the metabolic syndrome (MS) is one of the major public health challenges worldwide that is characterized by clustering of waist circumference, blood triglycerides, high density lipoprotein (HDL) cholesterol and fasting glucose levels. MS is also closely associated with NAFLD, and thus today, NAFLD is considered as a component of the MS (reviewed in Saidijam et al., 2014). MS affects approximately 25 per cent of the adult population in Western countries and also is quickly increasing in young populations. Accordingly, also NAFLD incidence is further increasing and effective strategies

TABLE 1 | Beneficial effects of Xanthohumol on liver injury.

Finding made in	Model	Biological activity	References
In vitro (cell culture model)	Hepatitis C virus replication in cell culture	XN reduced hepatitis C virus RNA levels	Lou et al., 2014
	Cultivated human hepatocytes and hepatic stellate cells	In both cell types XN inhibited activation of the transcription factor NF-κB and expression of NF-κB dependent proinflammatory genes	Dorn et al., 2010a
	Hepatocellular carcinoma cell lines (HepG2 and Huh7)	XN induced apoptosis and repressed proliferation and migration as well as TNF-induced NF-κB activity	Dorn et al., 2010b
	Comet assay in cultured human hepatoma cell line HepG2	XN prevents the formation of DNA strand breaks, indicating that its protective effect is mediated by induction of cellular defense mechanisms against oxidative stress	Plazar et al., 2007
In vitro (Precision cut liver slices)	Precision-cut rat liver slices	XN completely prevented 2-amino-3-methylimidazo[4,5-f]quinoline- and benzo(a)pyrene-induced DNA damage	Plazar et al., 2008
In vivo (mice)	Ischemia-reperfusion (I/R) induced liver injury in BALB/c mice	Orally applied XN (1 mg/g body weight for 5 days before I/R-injury) reduced liver injury, NF-κB activation, expression of proinflammatory cytokines	Rauen et al., 1994
	Carbon tetrachloride-induced acute liver injury in 10 weeks old female BALB/c mice	XN inhibited pro-inflammatory and profibrogenic hepatic gene expression and decreased hepatic NF-κB activity	Dorn et al., 2012
	Western-type diet-fed ApoE-deficient mice	XN reduced plasma cholesterol concentrations, decreased atherosclerotic lesion area, and attenuated plasma concentrations of the proinflammatory cytokine monocyte chemoattractant protein 1	Doddapattar et al., 2013
	Mouse model of Non-Alcoholic Steatosis	XN reduced hepatic inflammation and expression of profibrogenic genes	Dorn et al., 2010a
In vivo (Tupaia)	Hepatitis C virus infected *Tupaia belangeri*	XN improves hepatic inflammation, steatosis and fibrosis through inhibition of oxidative reaction and regulation of apoptosis and suppression of hepatic stellate cell activation	Yang et al., 2013
In vivo (rat)	High fat diet in rats	XN inhibited the increase of body weight, liver weight, and triacylglycerol levels	Yui et al., 2014
	Orally administered hop extract and subcutaneously injection of XN in Sprague-Dawley rats over 4 days	XN display cytoprotective effects in the liver	Dietz et al., 2013
	Carbon tetrachloride-induced acute liver injury in rats	XN evolves hepatoprotective effects by its antioxidant properties and inhibition of lipid peroxidation and degradation of antioxidant enzymes that are induced by CCl_4 intoxication	Pinto et al., 2012
	Metabolic syndrome in 4 week old Zucker fa/fa rats	XN has beneficial effects on markers of metabolic syndrome such as body weight and plasma glucose levels	Legette et al., 2013
	Amino-3-methyl-imidazo[4,5-*f*]quinoline-induced preneoplastic foci formation in rat livers	XN protects against DNA damage and cancer	Pinto et al., 2012
	IR-induced hepatic injury in rats	XN reduced reactive oxygen species formation and NF-κB activity *in vitro* and lipid peroxidation was attenuated, while Bcl-X expression and caspase-3 like activity was decreased	Hartkorn et al., 2009

Abbreviations used are: I/R, Ischemia-reperfusion; NF-κB, nuclear factor-κB; XN, Xanthohumol.

to prevent the development and progression of NAFLD to its advanced form NASH are urgently needed. Of note, XN has been shown to exhibit a beneficial effect in different experimental NAFLD models. Yui et al. reported that feeding rats a high-fat diet enriched with XN extract (1% w/w equivalent to a dose of 100 mg/kg BW) inhibited the increase of body and liver weight, as well as triacylglycerol levels in the plasma and in the liver (Yui et al., 2014). The mechanisms were found to be related to the regulation of the hepatic fatty acid metabolism and an inhibition of fat absorption in the intestine (Doddapattar et al., 2013). In this study, XN also tended to reduce hepatic fatty acid synthesis through the reduction of hepatic sterol regulatory element-binding protein (SREBP) 1c mRNA expression in rats fed with a high-fat diet. Furthermore, it was observed that plasma

adiponectin levels tended to be elevated by dietary application of the XN-rich hop extract (Yui et al., 2014). Also in a second model, in which NASH was induced by feeding the mice with an NASH-inducing diet, XN exhibited anti-inflammatory and anti-fibrogenic effects (Dorn et al., 2010a). Here, XN was applied in the diet in a concentration of 1% W/W corresponding to a dose of approximately 1000 mg/kg BW. In this model, after 3 weeks feeding, the induction of hepatic inflammation and pro-fibrogenic gene expression was almost completely blunted in mice receiving XN-supplemented NASH diet compared to mice fed with the pure NASH-diet (Dorn et al., 2010a).

