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Loss of Prolyl-Hydroxylase 1 Protects against Biliary Fibrosis via Attenuated Activation of Hepatic Stellate Cells

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Liver fibrosis, eventually progressing to cirrhosis necessitating liver transplantation, poses a significant clinical problem. Oxygen shortage (hypoxia) and hypoxia-inducible transcription factors (HIFs) have been acknowledged as important drivers of liver fibrosis. The significance of oxygen-sensing HIF prolyl-hydroxylase (PHD) enzymes in this context has, however, remained elusive. In this study, we demonstrate that loss of PHD1 (PHD1^{-/-}) attenuates the development of liver fibrosis in mice subjected to chronic bile duct injury, induced by 3,5-diethoxycarbonyl-1,4-dihydrocollidine. This effect was accompanied with reduced recruitment of inflammatory leukocytes and attenuated occurrence of profibrotic myofibroblasts in PHD1^{-/-} livers. Further analyses focused on the significance of PHD1 in the activation of hepatic stellate cells (HSCs), which represent the driving force in liver fibrosis. Primary HSCs isolated from PHD1^{-/-} mice displayed significantly attenuated myofibroblast differentiation and profibrogenic properties compared with HSCs isolated from wild-type mice. Consistently, the expression of various profibrogenic and prometogenic factors was reduced in PHD1^{-/-} HSCs, without alterations in HIF-1 α protein levels. Of importance, PHD1 protein was expressed in HSCs within human livers, and PHD1 transcript expression was significantly increased with disease severity in hepatic tissue from patients with liver fibrosis. Collectively, these findings indicate that PHD1 deficiency protects against liver fibrosis and that these effects are partly due to attenuated activation of HSCs. PHD1 may represent a therapeutic target to alleviate liver fibrosis. (*Am J Pathol* 2018, 188: 2826–2838; <https://doi.org/10.1016/j.ajpath.2018.08.003>)

Liver cirrhosis accounts for approximately 60% of all indications for liver transplantation. Thus, there is a tremendous need for innovative therapeutic approaches to prevent progression from reversible liver fibrosis to liver cirrhosis and eventually liver cancer.^{1,2} Liver fibrosis is characterized by excess deposition of extracellular matrix within hepatic parenchyma.¹ This process is crucially triggered by hepatic stellate cells (HSCs), which become activated by cytokines such as profibrogenic transforming growth factor (TGF)- β to form myofibroblasts.^{3,4} In mice, these processes can be experimentally induced by feeding with xenobiotics such as 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC).^{5,6}

Several studies have highlighted the significance of hypoxia in the development of liver fibrosis after chronic

injury.^{7–9} The effects of hypoxia on cells and organs are crucially mediated by hypoxia-inducible transcription factors (HIF-1 α , HIF-2 α), which are continuously degraded under normoxia but stabilized in hypoxic conditions.^{10–12} HIF prolyl-hydroxylase domain-containing enzymes (PHD1, PHD2, and PHD3) are molecular oxygen sensors

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that regulate the activity of HIFs and other (yet only partly identified) molecular targets. The three PHD enzymes exert specific and nonredundant functions and are implicated in various pathologic disorders characterized by hypoxia.^{13,14} The expression of HIF-1 α increases gradually with the severity of liver fibrosis,^{15,16} and the significance of HIF-1 α in the pathogenesis of liver fibrosis has been well documented.^{8,16} Specific functions of the HIF-PHD enzymes in this context are, by contrast, widely unknown.

However, several reports have highlighted a specific role of PHD1 in liver pathology.¹⁷ Loss of PHD1 reduces the oxygen consumption of hepatocytes exposed to ischemia, thus increasing their hypoxia tolerance and protecting liver tissue against ischemic damage.¹⁸ PHD1 deficiency likewise enhances hepatocyte proliferation and liver regeneration after partial hepatectomy.¹⁹ Of interest, this effect can be mimicked by treatment with pharmacologic PHD enzyme inhibitors.^{19–21}

Given these specific functions of PHD1 in the liver, we aimed to assess the significance of PHD1 in the pathogenesis of liver fibrosis, applying a mouse model of biliary fibrosis.

Materials and Methods

Animal Models

All animal experiments were approved by the ethical commission of the local government and conducted under the NIH's *Guide for the Care and Use of Laboratory Animals*.²² The generation of PHD1 knockout mice has been described elsewhere.²³ Wild-type (WT), globally PHD1-deficient (PHD1^{-/-}), PHD2-deficient (PHD2^{+/-}), and PHD3-deficient (PHD3^{-/-}) mice aged 10 to 12 weeks were used for experiments. Mice were treated with DDC (2.5 mg p.o. diluted in 2 mL/kg body weight corn oil 2 times/week) or vehicle (corn oil, 2 mL/kg body weight 2 times/week) for 42 days. Mice were sacrificed 6 weeks after initial treatment under anesthesia to harvest livers and blood samples under standardized conditions for further examination.

