

Hypoxia-Inducible Factor Activation in Myeloid Cells Contributes to the Development of Liver Fibrosis in Cholestatic Mice

Bryan L. Copple, Sophia Kaska, and Callie Wentling

Department of Pharmacology, Toxicology, and Experimental Therapeutics, University of Kansas Medical Center, Kansas City, Kansas

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ABSTRACT

Macrophages play an integral role in the development of liver fibrosis by releasing mediators, such as platelet-derived growth factor-B (PDGF-B) and transforming growth factor- β 1, which stimulate hepatic stellate cell proliferation, chemotaxis, and collagen production. However, the mechanism by which chronic liver injury stimulates macrophages to release these mediators is not completely understood. We tested the hypothesis that chronic liver injury activates hypoxia-inducible factor (HIF) transcription factors in macrophages that regulate the production of mediators that promote fibrosis. To test this hypothesis, Cre/lox technology was used to generate myeloid cell-specific HIF-1 α or HIF-1 β knockout mice. When these mice were subjected to bile duct ligation (BDL), levels of α -smooth muscle actin and type I collagen in the liver were reduced

compared with those of mice with normal levels of HIFs. The deficiency of HIFs in macrophages did not affect liver injury or inflammation after BDL but reduced PDGF-B mRNA and protein, suggesting that HIF activation in macrophages may promote fibrosis by regulating the production of PDGF-B. Consistent with a role for HIFs in liver fibrosis in cholestatic liver disease, nuclear HIF-1 α protein was present in macrophages, hepatocytes, and fibroblasts in the livers from patients with primary biliary cirrhosis and primary sclerosing cholangitis. These studies demonstrate that HIFs are important regulators of profibrotic mediator production by macrophages during the development of liver fibrosis and suggest that HIFs may be a novel therapeutic target for the treatment of chronic liver disease in patients.

Introduction

Humans are exposed to a variety of chemicals, pathogens, or conditions that produce chronic liver injury, including alcohol, hepatitis viruses, autoimmune diseases, and genetic disorders. The liver responds to injury by producing soluble mediators, such as growth factors, that facilitate liver repair (Fausto, 2000). This process is essential for the restoration of liver homeostasis after acute injury. During chronic injury, however,

persistent activation of these pathways promotes pathological processes, including fibrosis and cancer (Friedman, 2008).

Macrophages play an integral role in the development of liver fibrosis. Studies have shown that the depletion of macrophages slows the progression of fibrosis in animal models (Rivera et al., 2001; Duffield et al., 2005). The mechanism by which macrophages promote fibrosis is not completely understood, although studies have shown that these cells express key mediators of fibrosis, such as platelet-derived growth factor-B (PDGF-B) and transforming growth factor- β (TGF- β) (Nakatsukasa et al., 1990; Faiz Kabir Uddin Ahmed et al., 2000). Accordingly, macrophages may promote fibrosis by releasing profibrotic growth factors that stimulate hepatic stellate cells and peribiliary fibroblasts to become activated and to produce collagen. However, what remains to be identified is the mechanism by which chronic liver injury stimulates macrophages to produce mediators that promote fibrosis. Our recent studies suggest that hepatocellular hypoxia may be important for this process.

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ABBREVIATIONS: PDGF-B, platelet-derived growth factor-B; α -SMA, α -smooth muscle actin; ALT, alanine aminotransferase; Ang, angiotensin; BDL, bile duct ligation; FGF-2, fibroblast growth factor-2; HIF, hypoxia-inducible factor; KC, keratinocyte-derived chemotactic factor; MCP-1, monocyte chemoattractant protein-1; MIP-2, macrophage inflammatory protein-2; PAI-1, plasminogen activator inhibitor-1; PBC, primary biliary cirrhosis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PP, periportal; PSC, primary sclerosing cholangitis; TGF- β , transforming growth factor- β ; TNF- α , tumor necrosis factor- α ; VEGF-A, vascular endothelial growth factor-A.

Several studies have demonstrated that hepatocellular damage causes regions of hypoxia to develop in the liver (Ji et al., 1982; Rosmorduc et al., 1999; Corpechot et al., 2002; Copple et al., 2004; Moon et al., 2009; Rosmorduc and Housset, 2010). We demonstrated recently that the exposure of Kupffer cells to hypoxia *in vitro* increased the expression of PDGF-B, a potent mitogen and chemotaxin for hepatic stellate cells, and monocyte chemoattractant protein-1 (MCP-1), a mediator of hepatic stellate cell chemotaxis (Copple et al., 2010). This study suggested that hypoxia may be a key driving force for the production of profibrotic mediators by macrophages during fibrosis. These studies further demonstrated that the up-regulation of PDGF-B and MCP-1 in hypoxic Kupffer cells required the transcription factor hypoxia-inducible factor-1 α (HIF-1 α) (Copple et al., 2010).

HIFs are a group of transcription factors activated in hypoxic cells (Semenza and Wang, 1992; Gaber et al., 2005; Coleman and Ratcliffe, 2007). The functional HIF transcription factor is composed of an α subunit, either HIF-1 α or HIF-2 α , and a β subunit, HIF-1 β . When cells become hypoxic, α subunits of the protein become stabilized and translocate to the nucleus where they heterodimerize with HIF-1 β and regulate the expression of genes that allow cells to adapt to a hypoxic environment (Cash et al., 2007). Our recent studies demonstrated that HIF-1 α is activated in macrophages in the livers of bile duct ligation (BDL) mice, an animal model of peribiliary fibrosis (Moon et al., 2009). However, whether the activation of HIF-1 α in hepatic macrophages *in vivo* is a key event in the development of liver fibrosis is not known. Accordingly, in the present study, the hypothesis was tested that HIF-1 α activation in macrophages is critical for the up-regulation of profibrotic mediators and the development of liver fibrosis *in vivo*.

Materials and Methods

Animals. To selectively reduce HIF-1 α or HIF-1 β levels in myeloid cells, HIF-1 α ^{fl/fl} and HIF-1 β ^{fl/fl} mice, described in detail previously (Tomita et al., 2000, 2003), were crossed with mice expressing Cre recombinase under the control of the lysozyme M promoter (LysMCre mice; The Jackson Laboratory, Bar Harbor, ME) (Clausen et al., 1999). In these mice, Cre recombinase is expressed in monocytes, macrophages, and neutrophils (Clausen et al., 1999). The offspring of the HIF-1 α ^{fl/fl} mouse breeding were HIF-1 α ^{fl/fl}LysMCre⁺ (i.e., myeloid cells deficient in HIF-1 α) or HIF-1 α ^{fl/fl}LysMCre⁻ (i.e., normal HIF-1 α levels in myeloid cells). The offspring of the HIF-1 β ^{fl/fl} mouse breeding were HIF-1 β ^{fl/fl}LysMCre⁺ (i.e., myeloid cells deficient in HIF-1 β) or HIF-1 β ^{fl/fl}LysMCre⁻ (i.e., normal

HIF-1 β levels in myeloid cells). Polymerase chain reaction (PCR) of genomic DNA was used to detect the floxed HIF-1 α gene, floxed HIF-1 β gene, and the Cre transgene as described previously (Tomita et al., 2000, 2003; Copple et al., 2009).

All of the mice were maintained on a 12-h light/dark cycle under controlled temperature (18–21°C) and humidity. Food (Rodent Diet; Harlan Teklad, Madison, WI) and tap water were allowed *ad libitum*. All of the procedures on animals were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* promulgated by the National Institutes of Health (Institute of Laboratory Animal Resources, 1996).

BDL. Mice were subjected to BDL as described previously (Kim et al., 2006). At 10 days after surgery, mice were anesthetized with sodium pentobarbital and euthanized by exsanguination.

Real-Time PCR. RNA was isolated using TRIzol reagent (Sigma-Aldrich, St. Louis, MO), and contaminating DNA was removed using the TURBO DNA-free kit (Applied Biosystems, Foster City, CA). The RNA then was reverse-transcribed into cDNA as described previously by us (Kim et al., 2006). Real-time PCR was performed on a 7900 real-time PCR instrument (Applied Biosystems) with the SYBR green DNA PCR kit (Applied Biosystems). Real-time PCR was used to quantify mRNA levels on a Prism 7300 real-time PCR instrument (Applied Biosystems) using the SYBR green DNA PCR kit (Applied Biosystems) as described previously (Kim et al., 2006). Primer sequences for real-time PCR are shown in Table 1.