Moreover, ApoE$^{-/-}$ mice showed decreased hepatic triglyceride and cholesterol content, activation of AMP-activated protein kinase, phosphorylation and inactivation of acetyl-CoA carboxylase, and reduced expression levels of mature SREBP-1c upon 8 weeks XN feeding (300 mg/kg BW/day) (Doddapattar et al., 2013).

In summary, XN has been shown to beneficially affect several components of the metabolic syndrome. Furthermore, it positively affects other obesity associated pathological factors such as misbalance of adipokine levels, which are known to promote NAFLD development and progression. Fitting to this is the beneficial effect of XN on hepatic steatosis, inflammation and fibrosis in experimental NASH models. In addition to NAFLD/NASH, XN revealed positive effects in other experimental models of chronic liver injury such as HCV, which can be explained by the above described inhibitory effects on fibrogenic and inflammatory gene expression or anti-bacterial effects. Still, it has to be noted that in the published studies, XN was applied in a preventive experimental setting. Future studies are warranted to analyze the potential of XN to treat, i.e., stop or reverse liver fibrosis.

Pharmacokinetics and Effective Dose of XN

Recent studies enhanced our knowledge regarding metabolisms and pharmacokinetics of XN (Nookandeh et al., 2004; Legette et al., 2012, 2014). Investigations using human liver microsomes showed that the hydroxylation of a prenyl methyl group is the primary route of the oxidative metabolism. Furthermore, XN and its metabolites were found to be excreted mainly in feces within 24 h of administration, when XN was fed to rats up to a dose of 500 mg/kg BW (Avula et al., 2004; Hanske et al., 2010). Twenty two metabolites were identified in the feces, most of them confined to modified chalcone structures and flavanone derivatives (Nookandeh et al., 2004). Still, most of the XN remained unchanged in the intestinal tract (89%), and only 11% were found to be metabolites (Nookandeh et al., 2004). Furthermore, two phase I metabolites and five phase II metabolites were identified in rats revealing oxidation, demethylation, hydration and sulfatation reactions (Jirasko et al., 2010). The bioavailability of XN in rats was found to be dose-dependent (0.33, 0.13, and 0.11) upon oral administration of single XN doses (1.86, 5.64, and 16.9 mg/kg BW) (Legette et al., 2012).

Despite the poor bioavailability, the highest XN concentrations are reached in intestinal cells and non-parenchymal liver cells upon oral uptake. Here, the anatomical situation of the liver has to be considered. Thus, it can be expected, that after oral intake of XN its concentration in the portal vein is higher than in the systemic circulation. Further, HSC are located in the liver in the space of Disse (perisinusoidal space), i.e., between the sinusoids and the hepatocytes. Herewith, HSC are directly exposed to XN concentration reaching the liver *via* the portal vein irrespective of the (subsequent) metabolism in hepatocytes. Anti-fibrogenic effects have been observed at concentrations as low as 5 μM (Dorn et al., 2010a), and in previous studies we found that these concentration levels are reached in the murine hepatic tissue upon oral administration of XN at dose of 1000 mg/kg BW (Dorn et al., 2013). Furthermore, it has to be considered that XN concentrations reaching HSC in the space between the endothelial cells and the hepatocytes (i.e., space of Disse) likely are significantly higher than the levels in whole liver tissue.

Still, the applied dose of 1000 mg/kg BW in this study (Dorn et al., 2013) was quite high and it has not yet been exactly defined what XN doses are required to achieve hepatoprotective effects. In the above described studies revealing beneficial effects in models of acute and chronic liver injury, XN was applied in the dose-range of approximately 100–1000 mg XN/kg BW to mice and rats. Effective doses with regards to beneficial effects on other components of the metabolic syndrome were achieved with doses in the range of 15–300 mg XN /kg BW in rats and mice (Doddapattar et al., 2013; Legette et al., 2013; Yang et al., 2013; Yui et al., 2014). In addition, there are several further studies in other experimental disease models, which revealed beneficial effects in doses as low as 0.2–9 mg XN/kg BW (Benelli et al., 2012; Negrao et al., 2012; Rudzitis-Auth et al., 2012; Yen et al., 2012; Costa et al., 2013). For example, Costa et al. have shown that XN modulates inflammation, oxidative stress, and angiogenesis in a type 1 diabetic rat skin wound healing model by supplementing a stout beer with 10 mg XN/L, which corresponds to approximately 1 mg XN/kg BW/day (Costa et al., 2013). Moreover, Benelli et al. reported that XN revealed beneficial effects in a murine model of leukemia in doses as low as 2 mg XN/kg BW (Benelli et al., 2012).

Potential Forms of XN Application

Elegant studies by Legette et al. (2012) demonstrated the similarity of XN metabolisms and pharmacokinetics between rodents and humans, which allows the translation of data generated in murine and rat models into clinical studies/application. To convert an animal dose (mg/kg BW) to the human equivalent dose (HED), the murine dose should be either divided by 12.3 or multiplied by 0.08. Oral dose ratio can be calculated using allometric interspecies scaling [1].

[1]Guidance for Industry, Estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers. This guidance can be downloaded at http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM078932.pdf (last downloaded March 20, 2015).