Histology and Immunohistochemistry

Paraffin-embedded liver sections were routinely stained with hematoxylin and eosin (Merck, Darmstadt, Germany). Histomorphometric quantification of cirrhotic liver areas was performed by applying computer-assisted image analysis of 12 standardized high-power fields per specimen by two individual investigators using Zeiss KS300 morphometry software (Zeiss, Jena, Germany) and was expressed as percentage of the total parenchymal area. Porphyrin pigment plugs were counted in 12 high-power fields per section by two individual investigators of hematoxylin and eosin-stained liver sections.⁶ Masson-Trichrome-Goldner staining (Merck) was conducted to dye collagen fibers. For immunohistochemical staining of paraffin tissue,

antigens were retrieved with Target Retrieval Solution (Dako, Hamburg, Germany) and blocked with serum (Vector Laboratories, Burlingame, CA) with dilution of 1:5 in Tris-NaCl-blocking buffer. Primary antibody was incubated overnight at 4°C. The following primary antibodies were used: CD45 (dilution 1:50; BD Pharmingen, San Jose, CA), α -smooth muscle actin (α -SMA; dilution 1:200; Abcam, Cambridge, UK), and Desmin (dilution 1:200; Abcam). Appropriate secondary antibodies (Vector Laboratories) were added at dilution of 1:200 in 1.5% bovine serum albumin/phosphate-buffered saline and visualized with 3,3'-diaminobenzidine (Dako). Stained sections were analyzed by counting the total number of stained cells within 25 high-power fields. For double staining of human liver samples EnVision Doublestain System (Dako) was applied with PHD1 (dilution 1:100; Thermo Fisher, Mannheim, Germany) and α -SMA (dilution 1:100; Abcam) as primary antibodies.

For immunocytochemistry of isolated murine HSCs, cells were grown on coverslips, blocked with 3% bovine serum albumin/phosphate-buffered saline, and incubated with primary antibody overnight with the use of the following dilutions: α -SMA (dilution 1:100; Abcam), glial fibrillary acidic protein—cyanine 3 (dilution 1:300; Abcam), PHD1 (dilution 1:100; Abcam), PHD2 (dilution 1:50; Novus-Biologicals, Littleton, CO), and PHD3 (dilution 1:50; Abcam). For unconjugated antibodies appropriate secondary antibodies (dilution 1:100; Sigma-Aldrich, Taufkirchen, Germany) were added the next day, before mounting with Prolong Gold with DAPI (Thermo Fisher).

Quantitative Real-Time PCR

Total RNA was isolated from cells by using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Human paraffin-embedded liver tissue samples were deparaffinized by quenching in xylene (Carl Roth GmbH, Karlsruhe, Germany) before conducting RNA isolation by using the RNAqueous-Micro Kit (Life Technologies, Carlsbad, CA).²⁴ Reverse transcription was performed with ImProm-II Reverse Transcription System (Promega Corporation, Madison, WI) and quantitative RT-PCR (RT-qPCR) was conducted by using the LightCycler 480 SYBR Green I Master Mix Kit (Roche, Mannheim, Germany), applying specific primers (Table 1). 18S was used as housekeeping gene.

Cell Culture Experiments

Primary HSCs were isolated from livers of mice by a modified two-step collagenase perfusion as described previously.²⁵ Briefly, a 22-gauge cannula was inserted into the portal vein. The liver was then perfused at 10 mL/minute with wash solution (Gey's balanced salt solution; Sigma-Aldrich) until completely pale. Afterward the liver was perfused at 5 mL/minute with digestion solution (Gey's

Table 1 mRNA Primer Sequences for Quantitative RT-PCR

Gene	Synonym	Forward sequence	Reverse sequence
<i>Acta2</i>	α -SMA	5'-TCCTCCCTGGAGAAGAGCTAC-3'	5'-ATAGGTGGTTTCGTGGATGC-3'
<i>Col1a1</i>	Collagen I α	5'-CACCTATCACTGCAAGAACAGC-3'	5'-GACTGTCTTGCCCCAAGTTC-3'
<i>Ctgf</i>	CTGF	5'-CCACCCGAGTTACCAATGAC-3'	5'-GACAGGCTTGCGATTTTAG-3'
<i>Cxcl10</i>	CXCL-10	5'-CCACGTGTTGAGATCATTTGC-3'	5'-TGGCTTCACTCCAGTTAAGGAG-3'
<i>Pdgf</i>	PDGF	5'-GCACCGAAAGTTTAAGCACAC-3'	5'-GGGGCAATACAGCAAATACC-3'
<i>P4ha1</i>	P4HA1	5'-GAGCCGAGCTACAGTACATGA-3'	5'-GCCAAGCACTCTTAGATACTCTGT-3'
<i>P4ha2</i>	P4HA2	5'-TTTGACAGCGGCTCAAAC-3'	5'-AGCTTCGACATCGCTCATATAGT-3'
<i>EGLN2</i>	PHD1	5'-CTTTGACCGGTTGCTCATTT-3'	5'-TAGGCGGCTGTGATACAGGT-3'
<i>EGLN1</i>	PHD2	5'-CGACCTGATACGCCACTGTA-3'	5'-CCTTGGCATCCAGTCTTTA-3'
<i>EGLN3</i>	PHD3	5'-AGATCGTAGGAACCCACACG-3'	5'-CAGATTTTCAGAGCAGGTCA-3'
<i>Il6</i>	IL-6	5'-ACAAAGCCAGAGTCCTTCAGAG-3'	5'-TCCTTAGCCACTCCTTCTGTG-3'
<i>Tgfb1</i>	TGF- β 1	5'-TCCAAACAGATGGCAGAGC-3'	5'-TTCTGTGGCTGAGTTGTG-3'
<i>Tnf</i>	TNF α	5'-GGCATGGATCTCAAAGACAACC-3'	5'-CAGGTATATGGGCTCATACCAG-3'
<i>Vegfa</i>	VEGF	5'-TCACCAAAGCCAGCACATAG-3'	5'-ATGCTTTCTCCGCTCTGAAC-3'
<i>Serpine1</i>	PAI-1	5'-TAGCACAGGCACTGCAAAAG-3'	5'-GTTGTGCCGAACCAAAAG-3'