Kupffer Cell Isolation. Kupffer cells were isolated from mice and exposed to hypoxia as described previously by us (Copple et al., 2010).

Western Blot Analysis. Nuclear extracts were isolated from Kupffer cells as described previously (Copple et al., 2010). For Western blot analysis, aliquots (15 μ g) of nuclear extracts were subjected to 10% SDS-polyacrylamide gel electrophoresis, and proteins were transferred to Immobilon polyvinylidene difluoride transfer membranes (Millipore Corporation, Billerica, MA). The membranes then were probed with rabbit polyclonal anti-HIF-1 α antibody (NB100-449; Novus Biologicals, Inc., Littleton, CO) diluted 1:1000 or mouse monoclonal anti-HIF-1 β (Millipore Corporation) diluted 1:1000 followed by incubation with goat anti-rabbit antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for HIF-1 α or goat anti-mouse antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Inc.) for HIF-1 β .

For TGF- β 1 Western blot analysis, aliquots of liver extract (50 μ g) were separated on a 10% SDS-polyacrylamide gel under nonreducing conditions. TGF- β 1 was detected using a monoclonal anti-TGF- β 1 antibody (clone 9016; R&D Systems, Minneapolis, MN).

Assessment of Hepatic Injury and Serum Alkaline Phosphatase, Bilirubin, and Bile Acid Concentrations. Hepatocyte injury was evaluated by measuring the activity of alanine aminotransferase (ALT) (Pointe Scientific, Inc., Lincoln Park, MI). Serum bile acid concentrations were determined by using a commercially available kit (Colorimetric Total Bile Acids Assay Kit; Bio-Quant,

TABLE 1
Primer sequences from real-time PCR analysis

Gene Name	Forward Primer	Reverse Primer
18S	5'-TTGACGGAAGGGCACCACCAG-3'	5'-GCACCACCACCCACGGAATCG-3'
α -SMA	5'-CCACCGCAAATGCTTCTAAGT-3'	5'-GGCAGGAATGATTGGAAAGG-3'
Collagen type I α 1	5'-TGTGTTCCCTACTCAGCCGTCT-3'	5'-CATCGGTCATGCTCTCTCCAA-3'
MCP-1	5'-ACATTCGGCGGTTGCTCTAGA-3'	5'-ACATCCTGTATCCACACGGCAG-3'
PAI-1	5'-AGTCTTTCGACCAAGAGCA-3'	5'-ATCACTTGCCCATGAAGAG-3'
PDGF-B	5'-CCCACAGTGGCTTTTCATTT-3'	5'-GTGGAGGAGCAGACTGAAGG-3'
FGF-2	5'-AGCGACCCACACGTCAAACACTAC-3'	5'-CAGCCGTCCATCTTCTTCATA-3'
KC	5'-TGGCTGGGATTACCTCAAG-3'	5'-GTGGCTATGACTTCGGTTTGG-3'
MIP-2	5'-CTTTGGTTCTTCCGTTGAGG-3'	5'-CAAAAAGTTTGCCTTGACCC-3'
TNF- α	5'-GACCTCACACTCAGATCATCTTCT-3'	5'-CCTCCACTTGGTGGTTTGGT-3'
VEGF-A	5'-CATCTTCAAGCCGCTCTGTGT-3'	5'-CTCCAGGGCTTCATCGTTACA-3'
Ang-1	5'-TTTGCAATCTTCGCTGCCA-3'	5'-GGCATTGCCCCATGTTGA-3'
Ang-2	5'-ACCTTCAGAGACTGTGCGGAAA-3'	5'-CGTCCATGTCACAGTAGGCCTT-3'

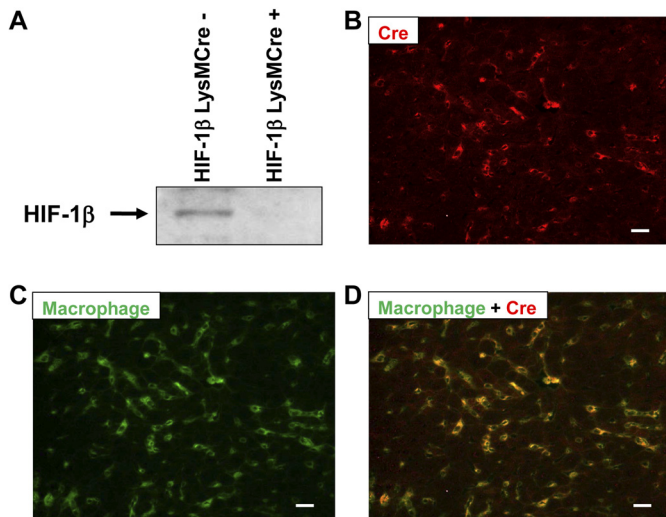


Fig. 1. Decreased HIF-1 β protein in Kupffer cells isolated from HIF-1 β LysMCre⁺ mice. A, Kupffer cells were isolated from HIF-1 β LysMCre⁻ and HIF-1 β LysMCre⁺ mice, and HIF-1 β was detected by Western blot analysis. HIF-1 β LysMCre⁺ mice were subjected to BDL. B–D, 10 days after surgery, immunohistochemistry was used to detect Cre recombinase (red staining in B and D) and macrophages (green staining in C and D). B, C, and D show the same field. Yellow staining in D demonstrates the colocalization of Cre recombinase and macrophages. Scale bar, 50 μ m.

San Diego, CA). Alkaline phosphatase (ALP) and bilirubin were quantified in serum by using commercially available kits (Pointe Scientific, Inc.).

Quantification of Type I Collagen, Cre Recombinase, Macrophages, and Neutrophils in the Liver. Type I collagen in the liver was detected using immunohistochemistry and quantified morphometrically by analyzing the area of immunohistochemical staining of type I collagen as described previously by us (Kim et al., 2006; Moon et al., 2009). An increase in the area of type I collagen staining in the liver is an indicator of fibrosis. Fluorescent staining in liver sections was visualized on a BX41 microscope (Olympus, Tokyo, Japan). For morphometric analysis of the total area of type I collagen in a liver section, digital images of 10, randomly chosen, low-power (magnification 100 \times) fields per tissue section were captured using a DP70 camera (Olympus). Samples were coded such that the evaluator was not aware of the treatment, and the same exposure time was used for all of the captured images. Scion Image software (Scion Corporation, Frederick, MD) then was used to quantify the total area of type I collagen (number of positive pixels) using methods described in detail previously (Coppole et al., 2002). The staining is expressed as a fraction of the total area. The 10 random fields analyzed for each liver section were averaged and counted as a replicate (i.e., each replicate represents a different mouse).

Neutrophils were detected in liver sections and quantified as described previously (Kim et al., 2006).

For the quantification of macrophages in the liver, sections of frozen liver were fixed in 4% formalin and then incubated with rat anti-mouse CD68 and rat anti-mouse F4/80 antibodies diluted 1:100 (AbD Serotec, Raleigh, NC). The sections then were incubated with

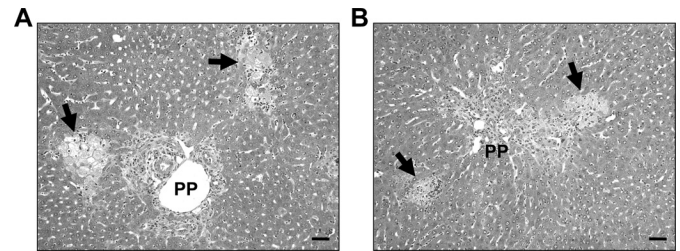


Fig. 2. Liver injury in HIF-1 β LysMCre⁻ and HIF-1 β LysMCre⁺ mice after BDL. HIF-1 β LysMCre⁻ and HIF-1 β LysMCre⁺ mice were subjected to sham operation or BDL. Ten days after surgery, liver sections were stained with hematoxylin and eosin. A and B, representative photomicrographs from HIF-1 β LysMCre⁻ (A) and HIF-1 β LysMCre⁺ (B) mice subjected to BDL. Arrows indicate areas of necrosis. Scale bar, 50 μ m.

goat anti-rat antibody conjugated to Alexa Fluor 488. The area of immunohistochemical staining then was quantified in liver sections as described above for collagen quantification.