For humans, beer is the major dietary source of XN. The beer content of XN varies significantly depending on the type of beer (in the range of 0.052–0.628 mg/l) (Chen et al., 2010). Lager and Pilsener beers have fairly low levels of this compound, and the highest levels of XN are found in Stout or Porte (Stevens et al., 1999). Moreover, it was possible to produce a dark beer enriched in XN (3.5 mg/l) (Magalhães et al., 2008), and a brewing process has been developed that produces a beer that contains 10 times the amount of XN as traditional brews (Wunderlich et al., 2005). Even upon consumption of such special types of beer, a daily beer consumption of approximately 150–1500 l would be necessary by a man (75 kg) to reach doses corresponding to 100–1000 mg XN/kg BW/day in mice, i.e., doses which have been shown to exhibit hepatoprotective effects. Thus, with regards to hepatoprotective effects, pharmacologically relevant XN concentrations cannot be reached in men by beer consumption. Moreover, there is certainly unanimous hesitancy among researchers to recommend drinking alcohol to avoid any kind of disease because of the fine line between moderate and binge drinking. Certainly, this is even more true in case of chronic liver disease.

However, XN can also be isolated from hops in large quantities, and different methods (e.g., extraction via liquid supercritical carbon dioxide) were developed to isolate XN from hop cones in large quantities. Thus, independent of beer intake, XN may be used as hepatoprotective dietary supplement. Therefore, pharmacological relevant concentrations can be reached by oral administration of XN enriched functional food, e.g., XN enriched beverages or solid foods. Methods or formulations for increasing the water solubility and bioavailability are presently object of numerous national or global patents[2]).

Safety of XN Application

One prerequisite for therapeutic application is the good safety profile of the used agent. Especially hepatotoxic properties have to be excluded, when the therapeutic agent must be taken by patients with liver disease. Hop has a long history as a medicinal plant and is known for its good tolerance. More recently, we and others have confirmed the safety of oral application of XN and XN-enriched hop extracts in rats and mice. Oral administration of XN (700 mg/kg/day BW) to mice for 4 weeks did neither affect the major organ functions, nor the protein, lipid, or carbohydrate metabolism (Vanhoecke et al., 2005). Similarly, mice fed with a XN enriched diet (1000 mg/kg BW) for 3 weeks exhibited no adverse effects (Dorn et al., 2010a). Histopathological evaluation of major organs (liver, kidney, colon, lung, heart, spleen, and thymus) as well as biochemical serum analysis, confirmed that XN did not negatively affect organ function and homoeostasis (Dorn et al., 2010c). Most recently, first in man studies confirmed the safety and good tolerability of XN or XN-enriched hop extracts, respectively. An escalating dose study (up to 1.35 mg XN/kg BW/day for 1 week) in menopausal women revealed that the XN enriched extract did neither affect the serum levels of sex hormones nor blood clotting. Also no other side effects were observed (van Breemen et al., 2014). A further study confirmed the safety of oral administration of a single XN dose (160 mg, i.e., approximately 2.5 mg XN/kg BW/day) in healthy volunteers (Legette et al., 2014).

Summary and Conclusion

In vitro and *in vivo* data indicate that the hop constituent xanthohumol (XN) affects several critical pathophysiological steps during the development and course of chronic liver disease,

[2]International application published under the patent cooperation treaty (PCT). A representative example can be downloaded at http://www.lens.org/images/patent/WO/2007016578/A2/WO_2007_016578_A2.pdf (last downloaded March 20, 2015).

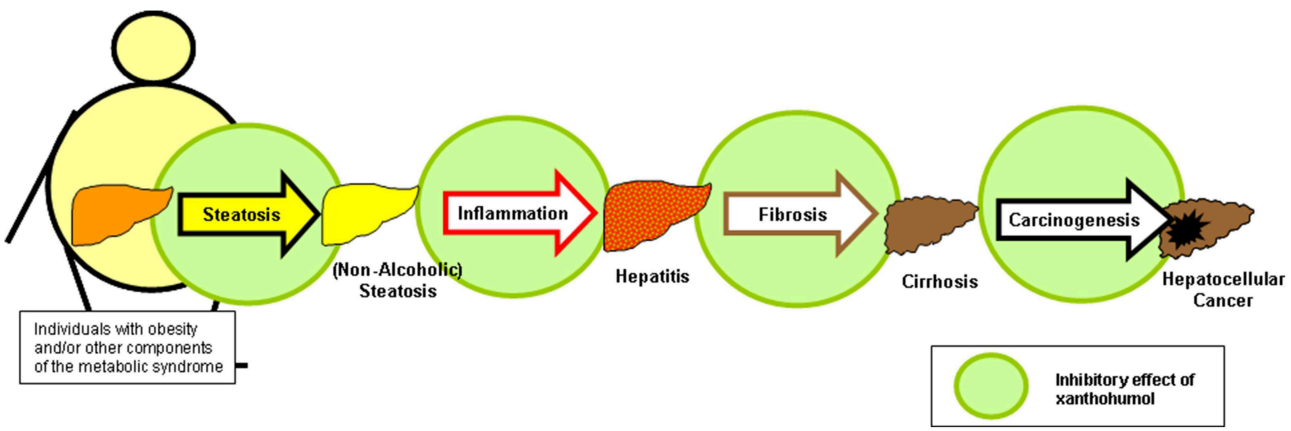

FIGURE 4 | Xanthohumol effects on different pathophysiological factors during the course of non-alcoholic fatty liver disease. Xanthohumol (XN) has the potential to inhibit hepatic steatosis, inflammation, fibrosis and even the causes of liver injury by interaction on different levels, i.e., pathogenesis of steatosis (Dorn et al., 2010a; Doddapattar et al., 2013; Yang et al., 2013; Yui et al., 2014), inflammation (Dorn et al., 2010a, 2013; Yang et al., 2013), fibrosis (Dorn et al., 2010a, 2013; Yang et al., 2013), and cancerogenesis (Gerhauser et al., 2002; Dorn et al., 2010b).