α -SMA, α -smooth muscle actin; CTGF, connective tissue growth factor; CXCL-10, chemokine (C-X-C motif) ligand 10; IL-6, interleukin-6; P4HA, prolyl-hydroxylase alpha; PAI, plasminogen activator inhibitor; PDGF, platelet-derived growth factor; PHD, HIF prolyl-hydroxylase domain-containing enzyme; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

balanced salt solution with Ca^{2+} and collagenase IV; Sigma-Aldrich). After the two-step collagenase perfusion the liver was excised, digested with DNase 1 solution (DNase 1: 30 $\mu\text{g}/\text{mL}$, 1 mol/L HEPES 0.5 mL; Sigma-Aldrich) and minced to prepare a cell suspension, which was subsequently repeatedly passed through a 100- μm cell strainer. The single-cell suspension was differentially centrifuged and resuspended, applying OptiPrep density gradient medium (Sigma-Aldrich) several times to isolate HSCs. HSCs were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich) with 10% fetal bovine serum (Sigma-Aldrich), 1% penicillin/streptomycin (Sigma-Aldrich), and 10 mmol/L HEPES.

NIH-3T3 murine embryonic fibroblasts were purchased from the ATCC (LGC Standards GmbH, Wesel, Germany) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Gibco, Darmstadt, Germany) and 1% penicillin/streptomycin at 37°C and 5% CO_2 . LX-2 human HSCs were obtained from Merck and maintained in Dulbecco's modified Eagle's medium with 2% fetal calf serum and 1% penicillin/streptomycin. RNA interference was conducted by using Lipofectamine2000 (Invitrogen, Darmstadt, Germany) and a PHD1-specific siRNA (Flexi Tube Gene solution; Qiagen), resulting in effective silencing of PHD1 (residual PHD1 mRNA expression levels in siPHD1-treated LX-2 cells: 24.37% $\pm$ 5.38% of levels in control-transfected cells; $P = 0.001$; $n = 3$). Transfected cells were starved overnight before performing an invasion assay by using a Matrigel-coated Boyden chamber (Greiner Bio-One, Kremsmünster, Austria) with fetal calf serum as chemoattractant. Invading cells were quantified by dissolving cell-bound crystal violet (Sigma-Aldrich) in 10% acetic acid (Carl Roth GmbH) and measuring the optical density at 540 nm. All cell culture experiments were performed in triplicates.

Detection of HIF-1 α Protein Levels

For quantification of HIF-1 α protein levels a commercially available enzyme-linked immunosorbent assay kit (Abcam) was used to detect DNA binding activity. HIF-1 α levels were assessed by using nuclear extracts from primary isolated HSCs of PHD1 $^{-/-}$ and WT mice under normoxic (21% oxygen) culture conditions and after hypoxia exposure (0.75% oxygen). In addition, HIF-1 α levels were determined in liver lysates from PHD1 $^{-/-}$ and WT mice under baseline conditions and after DDC exposure. Absorbance was measured at 450 nm. All experiments were performed in accordance with the instructions provided by the manufacturer and in triplicates.

Human Liver Samples

Tissue samples were provided by the tissue bank of the National Center of Tumor Diseases (NCT, Heidelberg, Germany) in accordance with the regulations of the tissue bank and approval of the ethics committee of the University of Heidelberg. In total, 81 specimens were analyzed. Histomorphologic changes of human liver samples were classified according to the Desmet score for staging of fibrosis in chronic hepatitis (Desmet score 0, no fibrosis, up to Desmet score 4, cirrhosis).²⁶

Statistical Analysis

All values are represented as arithmetic means $\pm$ SEM. Group differences were evaluated by two-tailed *t*-test or analysis of variance, followed by an appropriate *post hoc* test, including Bonferroni probabilities to compensate for multiple comparisons by using GraphPad Prism5 (GraphPad Software, La Jolla, CA) and Excel 2013 (Microsoft, Redman, WA). Overall statistical significance was set at $P < 0.05$.

Results

Loss of PHD1 Attenuates Biliary Fibrosis *in Vivo*

To determine the significance of PHD1 in liver fibrosis, WT and PHD1^{-/-} mice were exposed to an experimental animal model of biliary liver fibrosis, induced by feeding with DDC for 6 weeks.⁶ Microscopic analysis of liver sections stained with hematoxylin and eosin and Masson-Trichrome-Goldner histochemistry revealed normal appearance of WT and PHD1^{-/-} livers at baseline conditions before DDC feeding (Figure 1, A and B). DDC exposure expectedly

induced fibrotic deposits around the portal triads of WT livers (Figure 1, A and B). However, the extent of periportal fibrosis was strikingly reduced in DDC-treated PHD1^{-/-} mice (Figure 1, A and B). Morphometric quantification of fibrotic tissue areas confirmed a significant reduction of fibrotic tissue areas in livers from PHD1^{-/-} mice exposed to DDC (Figure 1C), which was not present in mice deficient for PHD2 (PHD2^{+/-}) or PHD3 (PHD3^{-/-}). At baseline conditions, periportal connective tissue areas tended to be slightly (but nonsignificantly) smaller in PHD1^{-/-} than in WT livers.