For the detection of Cre recombinase in the liver, sections of frozen liver were fixed in 4% formalin and then incubated with rabbit anti-Cre recombinase antibody diluted 1:4000 (Imgenex, San Diego, CA). The sections then were incubated with goat anti-rabbit antibody conjugated to Alexa Fluor 594.

Immunohistochemistry for PDGF-B in Sections of Mouse Liver. For PDGF-B immunostaining, livers were frozen in isopentane (Sigma-Aldrich) immersed in liquid nitrogen for 8 min. Sections of frozen liver were fixed in acetone for 10 min at -20°C . Sections were incubated with rabbit polyclonal anti-PDGF-B antibody (Bio-world Technology, Inc., St. Louis Park, MN) diluted 1:50 in phosphate-buffered saline (PBS) containing 3% goat serum at room temperature for 3 h. The sections were washed with PBS and then incubated with secondary antibody conjugated to Alexa Fluor 488 (green staining; Invitrogen, Carlsbad, CA). Macrophages were detected in the same sections as described above.

HIF-1 α Immunohistochemistry in Sections of Human Liver. Research involving human livers was reviewed by the University of Kansas Medical Center Human Research Protection Program, and the use of deidentified human liver samples was approved. The specimens used in this study were collected and provided by the KU Liver Center Tissue Bank at The University of Kansas Medical Center. Diseased liver tissue used was from patients with primary biliary cirrhosis (PBC) (2 females, ages 60, 61; 1 male, age 47) and patients with primary sclerosing cholangitis (PSC) (2 males, ages 45, 55; 1 female, age 70). Control liver sections were obtained from liver tissue taken from donor livers before transplantation. Five-micrometer frozen sections were fixed for 10 min in 4% neutral-buffered formalin, washed with PBS, and then blocked for 30 min in 10% goat serum. Sections then were incubated with primary antibodies, rabbit anti-human HIF-1 α (NB100-134; Novus Biologicals, Inc.) diluted 1:100 and mouse anti-human CD68 (Ab-3; Thermo Fisher Scientific, Waltham, MA) or mouse anti-human α -smooth muscle actin (α -SMA) (Millipore Bioscience Research Reagents, Temecula, CA) each diluted 1:100 overnight at 4°C . Sections were washed subsequently with PBS and then incubated

TABLE 2

Serum biomarkers of liver injury and cholestasis in HIF-1 β LysMCre⁻ and HIF-1 β LysMCre⁺ mice after BDL

HIF-1 β LysMCre⁻ and HIF-1 β LysMCre⁺ mice were subjected to sham operation or BDL. Ten days after surgery, ALT activity, bilirubin concentrations, ALP activity, and bile acid concentrations were quantified in serum. Data are expressed as mean \pm S.E.M.; $n = 8$.

Biomarker	Sham Operation		BDL	
	HIF-1 β LysMCre ⁻	HIF-1 β LysMCre ⁺	HIF-1 β LysMCre ⁻	HIF-1 β LysMCre ⁺
ALT, U/l	5.6 \pm 0.9	22.0 \pm 17.6	470.3 \pm 62.1 ^a	463.9 \pm 71.6 ^a
Bilirubin, mg/dl	0.2 \pm 0.1	0.1 \pm 0.1	15.0 \pm 3.4 ^a	15.9 \pm 3.6 ^a
ALP, U/l	124.2 \pm 9.8	123.1 \pm 11.6	846.0 \pm 261.5 ^a	970.8 \pm 381.1 ^a
Bile acids, μ M	5.6 \pm 0.9	22.0 \pm 17.6	428.9 \pm 25.3 ^a	414.6 \pm 31.8 ^a

^a Significantly different ($p < 0.05$) from sham-operated mice.

with secondary Alexa Fluor 594-conjugated rabbit anti-mouse and Alexa Fluor 488-conjugated goat anti-rabbit secondary antibodies, each diluted at 1:500 in blocking buffer for 1 h at room temperature. Sections were washed with PBS and counterstained with 4,6-diamidino-2-phenylindole.

Statistical Analysis. Results are presented as the mean \pm S.E.M. Data were analyzed by an analysis of variance. Data expressed as a fraction were transformed by arcsine square root before the analysis. Comparisons among group means were made using the Student-Newman-Keuls test. The criterion for significance was $p < 0.05$ for all of the studies.

Results

HIF-1 β Deletion in Myeloid Cells Does Not Affect Liver Injury or Hepatic Inflammation after BDL. To determine whether HIF-1 β in myeloid cells is critical for the development of liver fibrosis, HIF-1 $\beta^{\text{fl/fl}}$ mice were crossed with LysMCre mice. To confirm that HIF-1 β was deleted in hepatic macrophages (i.e., Kupffer cells), these cells were isolated from HIF-1 β LysMCre $^{-}$ and HIF-1 β LysMCre $^{+}$ mice, and HIF-1 β was detected by Western blot analysis. HIF-1 β was detected in Kupffer cells from HIF-1 β LysMCre $^{-}$ mice but not in Kupffer cells from HIF-1 β LysMCre $^{+}$ mice (Fig. 1A). Next, to confirm that Cre recombinase was only expressed in macrophages in the liver, Cre recombinase was detected by immunohistochemistry in liver sections from

HIF-1 β LysMCre $^{+}$ mice subjected to BDL (Fig. 1B, red staining). Macrophages then were identified in the same liver section by immunohistochemistry (Fig. 1C, green staining). When Cre recombinase immunostaining and macrophage immunostaining were overlaid, Cre recombinase was only detected in hepatic macrophages (Fig. 1D).

Next, HIF-1 β LysMCre $^{-}$ and HIF-1 β LysMCre $^{+}$ mice were subjected to BDL, and liver injury and inflammation were assessed at 10 days after surgery. ALT, bilirubin, ALP, and bile acids in serum were increased to a similar extent in HIF-1 β LysMCre $^{-}$ mice and HIF-1 β LysMCre $^{+}$ mice at 10 days after BDL (Table 2). In addition, the extent of necrosis was similar in these mice after BDL (Figs. 2).

To evaluate the extent of hepatic inflammation after BDL, macrophages and neutrophils were detected in liver sections by immunohistochemistry. In addition, levels of proinflammatory mediators were quantified by real-time PCR. Macrophage and neutrophil numbers were increased to a similar extent in the livers of HIF-1 β LysMCre $^{-}$ and HIF-1 β LysMCre $^{+}$ mice after BDL (Fig. 3). In addition, up-regulation of the chemokines keratinocyte-derived chemotactic factor (KC) and macrophage inflammatory protein-2 (MIP-2) and the cytokine tumor necrosis factor- α (TNF- α) were unaffected by the deficiency of HIF-1 β in myeloid cells (Table 3).