including hepatic inflammation and fibrosis, as well as the formation and progression of liver cancer (**Figure 4**). Also on the molecular level, XN ameliorates several mechanisms which play a critical role in the pathogenesis of acute and chronic liver injury. Strikingly, inhibitory XN effects on activated hepatic stellate cells (HSC)/myofibrobasts take place in a concentration range, which is significantly lower than the concentration which is shown to be safe for primary human hepatocytes *in vitro*. Furthermore, upon oral application, HSC are exposed to relatively high XN concentrations due to their location in the space of Disse irrespective of the hepatic metabolisms. This indicates these non-parenchymal liver cells as attractive targets for therapeutic XN application. Of note, XN also holds promise as a therapeutic agent for treating obesity, dysregulation of glucose metabolism and other components of the metabolic syndrome including hepatic steatosis. Thus, therapeutic XN application appears as promising strategy, particularly in obese patients, to inhibit the development as well as the progression of NAFLD.

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References

Albini, A., Dell'Eva, R., Vene, R., Ferrari, N., Buhler, D. R., Noonan, D. M., et al. (2006). Mechanisms of the antiangiogenic activity by the hop flavonoid xanthohumol: NF-kappaB and Akt as targets. *FASEB J.* 20, 527–529.

Amann, T., Bataille, F., Spruss, T., Muhlbauer, M., Gabele, E., Scholmerich, J., et al. (2009). Activated hepatic stellate cells promote tumorigenicity of hepatocellular carcinoma. *Cancer Sci.* 100, 646–653. doi: 10.1111/j.1349-7006.2009.01087.x

Araujo, J. R., Goncalves, P., and Martel, F. (2011). Chemopreventive effect of dietary polyphenols in colorectal cancer cell lines. *Nutr. Res.* 31, 77–87. doi: 10.1016/j.nutres.2011.01.006

Avula, B., Ganzera, M., Warnick, J. E., Feltenstein, M. W., Sufka, K. J., and Khan, I. A. (2004). High-performance liquid chromatographic determination of xanthohumol in rat plasma, urine, and fecal samples. *J. Chromatogr. Sci.* 42, 378–382. doi: 10.1093/chromsci/42.7.378

Bataller, R., and Brenner, D. A. (2001). Hepatic stellate cells as a target for the treatment of liver fibrosis. *Semin. Liver Dis.* 21, 437–451. doi: 10.1055/s-2001-17558

Benelli, R., Vene, R., Ciarlo, M., Carlone, S., Barbieri, O., and Ferrari, N. (2012). The AKT/NF-kappaB inhibitor xanthohumol is a potent anti-lymphocytic leukemia drug overcoming chemoresistance and cell infiltration. *Biochem. Pharmacol.* 83, 1634–1642. doi: 10.1016/j.bcp.2012.03.006

Buckwold, V. E., Wilson, R. J., Nalca, A., Beer, B. B., Voss, T. G., Turpin, J. A., et al. (2004). Antiviral activity of hop constituents against a series of DNA and RNA viruses. *Antiviral Res.* 61, 57–62. doi: 10.1016/S0166-3542(03)00155-4

Chen, L., Zhao, Q., Jin, H., Zhang, X., Xu, Y., Yu, A., et al. (2010). Determination of xanthohumol in beer based on cloud point extraction coupled with high performance liquid chromatography. *Talanta* 81, 692–697. doi: 10.1016/j.talanta.2010.01.004

Clark, J. M., Brancati, F. L., and Diehl, A. M. (2002). Nonalcoholic fatty liver disease. *Gastroenterology* 122, 1649–1657. doi: 10.1053/gast.2002.33573

Cobbold, J. F., Anstee, Q. M., and Taylor-Robinson, S. D. (2010). The importance of fatty liver disease in clinical practice. *Proc. Nutr. Soc.* 69, 518–527. doi: 10.1017/S0029665110001916

Colgate, E. C., Miranda, C. L., Stevens, J. F., Bray, T. M., and Ho, E. (2007). Xanthohumol, a prenylflavonoid derived from hops induces apoptosis and inhibits NF-kappaB activation in prostate epithelial cells. *Cancer Lett.* 246, 201–209. doi: 10.1016/j.canlet.2006.02.015

Costa, R., Negrao, R., Valente, I., Castela, A., Duarte, D., Guardao, L., et al. (2013). Xanthohumol modulates inflammation, oxidative stress, and angiogenesis in type 1 diabetic rat skin wound healing. *J. Nat. Prod.* 76, 2047–2053. doi: 10.1021/np4002898

Cuendet, M., Oteham, C. P., Moon, R. C., and Pezzuto, J. M. (2006). Quinone reductase induction as a biomarker for cancer chemoprevention. *J. Nat. Prod.* 69, 460–463. doi: 10.1021/np050362q

Dietz, B. M., Hagos, G. K., Eskra, J. N., Wijewickrama, G. T., Anderson, J. R., Nikolic, D., et al. (2013). Differential regulation of detoxification enzymes in hepatic and mammary tissue by hops (*Humulus lupulus*) *in vitro* and *in vivo*. *Mol. Nutr. Food Res.* 57, 1055–1066. doi: 10.1002/mnfr.201200534

Doddapattar, P., Radovic, B., Patankar, J. V., Obrowsky, S., Jandl, K., Nusshold, C., et al. (2013). Xanthohumol ameliorates atherosclerotic plaque formation, hypercholesterolemia, and hepatic steatosis in ApoE-deficient mice. *Mol. Nutr. Food Res.* 57, 1718–1728. doi: 10.1002/mnfr.201200794