Figure 1 Loss of prolyl-hydroxylase 1 (PHD1) protects mice against 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC)-induced fibrosis. **A** and **B**: Representative images of hematoxylin and eosin (HE) (**A**) and Masson-Trichrome-Goldner (MTG) staining (**B**) of wild-type (WT) and PHD1^{-/-} livers at baseline conditions and after treatment with DDC for 42 days. Note markedly reduced periportal fibrotic areas (dashed lines) in DDC-treated PHD1^{-/-} mice compared with WT animals. Asterisks depict portal vein branches. **C**: Quantification of fibrotic areas in livers from WT, PHD1^{-/-}, PHD2^{+/-}, and PHD3^{-/-} mice revealed the extent of periportal fibrotic areas in baseline conditions and after DDC treatment. **D**: Quantitative RT-PCR analysis revealed marked reduction of collagen Iα expression in liver lysates from PHD1^{-/-} mice but not from WT mice treated with DDC. Data are expressed as means ± SEM. *n* = 8 mice in each group (**A–C**); *n* = 5 mice per group (**D**). ****P* < 0.001. Scale bars = 100 μm (**A** and **B**).

The hepatic expression of type I α collagen was assessed as an alternative readout of liver fibrosis in DDC-treated mice. Consistent with the histologic findings outlined above, RT-qPCR of whole liver lysates revealed attenuated abundance of collagen I α transcripts in PHD1^{-/-} mice compared with WT mice treated with DDC (Figure 1D). At baseline conditions, however, collagen expression did not differ between PHD1^{-/-} and WT livers.

Serum markers of liver injury were determined to assess the extent of DDC-induced liver damage in WT and PHD1^{-/-} mice. DDC treatment expectedly increased serum levels of lactate dehydrogenase, glutamic oxaloacetic transaminase, and glutamic pyruvic transaminase compared with baseline conditions (Table 2). This DDC-induced increase in serum lactate dehydrogenase, glutamic oxaloacetic transaminase, and glutamic pyruvic transaminase levels was, however, significantly attenuated in PHD1^{-/-} mice (Table 2). Serum levels of alkaline phosphatase, a marker of biliary obstruction, were likewise increased in DDC-treated animals but not significantly altered in PHD1^{-/-} mice (Table 2). Variables that indicated hepatic protein synthesis, such as albumin or choline esterase, remained unaffected by DDC treatment or loss of PHD1 gene function (Table 2).

Development of biliary fibrosis in the DDC model was triggered by biliary porphyrin secretion.⁶ Of note, histomorphologic analysis revealed that the abundance of intraductal porphyrin pigment plugs in periportal fields was significantly attenuated in livers from DDC-treated PHD1^{-/-} mice compared with their WT littermates (Supplemental Figure S1, A and B). To assess whether this was due to putative effects of PHD1 deficiency on iron homeostasis, plasma levels of iron and transferrin were

likewise analyzed. Markers of iron homeostasis were, however, unaltered in PHD1^{-/-} mice at baseline conditions and after DDC exposure (Supplemental Figure S1, C and D, and Table 2). Consistently, enzyme-linked immunosorbent assays revealed comparable HIF-1 α protein levels in liver lysates from WT and PHD1^{-/-} mice (Supplemental Figure S1E).

Collectively, these results indicated a striking HIF-1-independent protection of PHD1^{-/-} mice against liver fibrosis in a murine model of biliary fibrosis.

Loss of PHD1 Reduces Leukocyte- and Myofibroblast-Infiltration after DDC Treatment

Biliary fibrosis was crucially triggered by interactions between infiltrating leukocytes (CD45⁺ cells) and HSCs, which become activated by cytokines such as TGF- β to form myofibroblasts.^{5,6} Immunohistochemistry and morphometric quantification were performed to determine the effects of PHD1 deficiency on the abundance of these cells in livers from healthy and DDC-treated mice. At baseline conditions, the abundance of CD45⁺ leukocytes was similarly low in WT and PHD1^{-/-} livers (Figure 2, A and B). DDC treatment led to marked accumulation of infiltrating leukocytes in periportal regions of WT livers, which was almost absent in livers from PHD1^{-/-} mice exposed to DDC (Figure 2, A and B). α -SMA immunostaining was applied to assess the abundance of hepatic myofibroblasts.²⁷ At baseline conditions, α -SMA expression within periportal areas was mostly restricted to the vascular smooth muscle cells of larger vessels in both WT and PHD1^{-/-} mice (Figure 2C). After DDC exposure, multiple α -SMA-positive myofibroblasts were detected in

Table 2 Plasma Concentrations of Liver Function Variables in Mice at Baseline Conditions and after 42 Hours of DDC Treatment ($n = 8$ in each group)

