cmgh

CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

REVIEW

Role of Hypoxia-Inducible Factors in the Development of Liver Fibrosis

Katherine J. Roth and Bryan L. Copple

Department of Pharmacology and Toxicology, the Institute for Environmental Toxicology, and the Program in Cellular and Molecular Biology, Michigan State University, East Lansing, Michigan

SUMMARY

Hypoxia-inducible factors (HIFs) play a critical role in the development of liver fibrosis. We summarize the important functions of HIFs in liver fibrosis and focus on the cell-specific role of these transcription factors in disease development.

Liver fibrosis remains a significant clinical problem in the United States and throughout the world. Although important advances in the understanding of this disease have been made, no effective pharmacologic agents have been developed that directly prevent or reverse the fibrotic process. Many of the successes in liver fibrosis treatment have been targeted toward treating the cause of fibrosis, such as the development of new antivirals that eradicate hepatitis virus. For many patients, however, this is not feasible, so a liver transplant remains the only viable option. Thus, there is a critical need to identify new therapeutic targets that will slow or reverse the progression of fibrosis in such patients. Research over the last 16 years has identified hypoxia-inducible factors (HIFs) as key transcription factors that drive many aspects of liver fibrosis, making them potential targets of therapy. In this review, we discuss the latest work on HIFs and liver fibrosis, including the cell-specific functions of these transcription factors in the development of liver fibrosis. *(Cell Mol Gastroenterol Hepatol 2015;1:589–597; http://dx.doi.org/10.1016/j.jcmgh.2015.09.005)*

Keywords: Hypoxia-Inducible Factors; Liver Fibrosis; Kupffer Cells; Hepatic Stellate Cells.

Liver fibrosis has many causes, including alcohol, drugs, viruses, and genetic disorders, among others. All these agents produce hepatocellular injury to some extent, which initiates a reparative process. Part of this process involves differentiation of hepatic stellate cells (HSCs) into myofibroblasts, a process called "activation."[1,2] In instances of biliary injury, in addition to HSCs, portal fibroblasts become activated.[3,4] Once this occurs, these cells begin to proliferate, migrate, and synthesize collagen, which provides the initial matrix for repair. After an acute hepatic insult, the excess collagen is removed, and the myofibroblasts revert back to a quiescent phenotype. If liver injury persists, however, collagen continues to become deposited, resulting in fibrosis and ultimately cirrhosis that may lead to liver failure or cancer. Many of the mediators that regulate HSC activation have been identified (for a comprehensive review, see Friedman[2]), but the mechanisms that regulate production of these mediators during acute and chronic injury are not fully understood. Recent studies, however, have demonstrated that a group of transcription factors called hypoxia-inducible factors (HIFs) may be critical for this process.[5]

To adapt to varying levels of oxygen in the environment, organisms have developed oxygen-sensing systems that trigger adaptive transcriptional responses to maintain homeostatic conditions. These systems use HIF transcription factors, which are heterodimeric transcription factor complexes composed of α and β subunits.[6,7] During hypoxia, HIF transcriptional activity leads to enhanced expression of various genes involved in cellular functions aimed at maintaining homeostasis, such as metabolism, proliferation, and migration. There are three known HIF transcription factors: HIF-1α, HIF-2α, and HIF-3α.[8–10] Of these three, HIF-1α and HIF-2α are the best characterized. These α subunits heterodimerize with HIF-1β, also called the aryl hydrocarbon nuclear transporter, before they are able to regulate gene expression.[6,7] HIF-1β is constitutively expressed and present in excess, whereas the α subunit is regulated in an oxygen-dependent manner.

Under normoxic conditions, the HIF α subunit is immediately targeted for degradation by the 26S proteasome.[11] In hypoxic conditions, however, the mechanisms that target the HIF α subunit for degradation are inhibited, allowing the HIF α unit to become stabilized and translocate to the nucleus. Once it enters the nucleus, it dimerizes with HIF-1β, forming a HIF complex that binds to specific hypoxia-responsive elements in target genes.[7,8]

Abbreviations used in this paper: **BDL, bile duct ligation; CCl_4, carbon tetrachloride; Ccr, C-C chemokine receptor; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; HIFs, hypoxia-inducible factors; HSC, hepatic stellate cell; Jmjd, Jumonji domain-containing; PAI-1, plasminogen activator inhibitor-1; PDGF, platelet-derived growth factor; Rgs, regulator of G-protein signaling; α-SMA, α-smooth muscle actin; TGF-β, transforming growth factor β; VEGF, vascular endothelial growth factor.**

Most current article

2352-345X

http://dx.doi.org/10.1016/j.jcmgh.2015.09.005

Hypoxia and Hypoxia-Inducible Factor-1α Activation in the Liver During Injury

Several studies, including our own, have demonstrated that regions of hypoxia develop in the liver after acute liver injury. Early studies showed that hypoxia develops in the liver after alcohol treatment.[12,13] Later studies showed that damage to the liver with compounds such as monocrotaline or acetaminophen also produce regions of hypoxia.[14,15] The mechanism by which this occurs is not known, but it most likely results from disruption of the hepatic architecture, which impedes blood flow through damaged regions; activation of the coagulation system, which leads to fibrin clot formation in the vasculature; and possibly production of vasoactive mediators that modulate hepatic blood flow to regions of injury.[16] To identify regions of hypoxia, many of these studies have used a chemical called pimonidazole, marketed as hypoxyprobe.[17] In addition to this method, activation of HIF-1α has been used as a surrogate marker of hypoxia. HIF-1α has been detected in the livers of mice treated with ethanol, acetaminophen, or carbon tetrachloride (CCl_4).[13,18–20]

In addition to hypoxia, however, HIF-1α can be activated in cells by other mediators, including cytokines, growth factors, and oxidative stress. Accordingly, caution should be used before equating HIF-1α activation with hypoxia. In fact, HIF-1α is activated in the liver after acetaminophen and CCl_4 treatment before the development of hypoxia, suggesting an important role for other mechanisms of HIF-1α regulation in the liver after exposure to these toxicants.[18,20]

Similar to acute injury, studies have shown that hypoxia is present in the liver during chronic injury. Rosmorduc et al[21,22] originally showed hypoxia in the liver after chronic treatment of rats with diethynitrosamine or after bile duct ligation (BDL), both of which produce severe fibrosis. We later confirmed these findings and demonstrated that HIF-1α is activated in several cell types in the liver after BDL.[5] In particular, HIF-1α was activated in macrophages and hepatocytes within and at the periphery of regions of necrosis, both areas where hypoxia was present. In addition to animal models, we detected HIF-1α protein in hepatocytes and scar-associated macrophages near regions of bridging fibrosis in livers from patients with primary biliary cirrhosis or primary sclerosing cholangitis.[23] We also detected HIF-1α in α-smooth muscle actin (α-SMA) expressing myofibroblasts within regions of bridging fibrosis.[23] Because HIF-1α regulates a number of genes that have been implicated in fibrosis development, including, platelet-derived growth factor (PDGF),[24] fibroblast growth factor-2 (FGF-2),[25] vascular endothelial growth factor (VEGF),[26] plasminogen activator inhibitor-1 (PAI-1),[27] and many others, our laboratory and others have conducted studies to evaluate the role of HIF-1α in the development of fibrosis.