Dorn, C., Bataille, F., Gaebele, E., Heilmann, J., and Hellerbrand, C. (2010c). Xanthohumol feeding does not impair organ function and homoeostasis in mice. *Food Chem. Toxicol.* 48, 1890–1897. doi: 10.1016/j.fct.2010.04.030

Dorn, C., Heilmann, J., and Hellerbrand, C. (2012). Protective effect of xanthohumol on toxin-induced liver inflammation and fibrosis. *Int. J. Clin. Exp. Pathol.* 5, 29–36. doi: 10.1055/s-0031-1295738

Dorn, C., Kraus, B., Motyl, M., Weiss, T. S., Gehrig, M., Scholmerich, J., et al. (2010a). Xanthohumol, a chalcon derived from hops, inhibits hepatic inflammation and fibrosis. *Mol. Nutr. Food Res.* 54(Suppl. 2), S205–S213. doi: 10.1002/mnfr.200900314

Dorn, C., Massinger, S., Wuzik, A., Heilmann, J., and Hellerbrand, C. (2013). Xanthohumol suppresses inflammatory response to warm ischemia-reperfusion induced liver injury. *Exp. Mol. Pathol.* 94, 10–16. doi: 10.1016/j.yexmp.2012.05.003

Dorn, C., Weiss, T. S., Heilmann, J., and Hellerbrand, C. (2010b). Xanthohumol, a prenylated chalcone derived from hops, inhibits proliferation, migration and interleukin-8 expression of hepatocellular carcinoma cells. *Int. J. Oncol.* 36, 435–441. doi: 10.1055/s-0029-1246396

Douhara, A., Moriya, K., Yoshiji, H., Noguchi, R., Namisaki, T., Kitade, M., et al. (2015). Reduction of endotoxin attenuates liver fibrosis through suppression of hepatic stellate cell activation and remission of intestinal permeability in a rat non-alcoholic steatohepatitis model. *Mol. Med. Rep.* 11, 1693–1700. doi: 10.3892/mmr.2014.2995

Elpek, G. O. (2014). Cellular and molecular mechanisms in the pathogenesis of liver fibrosis: an update. *World J. Gastroenterol.* 20, 7260–7276. doi: 10.3748/wjg.v20.i23.7260

Elsharkawy, A. M., Oakley, F., and Mann, D. A. (2005). The role and regulation of hepatic stellate cell apoptosis in reversal of liver fibrosis. *Apoptosis* 10, 927–939. doi: 10.1007/s10495-005-1055-4

Fan, C., Zwacka, R. M., and Engelhardt, J. F. (1999). Therapeutic approaches for ischemia/reperfusion injury in the liver. *J. Mol. Med.* 77, 577–592. doi: 10.1007/s001099900029

Ferk, F., Huber, W. W., Filipic, M., Bichler, J., Haslinger, E., Misík, M., et al. (2010). Xanthohumol, a prenylated flavonoid contained in beer, prevents the induction of preneoplastic lesions and DNA damage in liver and colon induced by the heterocyclic aromatic amine amino-3-methyl-imidazo[4,5-f]quinoline (IQ). *Mutat. Res.* 691, 17–22. doi: 10.1016/j.mrfmmm.2010.06.006

Gerhauser, C. (2005). Broad spectrum anti-infective potential of xanthohumol from hop (*Humulus lupulus* L.) in comparison with activities of other hop constituents and xanthohumol metabolites. *Mol. Nutr. Food Res.* 49, 827–831. doi: 10.1002/mnfr.200500091

Gerhauser, C., Alt, A., Heiss, E., Gamal-Eldeen, A., Klimo, K., Knauft, J., et al. (2002). Cancer chemopreventive activity of Xanthohumol, a natural product derived from hop. *Mol. Cancer Ther.* 1, 959–969.

Gerhauser, C., and Frank, N. (2005). Xanthohumol, a new all-rounder? *Mol. Nutr. Food Res.* 49, 821–823. doi: 10.1002/mnfr.200590033

Gines, P., Cardenas, A., Arroyo, V., and Rodes, J. (2004). Management of cirrhosis and ascites. *N. Engl. J. Med.* 350, 1646–1654. doi: 10.1056/NEJMra035021

Grunhage, F., Fischer, H. P., Sauerbruch, T., and Reichel, C. (2003). Drug- and toxin-induced hepatotoxicity. *Z. Gastroenterol.* 41, 565–578.

Hanske, L., Loh, G., Sczesny, S., Blaut, M., and Braune, A. (2010). Recovery and metabolism of xanthohumol in germ-free and human microbiota-associated rats. *Mol. Nutr. Food Res.* 54, 1405–1413. doi: 10.1002/mnfr.200900517

Hartkorn, A., Hoffmann, F., Ajamieh, H., Vogel, S., Heilmann, J., Gerbes, A. L., et al. (2009). Antioxidant effects of xanthohumol and functional impact on hepatic ischemia-reperfusion injury. *J. Nat. Prod.* 72, 1741–1747. doi: 10.1021/np900230p

Hellerbrand, C., Jobin, C., Iimuro, Y., Licato, L., Sartor, R. B., and Brenner, D. A. (1998a). Inhibition of NFkappaB in activated rat hepatic stellate cells by proteasome inhibitors and an IkappaB super-repressor. *Hepatology* 27, 1285–1295. doi: 10.1002/hep.510270514

Hellerbrand, C., Jobin, C., Licato, L. L., Sartor, R. B., and Brenner, D. A. (1998b). Cytokines induce NF-kappaB in activated but not in quiescent rat hepatic stellate cells. *Am. J. Physiol.* 275(2 Pt 1), G269–G278.