Plasma value	Baseline, means $\pm$ SEM			42d DDC, means $\pm$ SEM		
	WT	PHD1 ^{-/-}	<i>P</i> value*	WT	PHD1 ^{-/-}	<i>P</i> value*
Liver injury						
LDH, U/L	238.0 $\pm$ 95.3	147.0 $\pm$ 26.0	0.340	905.2 $\pm$ 111.4	364.9 $\pm$ 98.2	0.003
GOT, U/L	65.0 $\pm$ 30.4	27.8 $\pm$ 3.6	0.210	534.3 $\pm$ 82.6	316.5 $\pm$ 46.8	0.036
GPT, U/L	25.8 $\pm$ 3.3	25.7 $\pm$ 2.5	0.970	234.8 $\pm$ 69.9	102.1 $\pm$ 11.6	0.087
Biliary damage						
AP, U/L	27.6 $\pm$ 4.0	30.2 $\pm$ 3.3	0.620	226.5 $\pm$ 36.4	206.6 $\pm$ 26.7	0.660
Liver function						
ChE, kU/L	2.9 $\pm$ 0.5	2.9 $\pm$ 0.4	0.960	3.2 $\pm$ 0.2	3.8 $\pm$ 0.4	0.260
Albumin, g/L	13.7 $\pm$ 0.5	15.7 $\pm$ 0.3	0.008	17.5 $\pm$ 0.7	16.9 $\pm$ 0.7	0.560
Blood count						
Leukocytes, /nL	2.9 $\pm$ 0.5	3.3 $\pm$ 0.4	0.540	5.5 $\pm$ 0.5	4.6 $\pm$ 0.3	0.120
Erythrocytes, /pL	8.6 $\pm$ 0.5	9.4 $\pm$ 0.7	0.342	9.2 $\pm$ 1.1	9.6 $\pm$ 0.5	0.6848
Hemoglobin, g/dL	14.1 $\pm$ 0.7	15.5 $\pm$ 1.0	0.265	13.6 $\pm$ 1.7	14.9 $\pm$ 0.7	0.471
Hematocrit, L/L	0.4 $\pm$ 0.0	0.4 $\pm$ 0.0	0.355	0.4 $\pm$ 0.1	0.5 $\pm$ 0.0	0.457

*Determined by *t*-test.

AP, alkaline phosphatase; ChE, cholinesterase; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; LDH, lactate dehydrogenase; WT, wild-type.

Figure 2 Attenuated leukocyte and myofibroblast infiltration in livers from 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC)-treated prolyl-hydroxylase 1-deficient (PHD1^{-/-}) mice. **A** and **B**: Representative images of CD45 immunostaining (**A**) and histomorphometric quantification (**B**) of leukocytes (arrows) in livers from wild-type (WT) or PHD1^{-/-} mice at baseline conditions and after DDC treatment for 42 days (**A**). **C** and **D**: Representative images of α -smooth muscle actin (α -SMA) immunostaining (**C**) and histomorphometric quantification (**D**) of myofibroblasts (arrows) in livers from WT or PHD1^{-/-} mice at baseline conditions and after DDC treatment for 42 days (**C**). **E** and **F**: Representative images of desmin immunostaining (**E**) and histomorphometric quantification (**F**) of hepatic stellate cells (HSCs) and fibroblasts (arrows) in livers from WT or PHD1^{-/-} mice at baseline conditions and after DDC treatment for 42 days (**E**). Asterisks in **A**, **C**, and **E** depict portal branches. Data are expressed as means $\pm$ SEM. $n = 5$ mice in each group. $^{**}P < 0.01$. Scale bars = 100 μ m (**A**, **C**, and **E**). HPF, high-power field.

periportal regions of WT livers (Figure 2C). However, the abundance of α -SMA-positive myofibroblasts in livers from DDC-treated PHD1^{-/-} mice was strikingly lower than in those from their WT counterparts (Figure 2, C and D). In addition, desmin immunostaining was performed to label HSCs and fibroblasts in murine livers.²⁸ Consistent with the

reduced occurrence of α -SMA-positive myofibroblasts, the abundance of desmin-positive HSCs and fibroblasts was likewise significantly reduced in livers of PHD1^{-/-} mice exposed to DDC (Figure 2, E and F).

Taken together, alleviated development of biliary fibrosis in PHD1^{-/-} mice occurred concomitantly with reduced

recruitment of inflammatory leukocytes and markedly attenuated occurrence of profibrotic myofibroblasts in periportal areas.

Loss of PHD1 Attenuates the Profibrogenic Activation of HSCs

HSCs contributed an important source of myofibroblasts, which triggered biliary fibrogenesis.⁵ It was therefore investigated whether loss of PHD1 altered the fibrogenic potential of HSCs.

The expression of the different HIF-PHD enzymes in HSCs was first assessed. Indeed, RT-qPCR revealed that PHD1 represented the most abundantly expressed PHD enzyme in the immortalized human HSC line LX-2 (Figure 3A).²⁹ Immunocytochemistry was likewise performed and confirmed the expression of PHD1 protein in LX-2 HSCs, whereas PHD2 and PHD3 protein appeared to be only faintly expressed (Figure 3B).

To determine the functional relevance of PHD1 in fibrogenesis triggered by HSCs, primary cultures of HSCs, isolated from WT and PHD1^{-/-} mice, were established. No morphologic differences were apparent between WT and PHD1^{-/-} HSCs cultured for 1 day (Figure 3C). Likewise, immunocytochemistry revealed comparable expression of glial fibrillary acidic protein, a marker labeling un-activated HSCs,³⁰ in WT and PHD1^{-/-} HSCs after 1 day in culture (Figure 3C). After 5 days in culture, however, PHD1^{-/-} HSCs appeared significantly less enlarged and formed less pseudopodia than their WT counterparts (Figure 3D). Consistently, immunocytochemistry revealed reduced expression of α -SMA, a marker labeling activated HSCs and myofibroblasts,²⁷ in PHD1^{-/-} HSCs cultured for 7 days (Figure 3D), collectively indicating attenuated activation and myofibroblast differentiation of PHD1-deficient HSCs.