Role of Hypoxia-Inducible Factor-1α in the Development of Liver Fibrosis

HIF-1α knockout mice die during embryonic development.[28] Accordingly, to test the hypothesis that HIF-1α contributes to the development of liver fibrosis, our laboratory used Cre-lox technology to knockout HIF-1α in adult mice. In this study, HIF-1α floxed mice were crossed with mice that express Cre recombinase under control of the Mx interferon-inducible promoter.[5] In these mice, treatment with polyinosinic-polycytidylic acid ubiquitously increases Cre recombinase in most cell types.[29] These mice and control mice that did not receive polyinosinic-polycytidylic acid were subjected to BDL. In HIF-1α-deficient mice, liver fibrosis was substantially reduced, which demonstrated for the first time a key role for HIF-1α in the development of liver fibrosis in vivo.[5] Deletion of HIF-1α prevented up-regulation of several key profibrotic mediators including PDGF-A, PDGF-B, PAI-1, and FGF-2.[5] All these proteins have been implicated in the development of liver fibrosis, suggesting that HIF-1α may promote fibrosis by regulating expression of these genes.[30–33] In fact, studies have shown that these genes are directly regulated by HIF-1α in some cell types.[24,25,27] Although this study indicated a key role for HIF-1α in the development of liver fibrosis, the cell-specific role of HIF-1α in the development of this disease remained unknown.

Profibrotic Function of Hypoxia-Inducible Factors in Hepatocytes

As discussed earlier, HIF-1α is activated in hepatocytes in mice subjected to BDL and in patients with primary biliary cirrhosis and primary sclerosing cholangitis.[5,23] Early studies by Kietzmann et al[27] demonstrated that HIF-1α is activated in hypoxic hepatocytes and that HIF-1α directly regulates PAI-1 in these cells. Our laboratory confirmed these findings and also showed that HIF-2α was activated in hypoxic hepatocytes and was needed for full induction of PAI-1 in these cells.[34] In addition to PAI-1, our studies demonstrated that hypoxia up-regulates VEGF, adrenomedullin-1 (ADM-1), and ADM-2 in a HIF-1α and HIF-2α-dependent manner.[34] Interestingly, although HIF-1α regulates PDGF-A and PDGF-B in some cell types, these were not increased in hypoxic hepatocytes.

Our studies also showed for the first time an interaction between the HIF and transforming growth factor β (TGF-β) signaling pathways. In this study, our laboratory demonstrated that hypoxic hepatocytes activate latent TGF-β1 in a HIF-dependent manner.[35] Although the mechanism by which this occurs is not fully understood, we have evidence that hypoxia increases expression of several matrix metalloproteinases and thrombospondin-1 in hepatocytes (Copple, unpublished observations), all of which can activate latent TGF-β1.[36–38]

Collectively, these in vitro studies demonstrated that HIF-1α and HIF-2α are activated in hypoxic hepatocytes and regulate expression of PAI-1 and VEGF as well as regulate activation of latent TGF-β1, which could promote the development of liver fibrosis (Figure 1). Since these studies, other laboratories have investigated the role of hepatocyte HIFs in the development of liver fibrosis in vivo.

In a study by Scott et al,[39] HIF-1β (aryl hydrocarbon nuclear transporter) floxed mice were crossed with mice

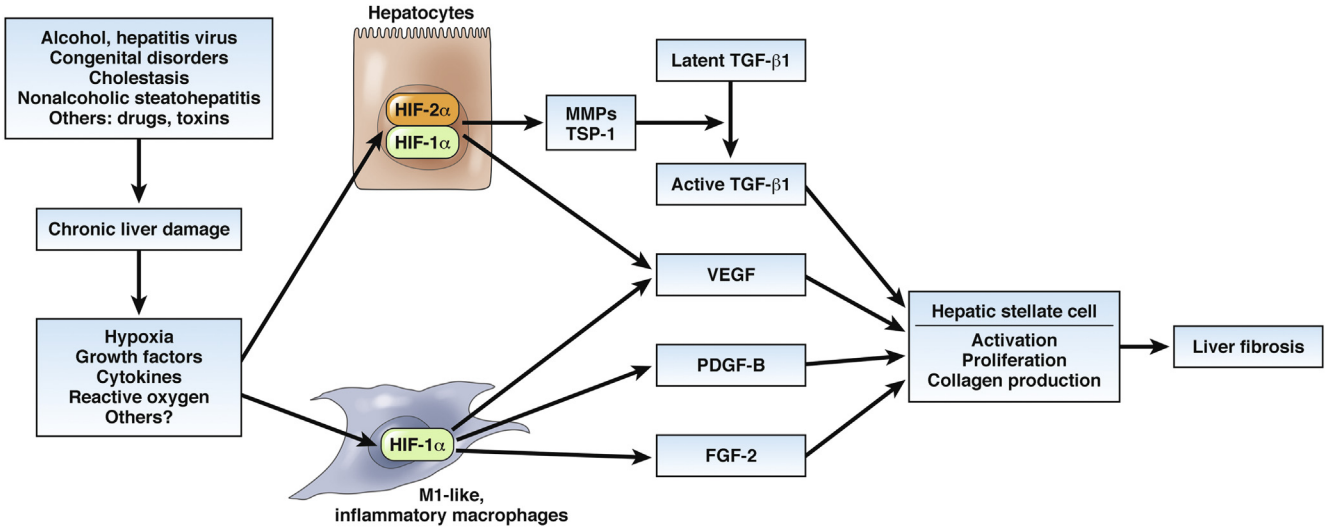

Figure 1. Potential profibrotic functions of hypoxia-inducible factors (HIFs) in hepatocytes and macrophages. Various hepatotoxicants produce hypoxia or stimulate release of mediators that activate HIFs in hepatocytes and M1-like inflammatory macrophages. In hepatocytes, HIF-1α and HIF-2α regulate vascular endothelial growth factor (VEGF). In addition, HIFs in hepatocytes regulate matrix metalloproteinases and thrombospondin 1 (TSP-1), which convert latent transforming growth factor β1 (TGF-β1) to active TGF-β1. In macrophages, HIF-1α regulates production of VEGF, platelet-derived growth factor B (PDGF-B), and fibroblast growth factor 2 (FGF-2), which promote fibrosis by affecting hepatic stellate cell activation, proliferation, and collagen production.