HIF-1 β Deletion in Myeloid Cells Reduces Liver Fibrosis after BDL. Next, we evaluated biomarkers of liver

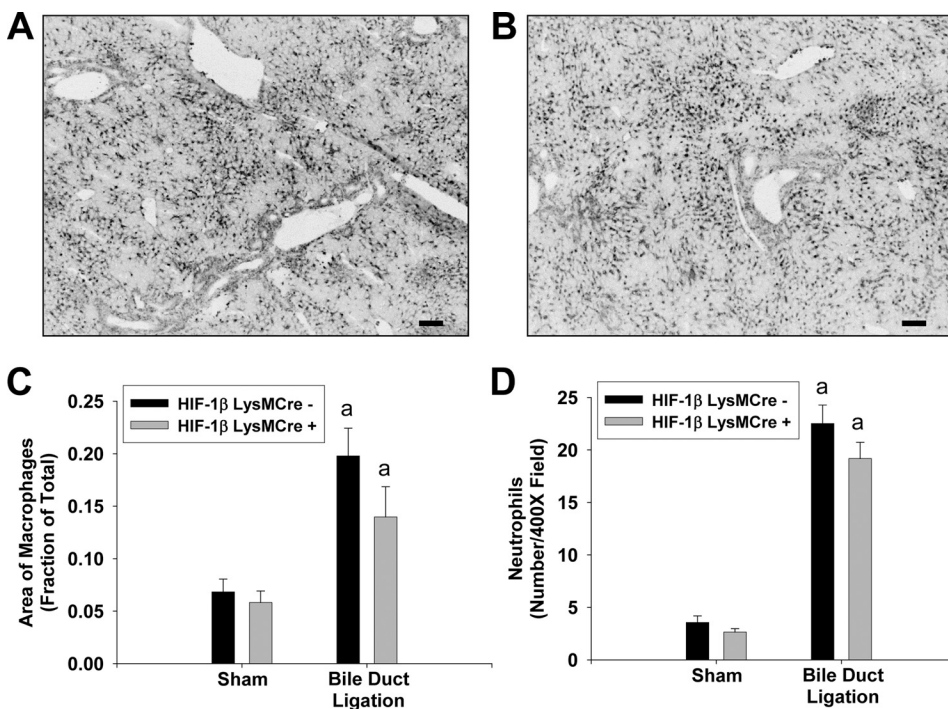


Fig. 3. Levels of macrophages and neutrophils in HIF-1 β LysMCre $^{-}$ and HIF-1 β LysMCre $^{+}$ mice after BDL. HIF-1 β LysMCre $^{-}$ and HIF-1 β LysMCre $^{+}$ mice were subjected to sham operation or BDL. Ten days after surgery, macrophages and neutrophils were detected in the liver by immunohistochemistry. A and B, representative photomicrographs of immunohistochemistry for macrophages in liver sections from HIF-1 β LysMCre $^{-}$ (A) and HIF-1 β LysMCre $^{+}$ (B) mice subjected to BDL. Positive staining appears black in the photomicrographs. Scale bar, 100 μ m. C, the area of macrophage immunostaining was quantified using morphometric techniques. D, neutrophils were quantified in liver sections. Data are expressed as mean \pm S.E.M.; $n = 8$. a, significantly different ($p < 0.05$) from sham-operated mice.

TABLE 3

mRNA levels of proinflammatory mediators in HIF-1 β LysMCre $^{-}$ and HIF-1 β LysMCre $^{+}$ mice after BDL

HIF-1 β LysMCre $^{-}$ and HIF-1 β LysMCre $^{+}$ mice were subjected to sham operation or BDL. Ten days after surgery, mRNA levels were quantified by real-time PCR. Data are expressed as mean \pm SEM; $n = 8$.

Gene Name/Function	Sham Operation		BDL	
	HIF-1 β LysMCre $^{-}$	HIF-1 β LysMCre $^{+}$	HIF-1 β LysMCre $^{-}$	HIF-1 β LysMCre $^{+}$
KC	1.0 \pm 0.2	2.4 \pm 1.0	8.3 \pm 1.9 ^a	8.2 \pm 0.8 ^a
MIP-2	1.0 \pm 0.3	2.9 \pm 1.1	14.2 \pm 0.3 ^a	20.6 \pm 0.3 ^a
TNF- α	1.2 \pm 0.6	1.2 \pm 0.4	4.7 \pm 1.0 ^a	3.4 \pm 0.7 ^a

^a Significantly different ($p < 0.05$) from sham-operated mice.

fibrosis to test the hypothesis that HIF activation in myeloid cells is required for the development of liver fibrosis after BDL. α -SMA mRNA levels were increased to a greater extent in the livers of HIF-1 β LysMCre⁻ mice after BDL compared with those of BDL HIF-1 β LysMCre⁺ mice (Fig. 4A). Type I collagen mRNA levels were increased in the livers of HIF-1 β LysMCre⁻ mice after BDL (Fig. 4B). Type I collagen mRNA levels were significantly lower in HIF-1 β LysMCre⁺

mice after BDL (Fig. 4B). Extensive deposition of type I collagen protein was observed in periportal (PP) regions of livers from HIF-1 β LysMCre⁻ mice after BDL (Fig. 4C). Lower levels of type I collagen were observed in BDL HIF-1 β LysMCre⁺ mice (Fig. 4D). A significant reduction in hepatic type I collagen protein in these mice was confirmed by the quantification of the area of immunohistochemical staining of type I collagen in liver sections (Fig.

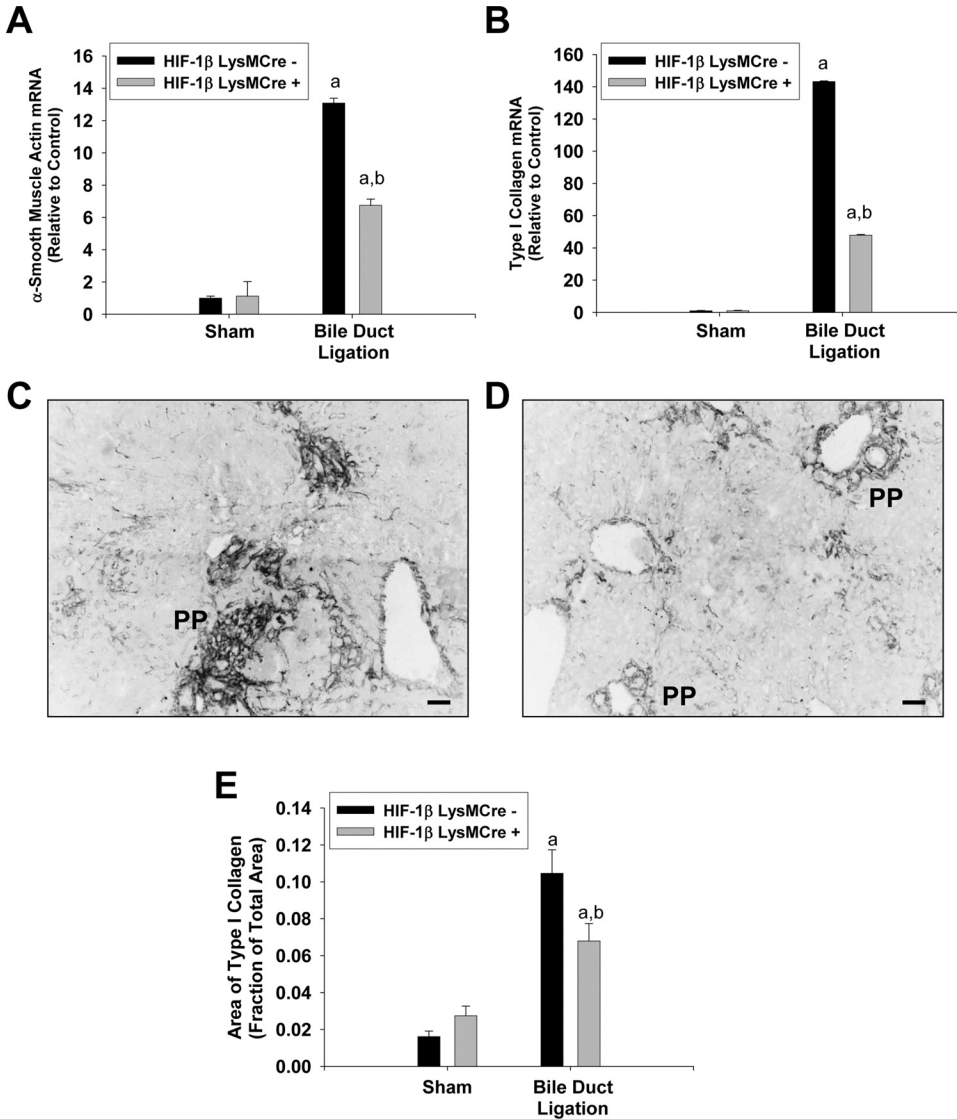


Fig. 4. Liver fibrosis in HIF-1 β LysMCre⁻ and HIF-1 β LysMCre⁺ mice after BDL. HIF-1 β LysMCre⁻ and HIF-1 β LysMCre⁺ mice were subjected to sham operation or BDL. A and B, ten days after surgery, mRNA levels of α -SMA (A) and type I collagen (B) were quantified by real-time PCR. C and D, representative photomicrographs of type I collagen immunohistochemistry in liver sections from BDL HIF-1 β LysMCre⁻ (C) and HIF-1 β LysMCre⁺ (D) mice. Positive staining appears dark gray in the photomicrographs. Scale bar, 50 μ m. E, the area of type I collagen immunostaining was quantified in liver sections. Data are expressed as mean \pm S.E.M.; *n* = 8. a, significantly different (*p* < 0.05) from sham-operated mice. b, significantly different (*p* < 0.05) from BDL HIF-1 β LysMCre⁻ mice.