It was also assessed whether PHD1 deficiency impaired the motility of fibrogenic cells. Because myofibroblast differentiation of PHD1^{-/-} HSCs was significantly delayed (see above), these experiments were performed, applying primary mouse embryonic fibroblasts harvested from WT and PHD1^{-/-} mice. Matrigel invasion assays revealed significantly impaired invasive potential of PHD1^{-/-} mouse embryonic fibroblasts compared with WT cells on stimulation with fetal calf serum (Figure 3E). Comparable results were obtained when performing siRNA-mediated knockdown of PHD1 in the commercially available mouse fibroblast cell line NIH3T3 or the human HSC line LX-2. Indeed, knockdown of PHD1 significantly impaired the invasive potential of NIH3T3 fibroblasts (Figure 3E) or LX-2 HSCs (Figure 3E) compared with control-treated cells.

Taken together, these findings indicated that loss of PHD1 attenuated myofibroblast differentiation and profibrogenic properties of HSCs.

Molecular Mediators of HSC Activation Affected by PHD1 Deficiency

RT-qPCR-based expression analysis of various profibrogenic and promitogenic factors was performed to substantiate the effects of PHD1 deficiency on HSC activation on the molecular level. Transcript expression levels of connective tissue growth factor, TGF- β 1, platelet-derived growth factor, CXCL-10, IL-6, and tumor necrosis factor α , all of which are autocrine drivers of HSC activation, invasion, proliferation, or transdifferentiation to myofibroblasts,^{4,31–33} were lower in PHD1^{-/-} HSCs than in corresponding WT cells maintained in culture for 7 days (Figure 4A). The same held true for the myofibroblast markers, α -SMA and collagen I α (Figure 4A).

The significance of putative molecular effectors, modulating HSC activation downstream PHD1, was also assessed. Of interest, transcript levels of distinct HIF target genes, such as vascular endothelial growth factor and plasminogen activator inhibitor 1, were not significantly altered in PHD1^{-/-} HSCs compared with corresponding WT cells (Figure 4A). Expression levels of prolyl-4-hydroxylase α 1 and prolyl-4-hydroxylase α 2, which represent procollagen-proline dioxygenases involved in collagen metabolism that are up-regulated by hypoxia in HSCs, were likewise unaltered in PHD1^{-/-} HSCs (Figure 4A). Consistently, enzyme-linked immunosorbent assay measurements revealed no significant differences regarding normoxic or hypoxic stabilization of HIF-1 α protein in WT and PHD1^{-/-} HSCs (Figure 4B).

Collectively, these analyses indicated that loss of PHD1 gene function attenuated HSC activation in a HIF-1-independent manner.

Hepatic PHD1 Expression Correlates with Severity of Liver Fibrosis in Human Patients

To assess whether PHD1 was likewise associated with the severity of liver fibrosis in human patients, its expression was determined in liver resection specimens from patients with absent or with mild (classified as Desmet score 0/1), with moderate (classified as Desmet score 2/3), or with severe liver fibrosis (classified as Desmet score 4).²⁶

RT-qPCR analysis of whole liver lysates revealed that hepatic expression of PHD1 transcripts was significantly higher in patients with severe liver fibrosis (Desmet stage 4) than in patients who displayed only moderate (Desmet score 2/3) or no fibrosis (Desmet score 0/1) (Figure 5A), whereas PHD2 and PHD3 transcript levels decreased or were not affected, respectively (Figure 5B). Consistently, abundant expression of PHD1 protein could be detected by immunohistochemistry in liver samples from patients with severe fibrosis (Desmet score 4) but not in samples from patients with nonfibrotic livers (Desmet score 0) (Figure 5C). Remarkably, within fibrotic livers, spatial expression of PHD1 protein was not only detected in

hepatocytes but was also likewise detected in dispersed spindle-like cells, which appeared to be myofibroblasts (Figure 5C). Indeed, immunohistochemical double-labeling revealed that these PHD1-positive cells

coexpressed α -SMA, identifying them as activated HSCs or myofibroblasts (Figure 5D).²⁷

Overall, these findings indicated that PHD1 was likewise implicated in human liver fibrosis, thus underlining the

Figure 3 Prolyl-hydroxylase 1 (PHD1) deficiency attenuated profibrogenic properties of hepatic stellate cells (HSCs) and motility of fibrogenic cells *in vitro*. **A:** Quantitative RT-PCR analysis revealed that PHD1 was more abundantly expressed than PHD2 or PHD3 in the human LX-2 HSC cell line. **B:** Representative images of immunocytochemistry for PHD1, PHD2, and PHD3 (red) and DAPI (cell nuclei, blue) confirmed abundant expression of PHD1 but not PHD2 or PHD3 protein in human LX-2 HSCs. **C:** Representative images of microscopy and cytochemistry for glial fibrillary acidic protein (GFAP; marker of un-activated HSCs, red) and DAPI (cell nuclei, blue) of primary HSCs isolated from wild-type (WT) or PHD1^{-/-} mice and cultured for 1 day. Note comparable morphologic appearance and GFAP expression of WT and PHD1^{-/-} HSCs. **D:** Representative images of microscopy and cytochemistry for α -smooth muscle actin (α -SMA; myofibroblast marker, green) and DAPI (cell nuclei, blue) of primary HSCs isolated from WT or PHD1^{-/-} mice and maintained in culture for 5 or 7 days. Note less myofibroblast-like morphology and attenuated α -SMA expression of PHD1^{-/-} HSCs. **E:** Matrigel assays revealed the migratory potential of primary mouse embryonic fibroblasts (MEFs) isolated from WT and PHD1^{-/-} mice, mouse NIH-3T3 fibroblasts with silenced PHD1 expression (siPHD1) versus control-transfected cells (Ctrl), and human LX-2 HSCs with siPHD1 versus Ctrl cells. Note that migratory potential on stimulation with fetal calf serum (FCS) was consistently impaired in PHD1-deficient or -underexpressing cells. Data are expressed as means $\pm$ SEM. $n = 3$ mice (**A**); $n = 1$ of 3 representative experiments (**E**). $^{**}P < 0.01$, $^{***}P < 0.001$. Scale bars = 50 μ m (**B**, **C**, and **D**).