that express Cre recombinase under control of the albumin promoter to generate hepatocyte-specific HIF-1β knockout mice. As discussed earlier, HIF-1β heterodimerizes with the basic helix-loop-helix-PER-ARNT-SIM (bHLH/PAS) transcription factor family, which includes HIFs, and is thus required for both HIF-1α and HIF-2α transcriptional activity.[6,9] Mice with and without deletion of HIF-1β in hepatocytes were treated with the hepatotoxicant thioacetamide to induce liver fibrosis. Although the pattern of fibrosis was similar in both, there was a decrease in macrophage infiltration as well as a decrease in the mRNA expression of profibrotic genes, including TGF-β1 and TGF-β2, collagen type 1 and 5, and tissue inhibitor of metalloproteinases 1 and 5 (Timp1 and 5) in hepatocyte-specific HIF-1β knockout mice.[39] These results provided support for the importance of HIFs in regulating profibrotic gene expression in hepatocytes in vivo after liver injury.

Further studies conducted by Roychowdhury et al[40] also suggested an important role for hepatocyte HIF-1α in the development of fibrosis. In their study, hepatocyte-specific HIF-1α–deficient mice were fed ethanol concomitantly with CCl_4 treatment, resulting in the amplification of CCl_4-induced hepatic fibrosis. Hepatocyte-specific HIF-1α–deficiency prevented the ethanol-induced increase in CCl_4-induced fibrosis compared with control mice, as measured by mRNA and protein levels of both type I collagen and α-SMA, indicating an important role for hepatocyte HIF-1α in the development of liver fibrosis in this model.[40] However, when mice were treated with CCl_4 alone without ethanol, HIF-1α deficiency in hepatocytes did not reduce liver fibrosis, which may indicate that hypoxia resulting from ethanol metabolism may enhance HIF-1α activation in hepatocytes after CCl_4 treatment, allowing HIF-1α to play a greater role in the development of liver fibrosis.[40]

Studies by Qu et al[41] found that HIF-2α in hepatocytes may also be important for progression of fibrosis. In these studies, hepatocyte-specific Vhl knockout mice were produced, which results in constitutively activate HIF-1α and HIF-2α in hepatocytes.[42] Gene expression profiles were then assessed in the livers of control mice and hepatocyte-specific Vhl knockout mice.[41] Numerous genes critical to the development of liver fibrosis were found to be upregulated. These included genes important for collagen formation, such as procollagen-lysine 2,oxoglutarate 5-dioxygenase 2 (PLOD2) and transglutaminase 2 (TGM2) as well as α-SMA.[41] Furthermore, *Vhl*-disrupted mice fed an ethanol diet produced an increase in fibrosis. This increase was completely prevented in hepatocyte-specific *Vhl-Hif-2α* double knockout mice, but not in hepatocyte-specific *Vhl-Hif-1α* double knockout mice.[41] Together, these data indicated that HIF-2α activation in hepatocytes is important for the development of liver fibrosis in this model.[41]

One caveat of this study, however, was that although deletion of HIF-2α did prevent an increase in fibrosis, it also resulted in reduced liver injury overall. Because liver injury is the driving factor leading to fibrosis, it is possible that HIF-2α did not play a direct role in the progression of fibrosis, but rather played a role in producing liver injury. Further studies are needed to more definitively elucidate the role of HIF-2α in hepatocytes in the development of fibrosis.

Kupffer Cells

Kupffer cells are resident macrophages of the liver. Although these cells perform key beneficial functions, they

also contribute to the pathogenesis of a number of liver disorders, including ischemia-reperfusion injury, toxin-induced liver injury, fatty liver disease, and liver fibrosis.[43,44] Rivera et al[43] were the first to show a key role for macrophages in the development of liver fibrosis in rats after chronic CCl_4 treatment. Subsequent studies have confirmed these results, reaffirming an essential role for these cells in the development of liver fibrosis.[45] Work from Karlmark et al[46] indicated that the macrophage population that promoted fibrosis was recruited from bone marrow in a manner dependent on C-C chemokine receptor type 2 (Ccr2) and type 6 (Ccr6). Immunophenotyping of these cells indicated that they were $CD11b^+F4/80^+Gr1^+$ monocyte-derived macrophages.

Although these studies have clearly indicated that macrophages are important for the development of fibrosis, the mechanism by which they contribute to this process was not known. Early studies by Friedman and Arthur[31] showed that conditioned medium from cultured Kupffer cells stimulated HSC proliferation in culture in a PDGF-dependent manner. In addition, studies provided evidence that intrahepatic $CD11b^+F4/80^+Gr1^+$ macrophages could stimulate HSCs to produce collagen in a TGF-β-dependent manner.[46]

Consistent with these studies, immunohistochemical studies and in situ hybridization techniques have shown that macrophages express TGF-β and PDGFs in the liver during the genesis of fibrosis.[47,48] Collectively, these studies demonstrated that macrophages are important for the development of fibrosis and that they may contribute to fibrosis by producing growth factors that stimulate HSC proliferation and collagen production. However, the mechanism by which chronic liver injury stimulated these cells to produce profibrotic mediators remained unknown.

As discussed earlier, our studies demonstrated that HIF-1α was activated in scar-associated macrophages in both mice and humans with liver fibrosis.[5,23] In subsequent studies, we showed that exposure of Kupffer cells to hypoxia in vitro activated HIF-1α but not HIF-2α, suggesting that HIF-1α signaling may predominate in Kupffer cells from nondiseased livers.[49] This, however, could potentially change in diseased livers. In the presence of certain cytokines, macrophages polarize to different phenotypes, including classically activated (M1) and alternatively activated (M2) macrophages. Takeda et al[50] showed that HIF-1α is up-regulated and HIF-2α is down-regulated in classically activated macrophages, whereas HIF-2α is up-regulated and HIF-1α down-regulated in alternatively activated macrophages. It is possible that Kupffer cells in nondiseased livers are skewed toward a classically activated phenotype, which may explain the lack of HIF-2α activation in these cells. In diseased livers, however, we have observed hepatic macrophages with activated HIF-1α or HIF-2α, indicating that both transcription factors can be activated in hepatic macrophages in vivo after injury (Copple, Mochizuki, and Roth, unpublished observation).

Because HIF-1α and HIF-2α regulate overlapping and distinct sets of genes, it would be interesting to evaluate the pattern of gene expression in hypoxic Kupffer cells either skewed toward a classic phenotype with activated HIF-1α or an alternative phenotype with activated HIF-2α. It is possible that these two macrophage populations may play very different roles in liver disease in part due to differential expression of HIF-1α and HIF-2α.