TABLE 4

mRNA levels of profibrotic and proangiogenic mediators in HIF-1 β LysMCre⁻ and HIF-1 β LysMCre⁺ mice after BDL

HIF-1 β LysMCre⁻ and HIF-1 β LysMCre⁺ mice were subjected to sham operation or BDL. Ten days after surgery, mRNA levels were quantified by real-time PCR. Data are expressed as mean \pm SEM; *n* = 8.

Gene Name/Function	Sham Operation		BDL	
	HIF-1 β LysMCre ⁻	HIF-1 β LysMCre ⁺	HIF-1 β LysMCre ⁻	HIF-1 β LysMCre ⁺
PAI-1	1.5 \pm 1.0	1.5 \pm 1.0	148.1 \pm 56.0 ^a	174.4 \pm 43.9 ^a
FGF-2	1.0 \pm 0.1	0.7 \pm 0.1	3.7 \pm 1.6 ^a	1.5 \pm 0.3
PDGF-B	1.0 \pm 0.1	1.0 \pm 0.8	14.5 \pm 0.3 ^a	4.7 \pm 0.4 ^{a,b}
MCP-1	1.0 \pm 1.3	1.0 \pm 1.7	12.3 \pm 0.3 ^a	3.8 \pm 0.6 ^{a,b}
VEGF-A	1.0 \pm 0.1	0.7 \pm 0.05 ^a	0.4 \pm 0.01 ^a	0.4 \pm 0.02 ^a
Ang-1	1.0 \pm 0.1	1.9 \pm 1.1	1.0 \pm 0.1	1.0 \pm 0.1
Ang-2	1.0 \pm 0.2	0.7 \pm 0.2	1.6 \pm 0.4	1.4 \pm 0.3

^a Significantly different (*p* < 0.05) from sham-operated mice.

^b Significantly different (*p* < 0.05) from BDL HIF-1 β LysMCre⁻ mice.

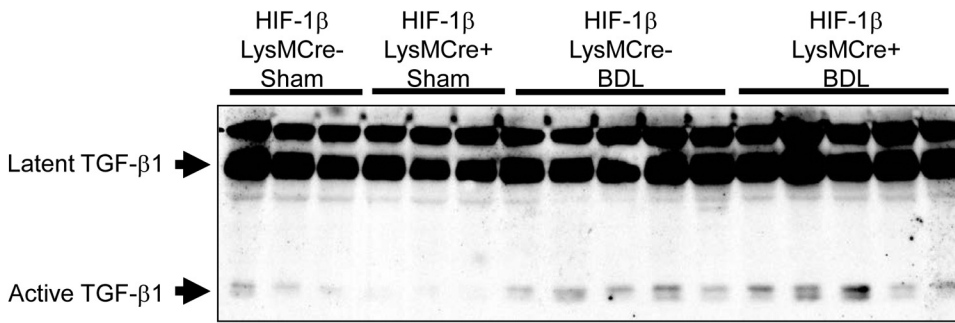


Fig. 5. TGF- β 1 protein levels in the livers of HIF-1 β LysMCre⁻ and HIF-1 β LysMCre⁺ mice after BDL. HIF-1 β LysMCre⁻ and HIF-1 β LysMCre⁺ mice were subjected to sham operation or BDL. Ten days after surgery, Western blot analysis was used to detect TGF- β 1 in liver homogenates.

4E). These data demonstrate that HIF signaling in myeloid cells is important for the development of liver fibrosis after BDL.

HIF-1 β in Myeloid Cells Is Required for Up-Regulation of PDGF-B after BDL. Next, we determined whether HIF-1 β deficiency affected the up-regulation of the mediators that are important for the development of fibrosis, such as plasminogen activator inhibitor-1 (PAI-1), fibroblast growth factor-2 (FGF-2), PDGF-B, MCP-1, and TGF- β 1. In addition, we determined the impact of HIF deficiency in myeloid cells on the levels of proangiogenic mediators, including vascular endothelial growth factor-A (VEGF-A), angiopoietin-1 (Ang-1), and Ang-2. PAI-1, FGF-2, PDGF-B, and MCP-1 mRNA levels were increased in HIF-1 β LysMCre⁻ mice after BDL (Table 4). PDGF-B and MCP-1 mRNA levels were significantly lower in HIF-1 β LysMCre⁺ after BDL compared with those in HIF-1 β LysMCre⁻ mice (Table 4). In contrast, VEGF-A, Ang-1, and Ang-2 mRNA levels were not increased significantly in the livers of either HIF-1 β LysMCre⁻ or HIF-1 β LysMCre⁺ mice after BDL (Table 4). To confirm this, we evaluated VEGF-A protein levels by Western blot and immunohistochemistry analyses. Consistent with VEGF-A mRNA levels, VEGF-A protein levels were not increased in the liver at 10 days after BDL (data not shown).

Levels of latent TGF- β 1 protein (75 kDa) and active TGF- β 1 protein (25 kDa) were increased to a similar extent in the livers of HIF-1 β LysMCre⁻ and HIF-1 β LysMCre⁺ mice after BDL (Fig. 5). Consistent with this, the ratio of active TGF- β 1 to latent TGF- β 1 was not different between HIF-1 β LysMCre⁻ and HIF-1 β LysMCre⁺ mice after BDL (HIF-1 β LysMCre⁻, 0.44 ± 0.07 , versus HIF-1 β LysMCre⁺, 0.53 ± 0.05).

Because myeloid cell-specific HIF-1 β deficiency largely prevented the increase in PDGF-B and MCP-1 mRNA levels in the liver, we next evaluated whether PDGF-B and MCP-1 protein levels were increased in a HIF-1 β -dependent manner. Immunohistochemistry was used to detect PDGF-B protein in hepatic macrophages. PDGF-B protein was not detected in the livers from sham-operated mice (data not shown). PDGF-B protein levels were increased in the livers of HIF-1 β LysMCre⁻ mice after BDL (Fig. 6B, green staining). Furthermore, PDGF-B protein (green staining) colocalized with hepatic macrophages (red staining) in these mice (Fig. 6C). In contrast, PDGF-B protein was not detected in hepatic macrophages from HIF-1 β LysMCre⁺ mice after BDL (Figs. 6, D–F). Next, serum concentrations of MCP-1 protein were quantified. In contrast to MCP-1 mRNA levels (Table 4), MCP-1 protein concentrations were increased to a similar extent in HIF-1 β LysMCre⁻ and HIF-1 β LysMCre⁺ mice after BDL (Fig. 6G).