Henderson, M. C., Miranda, C. L., Stevens, J. F., Deinzer, M. L., and Buhler, D. R. (2000). *In vitro* inhibition of human P450 enzymes by prenylated flavonoids from hops, *Humulus lupulus*. *Xenobiotica* 30, 235–251. doi: 10.1080/004982500237631

Huh, C. G., Factor, V. M., Sanchez, A., Uchida, K., Conner, E. A., and Thorgeirsson, SS. (2004). Hepatocyte growth factor/c-met signaling pathway is required for efficient liver regeneration and repair. *Proc. Natl. Acad. Sci. U.S.A.* 101, 4477–4482. doi: 10.1073/pnas.0306068101

Iredale, J. P. (2007). Models of liver fibrosis: exploring the dynamic nature of inflammation and repair in a solid organ. *J. Clin. Invest.* 117, 539–548. doi: 10.1172/JCI30542

Jaeschke, H. (1996). Chemokines, neutrophils, and inflammatory liver injury. *Shock* 6, 403–404. doi: 10.1097/00024382-199612000-00003

Jaeschke, H., Schini, V. B., and Farhood, A. (1992). Role of nitric oxide in the oxidant stress during ischemia/reperfusion injury of the liver. *Life Sci.* 50, 1797–1804. doi: 10.1016/0024-3205(92)90064-V

Jarrar, M. H., Baranova, A., Collantes, R., Ranard, B., Stepanova, M., Bennett, C., et al. (2008). Adipokines and cytokines in non-alcoholic fatty liver disease. *Aliment. Pharmacol. Ther.* 27, 412–421. doi: 10.1111/j.1365-2036.2007.03586.x

Jirasko, R., Holcapek, M., Vrublova, E., Ulrichova, J., and Simanek, V. (2010). Identification of new phase II metabolites of xanthohumol in rat *in vivo* biotransformation of hop extracts using high-performance liquid chromatography electrospray ionization tandem mass spectrometry. *J. Chromatogr. A* 1217, 4100–4108. doi: 10.1016/j.chroma.2010.02.041

Jongthawin, J., Techasen, A., Loilome, W., Yongvanit, P., and Namwat, N. (2012). Anti-inflammatory agents suppress the prostaglandin E2 production and migration ability of cholangiocarcinoma cell lines. *Asian Pac. J. Cancer Prev.* 13(Suppl.), 47–51.

Kisseleva, T., and Brenner, D. A. (2007). Role of hepatic stellate cells in fibrogenesis and the reversal of fibrosis. *J. Gastroenterol. Hepatol.* 22(Suppl. 1), S73–S78. doi: 10.1111/j.1440-1746.2006.04658.x

Krajka-Kuzniak, V., Paluszczak, J., and Baer-Dubowska, W. (2013). Xanthohumol induces phase II enzymes *via* Nrf2 in human hepatocytes *in vitro*. *Toxicol. In Vitro*. 27, 149–156. doi: 10.1016/j.tiv.2012.10.008

Kramer, B., Thielmann, J., Hickisch, A., Muranyi, P., Wunderlich, J., and Hauser, C. (2015). Antimicrobial activity of hop extracts against foodborne pathogens for meat applications. *J. Appl. Microbiol.* 118, 648–657. doi: 10.1111/jam.12717

Lang, A., and Brenner, D. A. (1999). Gene regulation in hepatic stellate cell. *Ital. J. Gastroenterol. Hepatol.* 31, 173–179.

Legette, L., Karnpracha, C., Reed, R. L., Choi, J., Bobe, G., Christensen, J. M., et al. (2014). Human pharmacokinetics of xanthohumol, an antihyperglycemic flavonoid from hops. *Mol. Nutr. Food Res.* 58, 248–255. doi: 10.1002/mnfr.201300333

Legette, L., Ma, L., Reed, R. L., Miranda, C. L., Christensen, J. M., Rodriguez-Proteau, R., et al. (2012). Pharmacokinetics of xanthohumol and metabolites in rats after oral and intravenous administration. *Mol. Nutr. Food Res.* 56, 466–474. doi: 10.1002/mnfr.201100554

Legette, L. L., Luna, A. Y., Reed, R. L., Miranda, C. L., Bobe, G., Proteau, R. R., et al. (2013). Xanthohumol lowers body weight and fasting plasma glucose in obese male Zucker fa/fa rats. *Phytochemistry* 91, 236–241. doi: 10.1016/j.phytochem.2012.04.018

Lou, S., Zheng, Y. M., Liu, S. L., Qiu, J., Han, Q., Li, N., et al. (2014). Inhibition of hepatitis C virus replication *in vitro* by xanthohumol, a natural product present in hops. *Planta Med.* 80, 171–176.

Magalhães, P. J., Dostalek, P., Cruz, J. M., Guido Ls, F, and Barros, A. A. (2008). The impact of a xanthohumol-enriched hop product on the behavior of xanthohumol and isoxanthohumol in pale and dark beers: a pilot scale approach. *J. Inst. Brewing* 114, 246–256. doi: 10.1002/j.2050-0416.2008.tb00335.x

Mehendale, H. M., Roth, R. A., Gandolfi, A. J., Klaunig, J. E., Lemasters, J. J., and Curtis, LR. (1994). Novel mechanisms in chemically induced hepatotoxicity. *FASEB J.* 8, 1285–1295.