Studies have shown that hypoxic Kupffer cells produce profibrotic mediators in vitro. Our laboratory showed that in vitro exposure of Kupffer cells to hypoxia increased expression of several genes involved in angiogenesis, including VEGF and angiopoietin-1, in a HIF-dependent manner.[49] In addition, PDGF-B was also up-regulated in hypoxic Kupffer cells in a HIF-dependent manner.[49] In mice subjected to BDL, knocking out HIF-1β selectively in macrophages using Cre/lox did not affect liver injury, markers of cholestasis, or hepatic inflammation, including up-regulation of cytokines and hepatic neutrophil and macrophage accumulation.[23] Deletion of HIF-1β did, however, reduce liver fibrosis as measured by type I collagen and α-SMA mRNA and protein levels.[23] In addition, knocking out HIF-1β in macrophages prevented an increase in PDGF-B mRNA and protein.[23] It also prevented up-regulation of FGF-2, another profibrotic growth factor, but did not affect levels of active TGF-β protein.[23] Similar results were observed in mice in which HIF-1α was knocked out in macrophages, suggesting that HIF-1α in macrophages is profibrotic (Figure 1).[23] This is consistent with reports suggesting that classically activated, inflammatory macrophages primarily promote fibrosis.[46] As mentioned earlier, HIF-1α is up-regulated in classically activated macrophages. It would be interesting, however, to investigate the role of HIF-2α in macrophages and determine whether it may play an opposing role and promotes fibrosis reversal.

Hepatic Stellate Cells

Despite being originally described in the 19th century, it was not until 1985 that Friedman et al[1] discovered that HSCs are the main collagen-producing cell type in the liver. Recent studies using sophisticated cell-tracing techniques have further confirmed the importance of these cells in collagen deposition during fibrosis.[51] During quiescence, these cells store and release retinoids. After liver injury, these cells become activated and differentiate into α-SMA–expressing myofibroblasts that produce collagen, proliferate, and migrate throughout the liver.[2] Because liver injury produces regions of hypoxia, Corpechot et al[22] tested the hypothesis that hypoxia stimulates HSCs to produce collagen. In this study, exposure of rat HSCs to hypoxia in vitro up-regulated type I collagen, indicating that hypoxia is a stimulus for collagen production by these cell types. In addition, we recently confirmed these findings in culture-activated, primary human HSCs (Copple, unpublished findings).

Although this process is not fully understood, it most likely evolved to provide an initial structural matrix to facilitate repair processes, such as angiogenesis. During chronic injury, however, persistent hypoxia provides a continuous stimulus for collagen production by HSCs, leading to fibrosis. A later study by Shi et al[52] confirmed that hypoxia up-regulates collagen in a human HSC line, LX-2

cells. In this study, they also observed an increase in TGF-β, suggesting that hypoxia stimulates autocrine production of TGF-β, which could stimulate collagen production.[52] This same group also observed an increase in matrix metalloproteinase 2, which could process TGF-β to its active form.[52] Whether these processes require HIFs and occur in vivo during fibrosis development, however, remain to be determined.

Because of the results described here, our laboratory next determined whether HIFs are activated in hypoxic HSCs. In these studies, exposure of culture-activated, primary mouse HSCs to hypoxia activated both HIF-1α and HIF-2α.[53] Hypoxia also increased the expression of several genes that could contribute to fibrosis development. For instance, hypoxia increased expression of two genes involved in collagen metabolism, prolyl-4-hypdroxylase α1 and prolyl-4-hydroxylase α2, which are key enzymes that contribute to the formation of stable collagen triple helices.[53] As expected, hypoxia also increased expression of several genes involved in angiogenesis, including VEGF, angiopoietin-like-4, placental growth factor, and macrophage-migration inhibitory factor.[53]

It has been proposed that angiogenesis is a critical factor in the development of liver fibrosis,[21] although this remains a rather controversial area. For instance VEGF has been shown to stimulate HSCs to produce collagen, to proliferate, and to migrate in vitro.[54] Furthermore, neutralization of VEGF was shown to reduce fibrosis in CCl_4-treated mice.[55] More recent studies, however, have shown that VEGF is critical for reversal of fibrosis.[56,57] The antifibrotic effects of VEGF are most likely due to effects of VEGF on sinusoidal endothelial cells rather than HSCs. Accordingly, the importance of VEGF and angiogenesis to liver fibrosis is complicated and remains an evolving field.

In addition to proangiogenic mediators, hypoxia increased the expression of receptors involved in HSC migration and activation. For instance, hypoxia increased the expression of the chemokine receptors Ccr1 and Ccr5.[53] In the presence of chemokines, activation of these receptors stimulates HSC migration. In support of a role for these receptors in the development of fibrosis, it was shown that Ccr1 and Ccr5 knockout mice have reduced liver fibrosis.[58] Two other receptors of interest were increased by hypoxia in HSCs. These were interleukin-13 receptor α1 and adrenergic receptor α2b, which are activated by interleukin-13 and catecholamines, respectively.[53] In vitro, activation of these receptors on HSCs stimulates collagen production, and in vivo both these pathways have been shown to play an important role in liver fibrosis development.[59–61]

Although up-regulation of some of the genes described here required HIF-1α, up-regulation of some did not require HIF-1α, suggesting an important role for HIF-2α or potentially other hypoxia-regulated transcription factors in regulation of these genes.[53] Of particular interest, up-regulation of many of these genes by hypoxia only occurred in culture-activated HSCs and did not occur in quiescent HSCs, suggesting that HSC activation increases the sensitivity of HSCs to HIF-mediated gene expression changes.[53] The mechanism by which this occurs is not known, but it may result from changes in histone modifications or DNA methylation changes that modify access of HIFs to the promoters of certain HIF-regulated genes in activated HSCs.

In addition to the genes already described, hypoxia also increased expression of genes that have the potential to globally affect HSC function in a profound manner. Two of these were the Jumonji domain-containing proteins Jmjd6 and Jmjd1a.[53] Jmjd6 regulates mRNA spicing, and Jmjd1a is a histone demethylase.[62,63] Up-regulation of Jmjd1a by HIFs could increase expression of a number of genes by modifying histone methylation, whereas up-regulation of Jmjd6 could promote the formation of alternatively spliced mRNAs that code for proteins with modified functions that promote fibrosis.