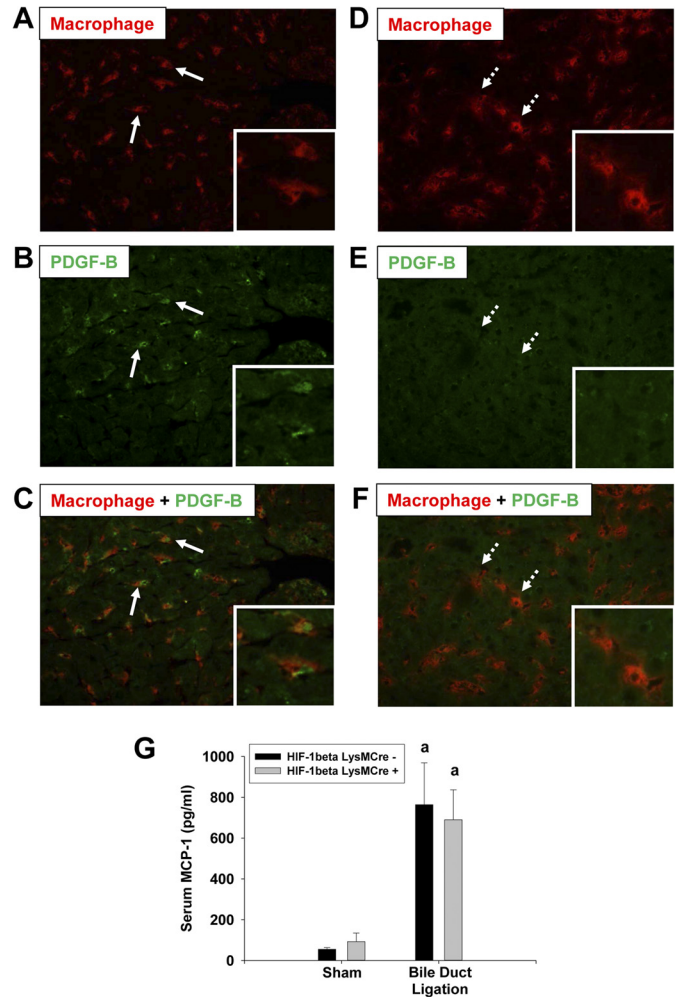


Fig. 6. Quantification of PDGF-B and MCP-1 protein in HIF-1 β LysMCre⁻ and HIF-1 β LysMCre⁺ mice after BDL. HIF-1 β LysMCre⁻ and HIF-1 β LysMCre⁺ mice were subjected to sham operation or BDL. A–F, 10 days after surgery, immunohistochemistry was used to detect macrophages (red staining in A, C, D, and F) and PDGF-B (green staining in B, C, E, and F) in liver sections from HIF-1 β LysMCre⁻ (A, B, and C) and HIF-1 β LysMCre⁺ (D, E, and F) mice subjected to BDL. Arrows indicate PDGF-B-positive macrophages from HIF-1 β LysMCre⁻ mice. Dashed arrows indicate PDGF-B-negative macrophages from HIF-1 β LysMCre⁺ mice. G, MCP-1 protein was quantified in serum. Data are expressed as mean \pm S.E.M.; $n = 8$. a, significantly different ($p < 0.05$) from sham-operated mice.

HIF-1 α in Myeloid Cells Contributes to the Development of Liver Fibrosis. During hypoxia, HIF-1 β heterodimerizes with either HIF-1 α or HIF-2 α to regulate gene expression (Cash et al., 2007). Because our results above demonstrate a role for myeloid HIF-1 β in liver fibrosis, we

determined whether HIF-1 α contributed to liver fibrosis and the up-regulation of profibrotic genes. To determine the role of HIF-1 α in myeloid cells, HIF-1 $\alpha^{fl/fl}$ mice were crossed with LysMCre mice. Kupffer cells were isolated from HIF-1 α LysMCre⁻ mice and HIF-1 α LysMCre⁺ mice and exposed to 1% oxygen, a concentration of oxygen that stimulates nuclear accumulation of HIF-1 α in these cells (Copple et al., 2010). HIF-1 α protein was detected in nuclear extracts from Kupffer cells isolated from HIF-1 α LysMCre⁻ mice (Fig. 7A). HIF-1 α protein levels were substantially lower in hypoxic Kupffer cells isolated from HIF-1 α LysMCre⁺ mice (Fig. 7A).

ALT activity was increased to a similar extent in HIF-1 α LysMCre⁻ and HIF-1 α LysMCre⁺ mice after BDL (Fig. 7B). In addition, pathological changes that occurred in the liver were similar (Fig. 7, C and D). mRNA levels of α -SMA, type I collagen, and PDGF-B were increased in HIF-1 α LysMCre⁻ mice after BDL (Fig. 8). mRNA levels of all of these genes were significantly lower in BDL HIF-1 α LysMCre⁺ mice (Fig. 8).

Activation of HIF-1 α in Macrophages, Hepatocytes, and Fibroblasts in the Livers of Patients with PBC and PSC. Immunohistochemistry was used to detect HIF-1 α in macrophages and fibroblasts in the livers from three patients with PBC, three patients with PSC, and three normal human livers. Nuclear accumulation of HIF-1 α was observed in macrophages in the liver of a patient with PBC (Fig. 9A). In addition, nuclear HIF-1 α was observed in hepatocytes adjacent to HIF-1 α -positive macrophages (Fig. 9A). HIF-1 α was detected in macrophages in the livers from all three patients with PBC. Likewise, nuclear HIF-1 α was observed in macrophages in all of the livers from patients with PSC (Fig. 9C). In contrast, HIF-1 α protein was not detected in the nuclei of macrophages in normal human livers (Fig. 9E). We also observed nuclear HIF-1 α protein in cells within fibrotic regions of livers from patients with PBC and PSC; therefore, we determined whether fibroblasts, identified by α -SMA im-

munostaining, were positive for HIF-1 α . As shown in Fig. 9G, HIF-1 α protein was detected in the nuclei of α -SMA-positive cells in all of the livers from patients with PBC and PSC. In addition, HIF-1 α protein was detected in the nuclei of hepatocytes adjacent to regions of bridging fibrosis (Fig. 9G).

Discussion

Our results demonstrate that HIF-1 β and HIF-1 α in myeloid cells contribute to the development of liver fibrosis after BDL. In LysMCre mice, Cre recombinase is expressed in macrophages and neutrophils (Clausen et al., 1999). Accordingly, it is possible that HIF-1 β and HIF-1 α deletion in macrophages and/or neutrophils was responsible for the reduction of fibrosis observed in our studies. However, a recent study showed that neutrophil depletion does not affect the development of liver fibrosis after BDL (Saito et al., 2003). Accordingly, it is unlikely that HIF activation in neutrophils contributes to the development of fibrosis after BDL. Therefore, our results indicate HIF-1 β and HIF-1 α in macrophages is important for liver fibrosis.

The mechanism by which the activation of HIFs in macrophages promoted fibrosis may be in part due to HIF-dependent production of PDGF-B by hepatic macrophages during chronic injury. PDGF-B is a potent mitogen and chemotaxin for hepatic stellate cells (Friedman and Arthur, 1989; Carloni et al., 1997; Marra et al., 1997). Furthermore, PDGF-B increases the expression of α -SMA in peribiliary fibroblasts, and the inhibition of PDGF-B signaling reduces fibrosis after BDL (Kinnman et al., 2003). Consistent with our results (Fig. 6), others have demonstrated that hepatic macrophages express PDGF-B in the liver during chronic injury (Pinzani et al., 1996). However, the mechanism by which chronic injury increases the expression of PDGF-B in hepatic macrophages has remained largely unknown. We demonstrated previously that HIF-1 α is activated in hepatic macrophages after BDL