Figure 4 Prolyl-hydroxylase 1 (PHD1) deficiency altered the expression of cytokines involved in hepatic stellate cell (HSC) activation. **A:** Quantitative RT-PCR analyses of HSCs isolated from wild-type (WT) or PHD1^{-/-} mice revealed attenuated expression of profibrogenic or promitogenic cytokines [connective tissue growth factor (CTGF), transforming growth factor (TGF)-β1, platelet-derived growth factor (PDGF), chemokine (C-X-C motif) ligand (CXCL)-10, IL-6, tumor necrosis factor (TNF)-α] and myofibroblast markers [α-smooth muscle actin (α-SMA), collagen Iα]. By contrast, transcript levels of prolyl-4-hydroxylase α1 (P4HA1), prolyl-4-hydroxylase α2 (P4HA2), vascular endothelial growth factor (VEGF), or plasminogen activator inhibitor (PAI)-1 were not affected by PHD1 deficiency. **B:** Enzyme-linked immunosorbent assay-based detection of hypoxia-inducible transcription factor (HIF)-1α protein levels in nuclear lysates from WT and PHD1^{-/-} HSCs revealed unaltered HIF-1α protein levels under normoxic and hypoxic culture conditions. Data are expressed as means ± SEM. *n* = 3 mice per group. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. Conc, concentration.

putative relevance of its fibrogenic effects in clinical practice.

Discussion

Here, we demonstrate that genetic loss of the HIF prolyl-hydroxylase (PHD) 1 protects against liver fibrosis in a mouse model of xenobiotic-induced bile duct injury without inducing HIF-1α protein levels. These findings collectively hint at a specific and pathologically relevant

function of the PHD1 oxygen sensor in HSCs, which triggers the development of biliary fibrosis in the applied animal model.⁶ It was also shown that HSCs within fibrotic human livers express PHD1 and that hepatic PHD1 expression correlates with the severity of liver fibrosis in human patients, indicating the putative clinical relevance of these findings.

Because liver fibrosis was not investigated in mice lacking PHD1 selectively in HSCs, it cannot be ruled out that other cell types likewise contribute to reduced biliary fibrosis in globally PHD1-deficient mice, which were used

Figure 5 Significance of prolyl-hydroxylase 1 (PHD1) expression in human liver fibrosis. **A:** Quantitative RT-PCR analysis revealed PHD1 mRNA expression levels in liver tissue from patients with absent/mild (Desmet score 0/1), moderate (Desmet score 2/3), or severe (Desmet score 4) liver fibrosis. Note that PHD1 expression was increased with disease severity. **B:** RT-qPCR analysis of PHD1, PHD2, and PHD3 mRNA expression levels in liver tissue of an additional patient cohort with absent/mild (Desmet score 0/1), moderate (Desmet score 2/3), or severe (Desmet score 4) liver fibrosis. Note that expression of PHD1, but not PHD2 or PHD3 transcripts, was increased with disease severity. **C:** Representative images of PHD1 immunostaining revealed abundant expression of PHD1 protein in livers from patients with severe liver fibrosis (Desmet 4) but not in healthy livers (Desmet 0). Note PHD1 expression in putative periportal myofibroblasts (arrows). **D:** Representative images of immunohistochemistry double staining of periportal regions from healthy (Desmet 0) and fibrotic (Desmet 4) tissues for PHD1 (brown) and α -smooth muscle actin (α -SMA; myofibroblast marker, blue). Note PHD1 expression in myofibroblasts (arrows and inset). Asterisks indicate portal vein branches. Boxed area is shown at higher magnification in the inset. Data are expressed as means $\pm$ SEM. $n = 22$ patients with absent/mild liver fibrosis (**A**); $n = 28$ patients with moderate liver fibrosis (**A**); $n = 10$ patients with severe liver fibrosis (**A**); $n = 9$ patients with absent/mild liver fibrosis (**B**); $n = 7$ patients with moderate liver fibrosis (**B**); $n = 5$ patients with severe liver fibrosis (**B**). $*P < 0.05$. Scale bars = 100 μ m (**C**); 50 μ m (**D**).

in this study. Indeed, previous analyses revealed that PHD1 deficiency alleviates hepatic ischemia/reperfusion injury¹⁸ and promotes liver regeneration after surgical resection.^{19,20} Moreover, it has been shown that loss of PHD1 conveys attenuated apoptosis of hepatocytes, most probably by increasing basal NF- κ B activity.³⁴ Mechanistically, these beneficial effects were attributable to loss of PHD1 in the hepatocytes themselves, rather than in other resident liver cells. Although it is likely that hepatocyte-specific effects of PHD1 deficiency support the phenotype described in this study, the present work unravels the HSC as a major player, conveying protective effects of PHD1 deficiency against biliary fibrosis. Indeed, HSCs represent the key trigger of liver fibrosis³⁵ and give rise to an overwhelming majority of collagen-producing cells.³ In this study, attenuated biliary fibrosis of PHD1^{-/-} livers was accompanied by

significantly reduced accumulation of HSCs and, likely as a consequence,^{1,36} attenuated recruitment of proinflammatory cells.