In addition to Jumoni proteins, hypoxia increased expression of regulator of G-protein signaling 2 (Rgs2) and Rgs4.[53] These proteins act as GTPase-activating proteins for G protein α subunits, thereby decreasing signaling by Giα, Goα, and Gqα subtypes.[64] Up-regulation of Rgs proteins could substantially affect signaling by G protein-coupled receptors in HSCs. The importance of this in regulating HSC function during the development of fibrosis remains to be determined. The various processes regulated by HIFs in HSCs that may contribute to fibrosis are summarized in Figure 2.

Although numerous genes were increased in hypoxic HSCs, surprisingly few were decreased by hypoxia. Two genes of particular interest that were decreased by hypoxia were hepatocyte growth factor (HGF) and α1-integrin.[53] HGF is important for liver regeneration, and studies have shown that HSCs are a source of HGF during regeneration.[65–67] In addition to our study, Corpechot et al[68] demonstrated in 2002 that hypoxia decreases HGF in hypoxic HSCs. In this study, they proposed that decreased HGF expression in hypoxic HSCs may contribute to liver regeneration failure in cirrhotic livers.[68] Alpha-1-integrin is a receptor for collagen, and studies have shown that collagen production by fibroblasts from α1-integrin knockout mice is enhanced, suggesting a role for this receptor in feedback inhibition of collagen production.[69] The importance of the decrease in α1-integrin in hypoxic HSCs, however, remains to be determined. Furthermore, whether HIF-1α contributes to the reduction in HGF and α1-integrin is not known.

In consideration of the impact of hypoxia and HIF activation on the function of HSCs in vitro, studies were conducted to evaluate the role of HIF-1α in HSCs in vivo on the development of liver fibrosis. In these studies, HIF-1α floxed mice were crossed with mice that express Cre recombinase under the glial fibrillary acidic protein promoter, which, in the liver, is only expressed by HSCs.[20] This resulted in deletion of HIF-1α in HSCs but no other liver cell types. Treatment of control mice in this study with a single hepatotoxic dose of CCl_4 increased the expression of types I, III, and IV collagens by 48 hours after treatment.[20] Deletion of HIF-1α in HSCs completely prevented up-regulation of all three collagens in the liver at this time point.[20] Surprisingly,

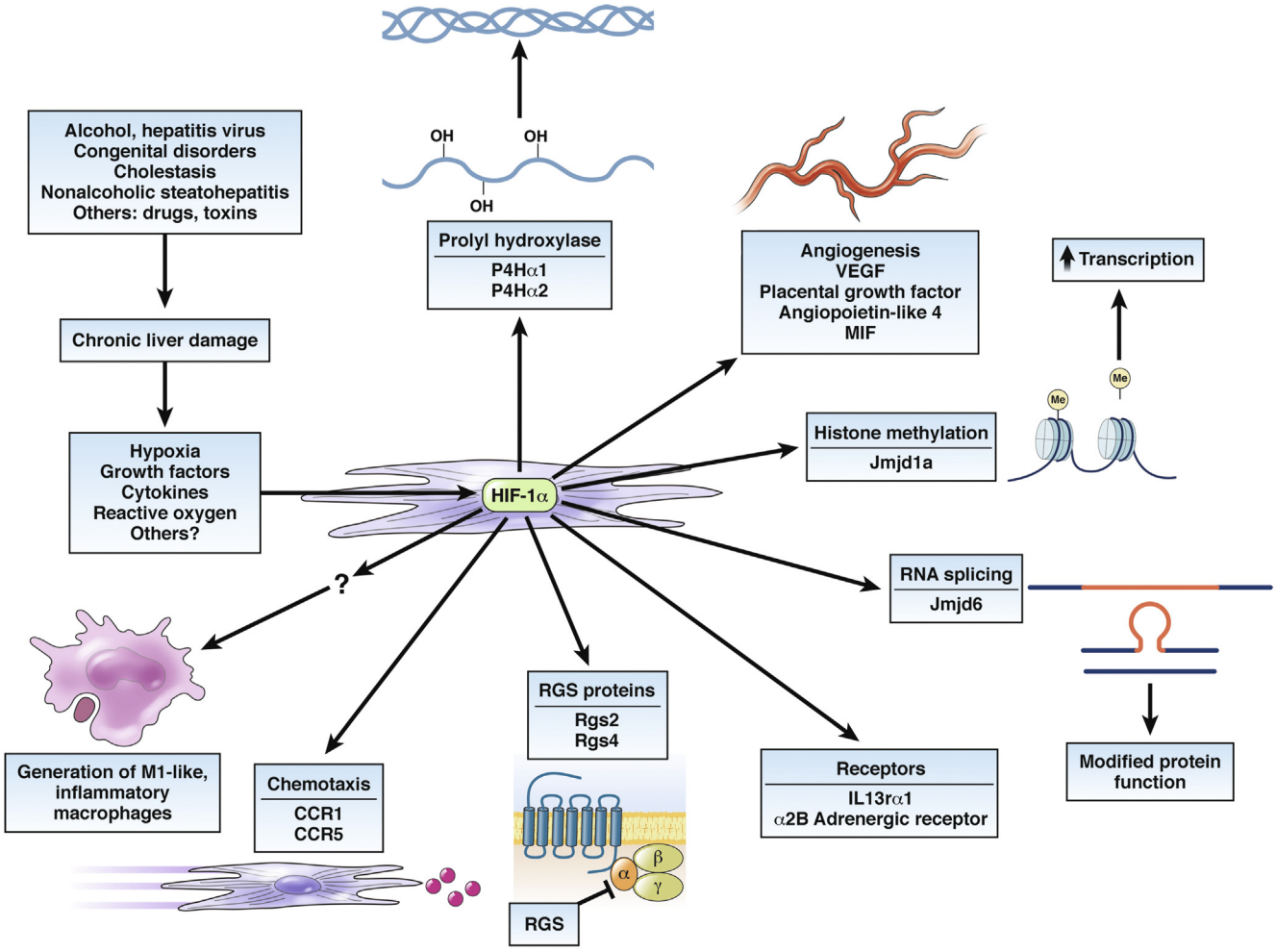

Figure 2. Genes regulated by hypoxia-inducible factor 1α (HIF-1α) in hepatic stellate cells that may promote fibrosis. (See the text for full details.)

by 72 hours after CCl_4 administration, the levels of type I collagen mRNA and protein were much greater in the livers of mice in which HIF-1α was knocked out in HSCs.[20] In addition, there were greater levels of α-SMA mRNA and protein, indicating enhanced HSC activation.[20]

These results were quite contrary to in vitro studies and suggested a potential role for HIF-1α in limiting HSC activation and collagen production in vivo after liver injury. Further analysis, however, demonstrated that there was a failure of macrophages to become activated in the livers of these mice, which prevented removal of necrotic hepatocytes from the liver.[20] We proposed that the persistence of necrotic cells most likely maintained HSC activation and enhanced production of collagen in mice deficient in HIF-1α in HSCs.