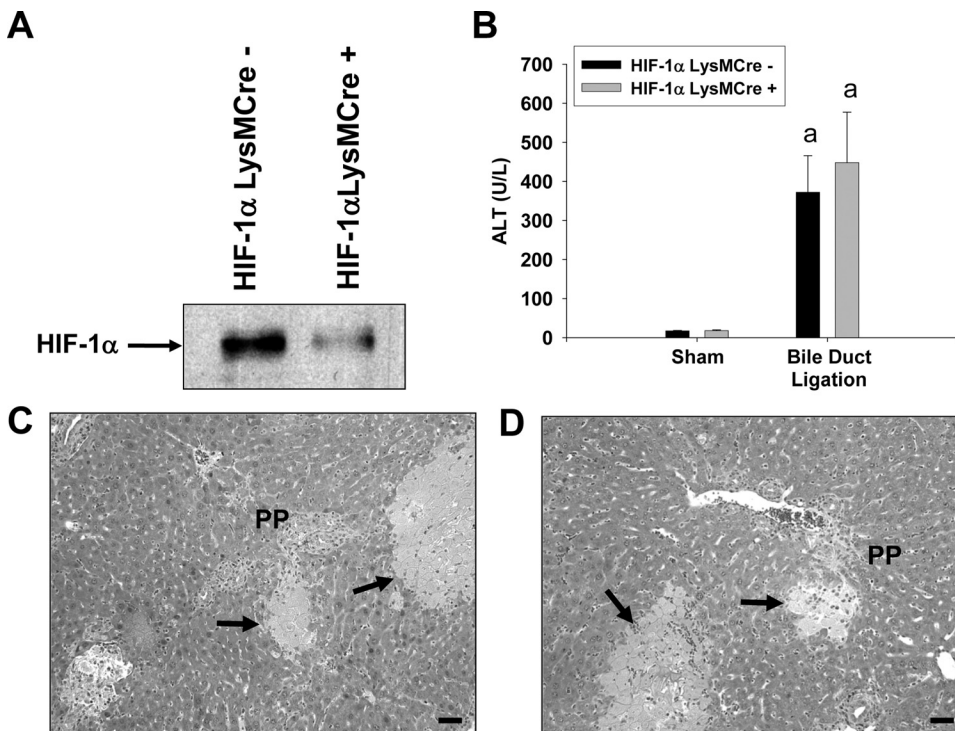


Fig. 7. Liver injury in HIF-1 α LysMCre⁻ and HIF-1 α LysMCre⁺ mice after BDL. **A**, Kupffer cells were isolated from HIF-1 α LysMCre⁻ and HIF-1 α LysMCre⁺ mice and exposed to 1% oxygen for 1 h. HIF-1 α was detected in nuclear extracts by Western blot analysis. HIF-1 α LysMCre⁻ and HIF-1 α LysMCre⁺ mice were subjected to sham operation or BDL. **B**, 10 days after surgery, ALT activity was measured in serum. Data are expressed as mean \pm S.E.M.; $n = 8$. **a**, significantly different ($p < 0.05$) from sham-operated mice. **C** and **D**, representative photomicrographs from HIF-1 α LysMCre⁻ (**C**) and HIF-1 α LysMCre⁺ (**D**) mice subjected to BDL. Arrows indicates area of necrosis. Scale bar, 50 μ m.

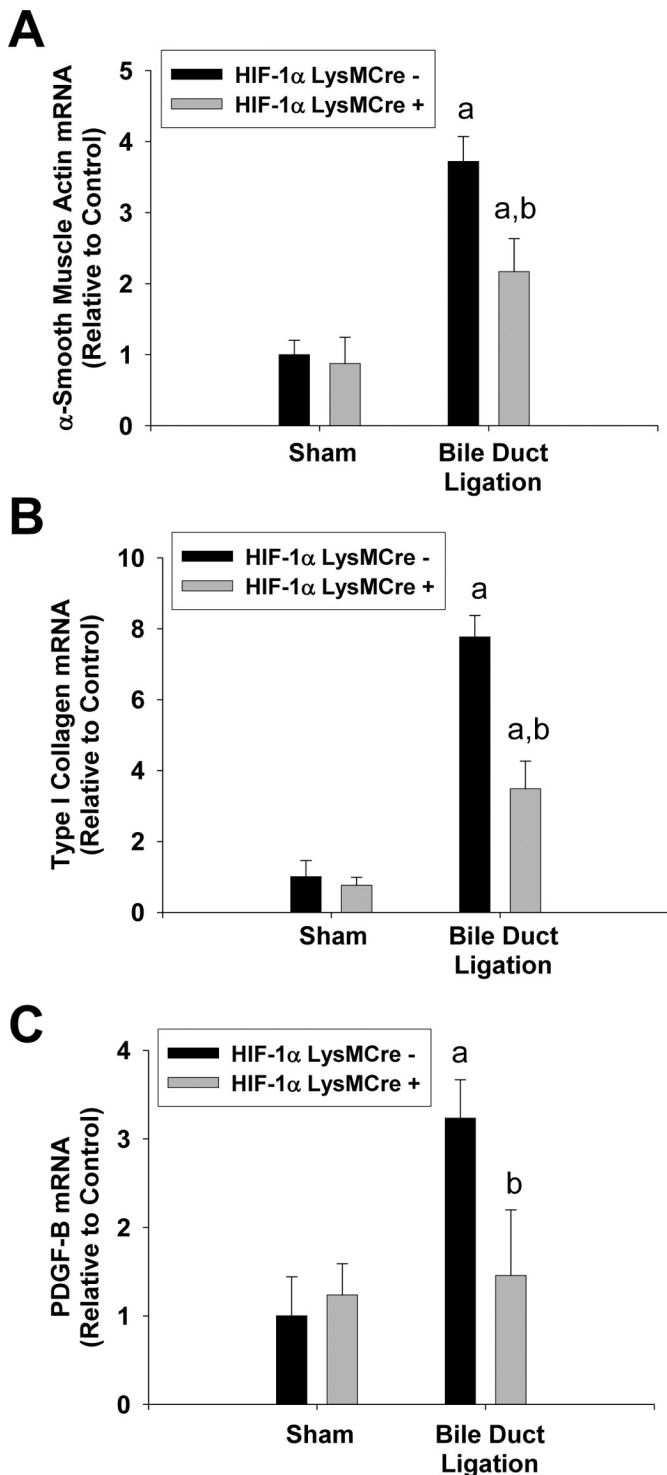


Fig. 8. Liver fibrosis in HIF-1 α LysMCre⁻ and HIF-1 α LysMCre⁺ mice after BDL. HIF-1 α LysMCre⁻ and HIF-1 α LysMCre⁺ mice were subjected to sham operation or BDL. A–C, 10 days after surgery, mRNA levels of α -SMA (A), type I collagen (B), and PDGF-B (C) were quantified by real-time PCR. Data are expressed as mean \pm S.E.M.; $n = 8$. a, significantly different ($p < 0.05$) from sham-operated mice. b, significantly different ($p < 0.05$) from BDL HIF-1 α LysMCre⁻ mice.

(Moon et al., 2009). We also demonstrated that the exposure of Kupffer cells in vitro to hypoxia up-regulates PDGF-B in a HIF-1 α -dependent manner (Copple et al., 2010). Collectively, these studies suggested that HIF-1 α may be an important regulator of PDGF-B in the liver during the development of

fibrosis. Consistent with this, our present study demonstrated that the deletion of either HIF-1 α or HIF-1 β in myeloid cells attenuated the increase in PDGF-B mRNA (Fig. 8; Table 4) and prevented the increase in PDGF-B protein in hepatic macrophages during the development of fibrosis in BDL mice (Fig. 6). This was associated with a marked reduction in α -SMA and type I collagen (Figs. 4 and 8). HIF-1 β deletion did not affect hepatic macrophage accumulation (Fig. 3), indicating that the decrease in PDGF-B was not simply due to a reduction in the numbers of hepatic macrophages. It is likely that HIF-1 α and HIF-1 β directly regulate PDGF-B in macrophages, because studies have shown that these HIFs directly regulate this gene in other cell types (Yoshida et al., 2006). Collectively, this suggests that, during chronic injury induced by cholestasis, hepatic macrophages accumulate in hypoxic regions of liver, where hypoxia stimulates the activation of HIF-1 α . HIF-1 α then heterodimerizes with HIF-1 β and regulates the production of PDGF-B that promotes fibrosis. Of importance to human disease, our studies demonstrated further that HIF-1 α is activated in hepatic macrophages in PBC and PSC patients with fibrosis (Fig. 9), suggesting that this mechanism also may be important for the regulation of PDGF-B and the progression of fibrosis in humans with cholestatic liver disease.

An interesting observation from these studies is that although a deficiency of either HIF-1 α or HIF-1 β in myeloid cells reduced liver fibrosis after BDL, it did not affect hepatocyte injury. This suggests that liver fibrosis after BDL is not dependent upon hepatocyte injury. Consistent with this observation, we demonstrated previously that early growth response factor-1 knockout mice had reduced liver injury and neutrophil accumulation after BDL compared with those of wild-type mice but had similar levels of fibrosis (Kim et al., 2006). Likewise, Fickert et al. (2009) demonstrated that farnesoid X receptor knockout mice have reduced liver fibrosis after BDL but have similar levels of liver injury compared with those of wild-type mice. Collectively, these studies indicate that hepatocyte injury after BDL is not an important stimulus for hepatic fibrosis. However, it remains possible that HIFs may contribute to the development of liver injury at earlier times after BDL. Our study focused on a late time point after BDL (i.e., 10 days). Much of the liver injury after BDL occurs within the first few days, and it is possible that the deletion of HIFs delayed the development of liver injury. This may have been sufficient to delay the development of liver fibrosis. Further studies are needed to evaluate this possibility, however.