Minguez, B., Tovar, V., Chiang, D., Villanueva, A., and Llovet, J. M. (2009). Pathogenesis of hepatocellular carcinoma and molecular therapies. *Curr. Opin. Gastroenterol.* 25, 186–194. doi: 10.1097/MOG.0b013e32832962a1

Miranda, C. L., Aponso, G. L., Stevens, J. F., Deinzer, M. L., and Buhler, D. R. (2000a). Prenylated chalcones and flavanones as inducers of quinone reductase in mouse Hepa 1c1c7 cells. *Cancer Lett.* 149, 21–29. doi: 10.1016/S0304-3835(99)00328-6

Miranda, C. L., Stevens, J. F., Ivanov, V., McCall, M., Frei, B., Deinzer, M. L., et al. (2000b). Antioxidant and prooxidant actions of prenylated and nonprenylated chalcones and flavanones *in vitro*. *J. Agric. Food Chem.* 48, 3876–3884. doi: 10.1021/jf0002995

Miranda, C. L., Yang, Y. H., Henderson, M. C., Stevens, J. F., Santana-Rios, G., Deinzer, M. L., et al. (2000c). Prenylflavonoids from hops inhibit the metabolic activation of the carcinogenic heterocyclic amine 2-amino-3-methylimidazo[4,5-f]quinoline, mediated by cDNA-expressed human CYP1A2. *Drug Metab. Dispos.* 28, 1297–1302.

Monteiro, R., Becker, H., Azevedo, I., and Calhau, C. (2006). Effect of hop (*Humulus lupulus* L.) flavonoids on aromatase (estrogen synthase) activity. *J. Agric. Food Chem.* 54, 2938–2943. doi: 10.1021/jf053162t

Motyl, M., Kraus, B., and Heilmann, J. (2012). Pitfalls in cell culture work with xanthohumol. *Pharmazie* 67, 91–04.

Negrao, R., Costa, R., Duarte, D., Gomes, T. T., Coelho, P., Guimaraes, J. T., et al. (2012). Xanthohumol-supplemented beer modulates angiogenesis and inflammation in a skin wound healing model. Involvement of local adipocytes. *J. Cell. Biochem.* 113, 100–109. doi: 10.1002/jcb.23332

Nookandeh, A., Frank, N., Steiner, F., Ellinger, R., Schneider, B., Gerhauser, C., et al. (2004). Xanthohumol metabolites in faeces of rats. *Phytochemistry* 65, 561–570. doi: 10.1016/j.phytochem.2003.11.016

Pan, L., Becker, H., and Gerhauser, C. (2005). Xanthohumol induces apoptosis in cultured 40-16 human colon cancer cells by activation of the death receptor- and mitochondrial pathway. *Mol. Nutr. Food Res.* 49, 837–843. doi: 10.1002/mnfr.200500065

Parks, D. A., and Granger, D. N. (1988). Ischemia-reperfusion injury: a radical view. *Hepatology* 8, 680–682. doi: 10.1002/hep.1840080341

Pinto, C., Duque, A. L., Rodríguez-Galdón, B., Cestero, J. J., Macías, P. (2012). Xanthohumol prevents carbon tetrachloride-induced acute liver injury in rats. *Food Chem. Toxicol.* 50, 3405–3412. doi: 10.1016/j.fct.2012.07.035

Plazar, J., Filipic, M., and Groothuis, G. M. (2008). Antigenotoxic effect of Xanthohumol in rat liver slices. *Toxicol. In Vitro* 22, 318–327. doi: 10.1016/j.tiv.2007.09.009

Plazar, J., Zegura, B., Lah, T. T., and Filipic, M. (2007). Protective effects of xanthohumol against the genotoxicity of benzo(a)pyrene (BaP), 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) and tert-butyl hydroperoxide (t-BOOH) in HepG2 human hepatoma cells. *Mutat. Res.* 632, 1–8. doi: 10.1016/j.mrgentox.2007.03.013

Possemiers, S., Heyerick, A., Robbens, V., De Keukeleire, D., and Verstraete, W. (2005). Activation of proestrogens from hops (*Humulus lupulus* L.) by intestinal microbiota; conversion of isoxanthohumol into 8-prenylnaringenin. *J. Agric. Food Chem.* 53, 6281–6288. doi: 10.1021/jf0509714

Rauen, U., Viebahn, R., Lauchart, W., and de Groot, H. (1994). The potential role of reactive oxygen species in liver ischemia/reperfusion injury following liver surgery. *Hepatogastroenterology* 41, 333–336.

Rozalski, M., Micota, B., Sadowska, B., Stochmal, A., Jedrejek, D., Wieckowska-Szakiel, M., et al. (2013). Antiadherent and antibiofilm activity of *Humulus lupulus* L. derived products: new pharmacological properties. *Biomed. Res. Int.* 2013:101089. doi: 10.1155/2013/101089

Rudzitis-Auth, J., Korbel, C., Scheuer, C., Menger, M. D., and Laschke, M. W. (2012). Xanthohumol inhibits growth and vascularization of developing endometriotic lesions. *Hum. Reprod.* 27, 1735–1744. doi: 10.1093/humrep/des095

Saidijam, M., Tootoonchi, A. S., Goodarzi, M. T., Hassanzadeh, T., Borzuei, S. H., Yadegarazari, R., et al. (2014). Expression of interleukins 7 & 8 in peripheral blood mononuclear cells from patients with metabolic syndrome: a preliminary study. *Indian J. Med. Res.* 140, 238–243.