Interestingly, this phenotype is also observed in plasminogen and urokinase plasminogen activator knockout mice, where there is a failure to clear necrotic hepatocytes, leading to enhanced collagen production.[70–72] Accordingly, while HIF-1α appeared to be important for collagen upregulation early after injury, it also stimulated HSCs to produce a HIF-1α–dependent factor(s) that activated macrophages, which is essential for phagocytic removal of necrotic hepatocytes. The mechanism by which this occurs is not known; however, considering the similar phenotype in mice deficient in plasmin, it is possible that HIF-1α activation in HSCs modulates plasmin formation after injury.

Summary and Perspectives for Targeting Hypoxia-Inducible Factors as a Therapy for Liver Fibrosis

Research over the past 16 years has provided important information on the role of HIFs in the development of liver fibrosis. This work has provided evidence that HIF-1α regulates a number of important profibrotic mediators in hepatocytes, HSCs, and Kupffer cells. It remains to be determined whether HIF-1α activation in other cell types contributes to this disease. For instance, VEGF, a key HIF-target gene, is increased in cholangiocytes after BDL.[73] Whether this requires HIF-1α is not known. HIF-1α also regulates the function of various immune cell types, including T cells, natural killer T cells, and B cells, which may be important for fibrosis.[74–77] Furthermore, very little

is known about the role of HIF-2α in liver fibrosis and in which cells HIF-2α is activated. Finally, there is a clear link between HIF-1α and HIF-2α activation and the development of cancer. Because cirrhosis can often progress to hepatocellular carcinoma, it would be interesting to determine whether HIFs are an important driving force for the progression of cirrhosis to cancer. Studies have shown that constitutive activation of HIF-1α and HIF-2α in hepatocytes produces hemangiomas in the liver, lending support for a role of HIFs in cancer development in the liver.[78] Over the next several years, research should begin to fill these knowledge gaps.

Also, it is unclear whether pharmacologic inhibition of HIFs would be an effective therapy for liver fibrosis. Only one study has addressed this topic thus far. In a rat model of transarterial chemoembolization in which rats are treated with CCl_4 and subjected to hepatic artery ligation, a putative HIF-1α inhibitor called LW6 prevented progression of liver fibrosis when started 2 weeks after the initiation of liver disease.[79] Although these results are exciting, additional studies with other models of liver fibrosis and other well-characterized HIF inhibitors need to be conducted before HIFs are validated targets for liver fibrosis therapy.

References

1. Friedman SL, Roll FJ, Boyles J, et al. Hepatic lipocytes: the principal collagen-producing cells of normal rat liver. Proc Natl Acad Sci USA 1985;82:8681–8685.
2. Friedman SL. Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. Physiol Rev 2008; 88:125–172.
3. Kinnman N, Francoz C, Barbu V, et al. The myofibroblastic conversion of peribiliary fibrogenic cells distinct from hepatic stellate cells is stimulated by platelet-derived growth factor during liver fibrogenesis. Lab Invest 2003;83:163–173.
4. Wells RG, Kruglov E, Dranoff JA. Autocrine release of TGF-beta by portal fibroblasts regulates cell growth. FEBS Lett 2004;559:107–110.
5. Moon JO, Welch TP, Gonzalez FJ, et al. Reduced liver fibrosis in hypoxia-inducible factor-1α-deficient mice. Am J Physiol Gastrointest Liver Physiol 2009; 296:G582–G592.
6. Wang GL, Jiang BH, Rue EA, et al. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. Proc Natl Acad Sci USA 1995;92:5510–5514.
7. Jiang BH, Rue E, Wang GL, et al. Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1. J Biol Chem 1996;271:17771–17778.
8. Semenza GL, Wang GL. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol Cell Biol 1992; 12:5447–5454.
9. Ema M, Taya S, Yokotani N, et al. A novel bHLH-PAS factor with close sequence similarity to hypoxia-inducible factor 1α regulates the VEGF expression and is potentially involved in lung and vascular development. Proc Natl Acad Sci USA 1997;94:4273–4278.
10. Gu YZ, Moran SM, Hogenesch JB, et al. Molecular characterization and chromosomal localization of a third alpha-class hypoxia inducible factor subunit, HIF3α. Gene Expr 1998;7:205–213.
11. Huang LE, Gu J, Schau M, et al. Regulation of hypoxia-inducible factor 1α is mediated by an O_2-dependent degradation domain via the ubiquitin-proteasome pathway. Proc Natl Acad Sci USA 1998; 95:7987–7992.
12. Ji S, Lemasters JJ, Christenson V, et al. Periportal and pericentral pyridine nucleotide fluorescence from the surface of the perfused liver: evaluation of the hypothesis that chronic treatment with ethanol produces pericentral hypoxia. Proc Natl Acad Sci USA 1982; 79:5415–5419.
13. Li J, French B, Wu Y, et al. Liver hypoxia and lack of recovery after reperfusion at high blood alcohol levels in the intragastric feeding model of alcohol liver disease. Exp Mol Pathol 2004;77:184–192.
14. Copple BL, Rondelli CM, Maddox JF, et al. Modes of cell death in rat liver after monocrotaline exposure. Toxicol Sci 2004;77:172–182.
15. Chaudhuri S, McCullough SS, Hennings L, et al. Acetaminophen hepatotoxicity and HIF-1α induction in acetaminophen toxicity in mice occurs without hypoxia. Toxicol Appl Pharmacol 2011;252:211–220.
16. Copple BL, Roth RA, Ganey PE. Anticoagulation and inhibition of nitric oxide synthase influence hepatic hypoxia after monocrotaline exposure. Toxicology 2006; 225:128–137.
17. Arteel GE, Thurman RG, Raleigh JA. Reductive metabolism of the hypoxia marker pimonidazole is regulated by oxygen tension independent of the pyridine nucleotide redox state. Eur J Biochem 1998;253:743–750.
18. James LP, Donahower B, Burke AS, et al. Induction of the nuclear factor HIF-1α in acetaminophen toxicity: evidence for oxidative stress. Biochem Biophys Res Commun 2006;343:171–176.
19. Sparkenbaugh EM, Saini Y, Greenwood KK, et al. The role of hypoxia-inducible factor-1α in acetaminophen hepatotoxicity. J Pharmacol Exp Ther 2011; 338:492–502.
20. Mochizuki A, Pace A, Rockwell CE, et al. Hepatic stellate cells orchestrate clearance of necrotic cells in a hypoxia-inducible factor-1α-dependent manner by modulating macrophage phenotype in mice. J Immunol 2014; 192:3847–3857.
21. Rosmorduc O, Wendum D, Corpechot C, et al. Hepatocellular hypoxia-induced vascular endothelial growth factor expression and angiogenesis in experimental biliary cirrhosis. Am J Pathol 1999;155:1065–1073.
22. Corpechot C, Barbu V, Wendum D, et al. Hypoxia-induced VEGF and collagen I expressions are associated with angiogenesis and fibrogenesis in experimental cirrhosis. Hepatology 2002;35:1010–1021.
23. Copple BL, Kaska S, Wentling C. Hypoxia-inducible factor activation in myeloid cells contributes to the