Studies indicate that angiogenesis is important for the development of hepatic fibrosis (Rosmorduc et al., 1999; Corpechot et al., 2002; Taura et al., 2008). Furthermore, a key function of HIFs is to regulate the production of proangiogenic mediators (Forsythe et al., 1996; Kelly et al., 2003; Yamakawa et al., 2003). Surprisingly, however, although HIF-1 α was activated in the liver after BDL, levels of proangiogenic mediators, such as VEGF-A and Angs, remained unchanged. We reported previously that hypoxia increases the expression of VEGF-A in primary mouse hepatocytes, Kupffer cells, and hepatic stellate cells in a HIF-1 α -dependent manner (Copple et al., 2009, 2010, 2011), demonstrating that HIF-1 α regulates the production of these mediators in these cell types. One possible explanation for the lack of induction of proangiogenic mediators may be that they are

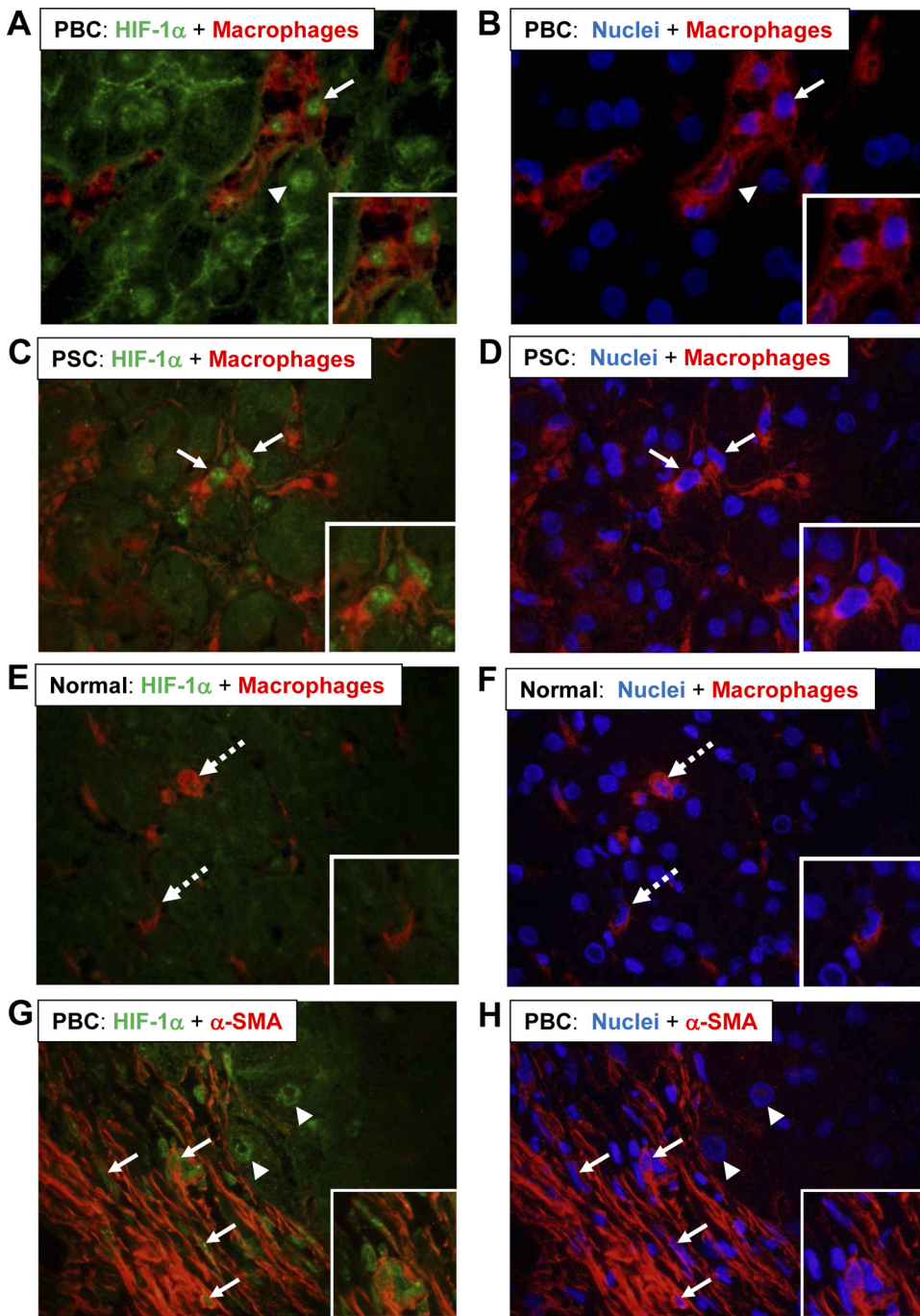


Fig. 9. HIF-1 α activation in the livers of patients with PBC and PSC. A–H, sections of frozen liver from patients with PBC (A, B, G, and H), patients with PSC (C and D), and normal human livers (E and F) were stained for HIF-1 α (green staining in A, C, E, and G) and either CD68 (red staining in A–F) or α -SMA (red staining in G and H). Nuclei were stained with 4,6-diamidino-2-phenylindole (blue staining in B, D, F, and H). The same fields in A, C, E, and G are shown in B, D, F, and H. Insets show higher-power fields. Arrows indicate HIF-1 α -positive nuclei in macrophages (A and C) or α -SMA-positive cells (G). Arrowheads indicate HIF-1 α -positive nuclei in hepatocytes. Dashed arrows indicate HIF-1 α -negative macrophages (E and F).

transiently produced and the time point that we chose did not coincide with the up-regulation of VEGF-A and Angs. In addition, it is possible that the up-regulation of VEGF-A occurs at a later time point in this model. Consistent with this, it was shown previously that the up-regulation of VEGF in the livers of rats after BDL required several weeks (Rosmorduc et al., 1999).

In addition to detecting nuclear HIF-1 α protein in macrophages in the livers of patients with PBC and PSC, we also detected nuclear HIF-1 α protein in α -SMA-positive cells (Fig. 9). We demonstrated recently that the exposure of primary mouse hepatic stellate cells to hypoxia increased the expression of a number of genes involved in the genesis of liver fibrosis, including the chemokine receptors Ccr1 and Ccr5,

prolyl-4-hydroxylase- α 1, prolyl-4-hydroxylase- α 2, and the proangiogenic mediators placental growth factor and Angi-like 4 (Coppole et al., 2011). Many of these were increased in hepatic stellate cells in a HIF-dependent manner (Coppole et al., 2011). Accordingly, it is possible that, in addition to macrophages, the activation of HIF-1 α in hepatic stellate cells may be important for the development of liver fibrosis.

Collectively, these studies demonstrate that HIF-1 α and HIF-1 β are important regulators of profibrotic mediator production by macrophages during the development of liver fibrosis in cholestatic mice. Furthermore, these studies indicate that this mechanism may be important for fibrosis development in humans because HIF-1 α was activated in macrophages in the livers of patients with PBC and PSC.

Accordingly, these studies suggest that therapeutic targeting of HIF-1 α may be effective at preventing the progression of liver fibrosis in patients with cholestatic liver disease and potentially in patients with liver fibrosis produced by other hepatic insults.

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Authorship Contributions

Participated in research design: Copple, Kaska, and Wentling.

Conducted experiments: Copple, Kaska, and Wentling.

Performed data analysis: Copple and Wentling.

Wrote or contributed to the writing of the manuscript: Copple and Kaska.

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Address correspondence to: Bryan L. Copple, Department of Pharmacology and Toxicology, Michigan State University, B403 Life Sciences Building, East Lansing, MI 48823. E-mail: copple@msu.edu