Seki, E., De, M. S., Osterreicher, C. H., Kluwe, J., Osawa, Y., Brenner, D. A., et al. (2007). TLR4 enhances TGF-beta signaling and hepatic fibrosis. *Nat. Med.* 13, 1324–1332. doi: 10.1038/nm1663

Shamoto, T., Matsuo, Y., Shibata, T., Tsuboi, K., Takahashi, H., Funahashi, H., et al. (2013). Xanthohumol inhibits angiogenesis through VEGF and IL-8 in pancreatic cancer. *Pancreatology* 13, S52–S53. doi: 10.1016/j.pan.2013.07.203

Stevens, J. F., and Page, J. E. (2004). Xanthohumol and related prenylflavonoids from hops and beer: to your good health! *Phytochemistry* 65, 1317–1330. doi: 10.1016/j.phytochem.2004.04.025

Stevens, J. F., Taylor, A. W., and Deinzer, M. L. (1999). Quantitative analysis of xanthohumol and related prenylflavonoids in hops and beer by liquid chromatography-tandem mass spectrometry. *J. Chromatogr. A* 832, 97–107. doi: 10.1016/S0021-9673(98)01001-2

van Breemen, R. B., Yuan, Y., Banuvar, S., Shulman, L. P., Qiu, X., Alvarenga, R. F., et al. (2014). Pharmacokinetics of prenylated hop phenols in women following oral administration of a standardized extract of hops. *Mol. Nutr. Food Res.* 58, 1962–1969. doi: 10.1002/mnfr.201400245

Vanhoecke, B. W., Delporte, F., Van, B. E., Heyerick, A., Depypere, H. T., Nuytinck, M., et al. (2005). A safety study of oral tangeretin and xanthohumol administration to laboratory mice. *In Vivo* 19, 103–107.

Vernon, G., Baranova, A., and Younossi, Z. M. (2011). Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. *Aliment. Pharmacol. Ther.* 34, 274–285. doi: 10.1111/j.1365-2036.2011.04724.x

Viegas, O., Zegura, B., Pezdric, M., Novak, M., Ferreira, I. M., Pinho, O., et al. (2012). Protective effects of xanthohumol against the genotoxicity of heterocyclic aromatic amines MeIQx and PhIP in bacteria and in human hepatoma (HepG2) cells. *Food Chem. Toxicol.* 50, 949–955. doi: 10.1016/j.fct.2011.11.031

Villanueva, A., Newell, P., Chiang, D. Y., Friedman, S. L., and Llovet, J. M. (2007). Genomics and signaling pathways in hepatocellular carcinoma. *Semin. Liver Dis.* 27, 55–76. doi: 10.1055/s-2006-960171

Wouters, K., van Gorp, P. J., Bieghs, V., Gijbels, M. J., Duimel, H., Lutjohann, D., et al. (2008). Dietary cholesterol, rather than liver steatosis, leads to hepatic inflammation in hyperlipidemic mouse models of nonalcoholic steatohepatitis. *Hepatology* 48, 474–486. doi: 10.1002/hep.22363

Wunderlich, S., Zurcher, A., and Back, W. (2005). Enrichment of xanthohumol in the brewing process. *Mol. Nutr. Food Res.* 49, 874–881. doi: 10.1002/mnfr.200500051

Yang, M., Li, N., Li, F., Zhu, Q., Liu, X., Han, Q., et al. (2013). Xanthohumol, a main prenylated chalcone from hops, reduces liver damage and modulates oxidative reaction and apoptosis in hepatitis C virus infected Tupaia belangeri. *Int. Immunopharmacol.* 16, 466–474. doi: 10.1016/j.intimp.2013.04.029

Yen, T. L., Hsu, C. K., Lu, W. J., Hsieh, C. Y., Hsiao, G., Chou, D. S., et al. (2012). Neuroprotective effects of xanthohumol, a prenylated flavonoid from hops (*Humulus lupulus*), in ischemic stroke of rats. *J. Agric. Food Chem.* 60, 1937–1944. doi: 10.1021/jf204909p

Yoshimaru, T., Komatsu, M., Tashiro, E., Imoto, M., Osada, H., Miyoshi, Y., et al. (2014). Xanthohumol suppresses oestrogen-signalling in breast cancer through the inhibition of BIG3-PHB2 interactions. *Sci. Rep.* 4, 7355. doi: 10.1038/srep07355

Yui, K., Kiyofuji, A., and Osada, K. (2014). Effects of xanthohumol-rich extract from the hop on fatty acid metabolism in rats fed a high-fat diet. *J. Oleo Sci.* 63, 159–168. doi: 10.5650/jos.ess13136

Zanoli, P., and Zavatti, M. (2008). Pharmacognostic and pharmacological profile of *Humulus lupulus* L. *J. Ethnopharmacol.* 116, 383–396. doi: 10.1016/j.jep.2008.01.011

Zhang, N., Liu, Z., Han, Q., Chen, J., Lou, S., Qiu, J., et al. (2009). Inhibition of bovine viral diarrhea virus *in vitro* by xanthohumol: comparisons with ribavirin and interferon-alpha and implications for the development of anti-hepatitis C virus agents. *Eur. J. Pharm. Sci.* 38, 332–340. doi: 10.1016/j.ejps.2009.08.005

Zhang, N., Liu, Z., Han, Q., Chen, J., and Lv, Y. (2010). Xanthohumol enhances antiviral effect of interferon alpha-2b against bovine viral diarrhea virus, a surrogate of hepatitis C virus. *Phytomedicine* 17, 310–316. doi: 10.1016/j.phymed.2009.08.005

Zimmerman, H. J., and Lewis, J. H. (1995). Chemical- and toxin-induced hepatotoxicity. *Gastroenterol. Clin. North Am.* 24, 1027–1045.

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