development of liver fibrosis in cholestatic mice. J Pharmacol Exp Ther 2012;341:307–316.
24. Yoshida D, Kim K, Noha M, et al. Hypoxia inducible factor 1-alpha regulates of platelet derived growth factor-B in human glioblastoma cells. J Neurooncol 2006; 76:13–21.
25. Calvani M, Rapisarda A, Uranchimeg B, et al. Hypoxic induction of an HIF-1α-dependent bFGF autocrine loop drives angiogenesis in human endothelial cells. Blood 2006;107:2705–2712.
26. Forsythe JA, Jiang BH, Iyer NV, et al. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol 1996; 16:4604–4613.
27. Kietzmann T, Roth U, Jungermann K. Induction of the plasminogen activator inhibitor-1 gene expression by mild hypoxia via a hypoxia response element binding the hypoxia-inducible factor-1 in rat hepatocytes. Blood 1999;94:4177–4185.
28. Iyer NV, Kotch LE, Agani F, et al. Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha. Genes Dev 1998;12:149–162.
29. Kuhn R, Schwenk F, Aguet M, et al. Inducible gene targeting in mice. Science 1995;269:1427–1429.
30. Neef M, Ledermann M, Saegesser H, et al. Oral imatinib treatment reduces early fibrogenesis but does not prevent progression in the long term. J Hepatol 2006; 44:167–175.
31. Friedman SL, Arthur MJ. Activation of cultured rat hepatic lipocytes by Kupffer cell conditioned medium. Direct enhancement of matrix synthesis and stimulation of cell proliferation via induction of platelet-derived growth factor receptors. J Clin Invest 1989;84:1780–1785.
32. Bergheim I, Guo L, Davis MA, et al. Critical role of plasminogen activator inhibitor-1 in cholestatic liver injury and fibrosis. J Pharmacol Exp Ther 2006;316:592–600.
33. Yu C, Wang F, Jin C, et al. Role of fibroblast growth factor type 1 and 2 in carbon tetrachloride-induced hepatic injury and fibrogenesis. Am J Pathol 2003; 163:1653–1662.
34. Copple BL, Bustamante JJ, Welch TP, et al. Hypoxia-inducible factor-dependent production of profibrotic mediators by hypoxic hepatocytes. Liver Int 2009; 29:1010–1021.
35. Copple BL. Hypoxia stimulates hepatocyte epithelial to mesenchymal transition by hypoxia-inducible factor and transforming growth factor-beta-dependent mechanisms. Liver Int 2010;30:669–682.
36. Young GD, Murphy-Ullrich JE. Molecular interactions that confer latency to transforming growth factor-beta. J Biol Chem 2004;279:38032–38039.
37. D'Angelo M, Billings PC, Pacifici M, et al. Authentic matrix vesicles contain active metalloproteases (MMP). a role for matrix vesicle-associated MMP-13 in activation of transforming growth factor-beta. J Biol Chem 2001; 276:11347–11353.
38. Maeda S, Dean DD, Gay I, et al. Activation of latent transforming growth factor beta1 by stromelysin 1 in extracts of growth plate chondrocyte-derived matrix vesicles. J Bone Miner Res 2001;16:1281–1290.
39. Scott C, Cha K, Rao R, et al. Hepatocyte-specific deletion of ARNT (aryl hydrocarbon receptor nuclear translocator) results in altered fibrotic gene expression in the thioacetamide model of liver injury. PLoS One 2015; 10:e0121650.
40. Roychowdhury S, Chiang DJ, McMullen MR, et al. Moderate, chronic ethanol feeding exacerbates carbon-tetrachloride-induced hepatic fibrosis via hepatocyte-specific hypoxia inducible factor 1α. Pharmacol Res Perspect 2014;2:e00061.
41. Qu A, Taylor M, Xue X, et al. Hypoxia-inducible transcription factor 2α promotes steatohepatitis through augmenting lipid accumulation, inflammation, and fibrosis. Hepatology 2011;54:472–483.
42. Haase VH, Glickman JN, Socolovsky M, et al. Vascular tumors in livers with targeted inactivation of the von Hippel-Lindau tumor suppressor. Proc Natl Acad Sci USA 2001;98:1583–1588.
43. Rivera CA, Bradford BU, Hunt KJ, et al. Attenuation of CCl_4-induced hepatic fibrosis by $GdCl_3$ treatment or dietary glycine. Am J Physiol Gastrointest Liver Physiol 2001;281:G200–G207.
44. Jaeschke H, Farhood A. Neutrophil and Kupffer cell-induced oxidant stress and ischemia-reperfusion injury in rat liver. Am J Physiol 1991;260:G355–G362.
45. Duffield JS, Forbes SJ, Constandinou CM, et al. Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. J Clin Invest 2005; 115:56–65.
46. Karlmark KR, Weiskirchen R, Zimmermann HW, et al. Hepatic recruitment of the inflammatory Gr1$^+$ monocyte subset upon liver injury promotes hepatic fibrosis. Hepatology 2009;50:261–274.
47. Faiz Kabir Uddin Ahmed A, Ohtani H, Nio M, et al. In situ expression of fibrogenic growth factors and their receptors in biliary atresia: comparison between early and late stages. J Pathol 2000;192:73–80.
48. Nakatsukasa H, Nagy P, Evarts RP, et al. Cellular distribution of transforming growth factor-beta 1 and procollagen types I, III, and IV transcripts in carbon tetrachloride-induced rat liver fibrosis. J Clin Invest 1990;85:1833–1843.
49. Copple BL, Bai S, Moon JO. Hypoxia-inducible factor-dependent production of profibrotic mediators by hypoxic Kupffer cells. Hepatol Res;40:530–539.
50. Takeda N, O'Dea EL, Doedens A, et al. Differential activation and antagonistic function of HIF-{alpha} isoforms in macrophages are essential for NO homeostasis. Genes Dev;24:491–501.
51. Brenner DA, Kisseleva T, Scholten D, et al. Origin of myofibroblasts in liver fibrosis. Fibrogenesis Tissue Repair 2012;5(Suppl):S17.
52. Shi YF, Fong CC, Zhang Q, et al. Hypoxia induces the activation of human hepatic stellate cells LX-2 through TGF-beta signaling pathway. FEBS Lett 2007; 581:203–210.
53. Copple BL, Bai S, Burgoon LD, et al. Hypoxia-inducible factor-1α regulates the expression of genes in hypoxic hepatic stellate cells important for collagen deposition and angiogenesis. Liver Int 2011;31:230–244.

54. Novo E, Cannito S, Zamara E, et al. Proangiogenic cytokines as hypoxia-dependent factors stimulating migration of human hepatic stellate cells. Am J Pathol 2007;170:1942–1953.
55. Yoshiji H, Kuriyama S, Yoshii J, et al. Vascular endothelial growth factor and receptor interaction is a prerequisite for murine hepatic fibrogenesis. Gut 2003;52:1347–1354.
56. Yang L, Kwon J, Popov Y, et al. Vascular endothelial growth factor promotes fibrosis resolution and repair in mice. Gastroenterology 2014;146:1339–1350.e1.
57. Kantari-Mimoun C, Castells M, Klose R, et al. Resolution of liver fibrosis requires myeloid cell-driven sinusoidal angiogenesis. Hepatology 2015;61:2042–2055.
58. Seki E, De Minicis S, Gwak GY, et al. CCR1 and CCR5 promote hepatic fibrosis in mice. J Clin Invest 2009; 119:1858–1870.
59. Weng HL, Liu Y, Chen JL, et al. The etiology of liver damage imparts cytokines transforming growth factor beta1 or interleukin-13 as driving forces in fibrogenesis. Hepatology 2009;50:230–243.
60. Fallon PG, Richardson EJ, McKenzie GJ, et al. Schistosome infection of transgenic mice defines distinct and contrasting pathogenic roles for IL-4 and IL-13: IL-13 is a profibrotic agent. J Immunol 2000; 164:2585–2591.
61. Oben JA, Yang S, Lin H, et al. Norepinephrine and neuropeptide Y promote proliferation and collagen gene expression of hepatic myofibroblastic stellate cells. Biochem Biophys Res Commun 2003;302:685–690.
62. Webby CJ, Wolf A, Gromak N, et al. Jmjd6 catalyses lysyl-hydroxylation of U2AF65, a protein associated with RNA splicing. Science 2009;325:90–93.
63. Yamane K, Toumazou C, Tsukada Y, et al. JHDM2A, a JmjC-containing H3K9 demethylase, facilitates transcription activation by androgen receptor. Cell 2006; 125:483–495.
64. Zhong H, Neubig RR. Regulator of G protein signaling proteins: novel multifunctional drug targets. J Pharmacol Exp Ther 2001;297:837–845.
65. Skrtic S, Wallenius V, Ekberg S, et al. Insulin-like growth factors stimulate expression of hepatocyte growth factor but not transforming growth factor beta1 in cultured hepatic stellate cells. Endocrinology 1997;138: 4683–4689.
66. Skrtic S, Wallenius V, Ekberg S, et al. Hepatocyte-stimulated expression of hepatocyte growth factor (HGF) in cultured rat hepatic stellate cells. J Hepatol 1999; 30:115–124.
67. Nejak-Bowen K, Orr A, Bowen WC Jr, et al. Conditional genetic elimination of hepatocyte growth factor in mice compromises liver regeneration after partial hepatectomy. PLoS One 2013;8:e59836.
68. Corpechot C, Barbu V, Wendum D, et al. Hepatocyte growth factor and c-Met inhibition by hepatic cell hypoxia: a potential mechanism for liver regeneration failure in experimental cirrhosis. Am J Pathol 2002;160:613–620.
69. Gardner H, Broberg A, Pozzi A, et al. Absence of integrin $\alpha 1\beta 1$ in the mouse causes loss of feedback regulation of collagen synthesis in normal and wounded dermis. J Cell Sci 1999;112:263–272.
70. Bezerra JA, Bugge TH, Melin-Aldana H, et al. Plasminogen deficiency leads to impaired remodeling after a toxic injury to the liver. Proc Natl Acad Sci USA 1999; 96:15143–15148.
71. Pohl JF, Melin-Aldana H, Sabla G, et al. Plasminogen deficiency leads to impaired lobular reorganization and matrix accumulation after chronic liver injury. Am J Pathol 2001;159:2179–2186.
72. Currier AR, Sabla G, Locaputo S, et al. Plasminogen directs the pleiotropic effects of uPA in liver injury and repair. Am J Physiol Gastrointest Liver Physiol 2003; 284:G508–G515.
73. Gaudio E, Barbaro B, Alvaro D, et al. Vascular endothelial growth factor stimulates rat cholangiocyte proliferation via an autocrine mechanism. Gastroenterology 2006; 130:1270–1282.
74. Zhang J, Han C, Dai H, et al. Hypoxia-inducible factor-2α limits natural killer T cell cytotoxicity in renal ischemia/reperfusion injury. J Am Soc Nephrol 2015, Published online, http://dx.doi.org/10.1681/ASN.2014121248.
75. Lukashev D, Klebanov B, Kojima H, et al. Cutting edge: hypoxia-inducible factor 1α and its activation-inducible short isoform I.1 negatively regulate functions of $CD4^+$ and $CD8^+$ T lymphocytes. J Immunol 2006;177: 4962–4965.
76. Makino Y, Nakamura H, Ikeda E, et al. Hypoxia-inducible factor regulates survival of antigen receptor-driven T cells. J Immunol 2003;171:6534–6540.
77. Kojima H, Gu H, Nomura S, et al. Abnormal B lymphocyte development and autoimmunity in hypoxia-inducible factor 1α -deficient chimeric mice. Proc Natl Acad Sci USA 2002;99:2170–2174.
78. Rankin EB, Rha J, Unger TL, et al. Hypoxia-inducible factor-2 regulates vascular tumorigenesis in mice. Oncogene 2008;27:5354–5358.
79. Qu K, Yan Z, Wu Y, et al. Transarterial chemoembolization aggravated peritumoral fibrosis via hypoxia-inducible factor-1α dependent pathway in hepatocellular carcinoma. J Gastroenterol Hepatol 2015; 30:925–932.

Received August 19, 2015. Accepted September 16, 2015.

Correspondence
Address correspondence to: Bryan L. Copple, PhD, Department of Pharmacology and Toxicology, Michigan State University, 1355 Bogue Street, B403 Life Sciences Building, East Lansing, Michigan 48824. e-mail: copple@msu.edu.

Conflicts of interest
The authors disclose no conflicts.

Funding
This study was funded by National Institutes of Health grant 2 R01 DK073566 (to B.L.C.) and National Institutes of Health Training Grant T32 ES007255 (to K.J.R.).