The notion that the HSC represents the crucial cell type, mediating attenuated fibrosis in PHD1-deficient mice, is in accordance with several reports, highlighting the relevance of HSC activation and differentiation into myofibroblasts in the pathogenesis of liver fibrosis.^{1,31,37,38} The *in vitro* analyses presented in this study indicate that loss of PHD1 effectively interferes with these processes. For instance, the expression of the myofibroblast marker, α -SMA, was significantly reduced in differentiating cultures of PHD1-deficient HSCs. HSC activation, which is reflected by expression of α -SMA, is mainly driven by TGF- β and by platelet-derived growth factor.^{1,31} Of note, both cytokines can be secreted by HSCs in an autocrine fashion to

maintain profibrotic activation.^{4,31} Remarkably, it was found that expression levels of TGF- β and platelet-derived growth factor were significantly reduced in PHD1-deficient HSCs, along with similarly attenuated expression of various other cytokines involved in proliferation and migration of HSCs. Previous studies have shown that apoptosis of activated HSCs,³⁹ but also their deactivation to a quiescent state, result in the resolution of liver fibrosis.⁴⁰ It is in this context conceivable that PHD1 deficiency interferes with continued profibrotic signaling of activated HSCs, thus facilitating resolution of liver fibrosis.

It remains tempting to speculate on the molecular pathways behind these effects of PHD1 deficiency on HSC activation. Given the well-documented interplay between PHD1 and NF- κ B,³⁴ as well as the key significance of the NF- κ B pathway in liver fibrosis,⁴¹ it appears likely that NF- κ B is involved. The net effect of increased NF- κ B activity in HSCs could be assumed to be profibrogenic, which appears inconsistent with these findings of decreased HSC activation in PHD1-deficient mice.⁴¹ NF- κ B does, however, appear to play a dual role, especially in experimental models of chronic liver injury induced by hepatotoxins.^{41–43} For instance, NF- κ B1 (p50)-deficient mice develop more severe neutrophilic inflammation and fibrosis than WT littermates in a model of chronic liver injury induced by the hepatotoxin carbon tetrachloride.⁴³

Previous studies have highlighted the significance of hypoxia in liver fibrosis and, more specifically, delineated a profibrotic role of the HIFs in its development.^{8,16} Indeed, hypoxia levels are reportedly increased in fibrotic liver tissue,⁸ and interference with HIF-1 α and HIF-2 α stabilization by forced expression of von Hippel-Lindau protein attenuates hepatic fibrosis in mice.¹⁶ At first glance, it might thus appear paradoxical that global deletion of PHD1, which reportedly enhances hepatocellular HIF-2 α (and to a lesser extent HIF-1 α) levels,¹⁸ has opposite effects. However, the present study reveals that loss of PHD1 was not accompanied by an increase of HIF-1 α protein levels in HSCs. The observed hepatoprotective effects conferred by PHD1 deficiency in HSCs thus seem to be HIF-1 independent. Even in the event that loss of PHD1 induces HIF stabilization in other cell types than HSCs, these observations may be explicable by peculiarities related to the specific animal models that were applied to mimic chronic liver disease in these different studies. In particular, the antifibrotic functions of PHD1 deficiency in HSCs, which are outlined in the present study, may overshadow putatively opposite effects of HIF stabilization in hepatocytes and other resident liver cells. In fact, HSC activity plays a more prominent role in the DDC-induced model of biliary fibrosis, which was applied in this study, than in models of chronic bile duct ligation, which were applied in previous studies to address the significance of HIF in liver fibrosis.^{8,16} It is in

this context noteworthy that chronic feeding with the xenobiotic DDC causes pericholangitis and consecutive biliary liver fibrosis in mice^{5,6} and that the preclinical findings obtained in the present study might thus bear particular relevance about forms of human biliary liver fibrosis that are associated with sclerosing cholangitis.

Obviously, caution is warranted when speculating on the putative clinical significance of the present findings, which were generated in a transgenic mouse model. For instance, mice and humans differ substantially regarding specific proportions and absolute numbers of host immune cells that are present within the liver.⁵ Moreover, the genetic background of the applied transgenic mice may affect the responsiveness of the host immune system to toxic stimuli, such as DDC feeding.^{5,44,45} Of importance, the *in vivo* results, which were obtained in constitutively PHD1-deficient mice, may be biased by direct or indirect effects of global PHD1 deficiency on DDC metabolism, resulting in overall decreased DDC toxicity to the biliary system. In fact, the accumulation of biliary porphyrin pigment plugs, which represent a key trigger of liver fibrosis in the applied mouse model,^{6,46} was significantly attenuated in DDC-treated PHD1^{-/-} animals compared with WT littermates. It cannot be ruled out that reduced formation of porphyrin plugs is causally involved in the observed phenotype, regardless of the effects of PHD1 deficiency on HSC activation that was documented *in vitro*.

Given these shortcomings, it was important to obtain some initial evidence, indicating the putative significance of PHD1 in human liver fibrosis. Of importance, it was shown that PHD1 protein is not only abundantly expressed in human HSCs *in vitro* but likewise in resident HSCs of patients with liver fibrosis. Most strikingly, however, hepatic PHD1 (but not PHD2 or PHD3) expression levels correlated with the extent of liver fibrosis in human patients, raising the intriguing possibility that increased PHD1 enzyme activity in HSCs may contribute to the progression of human fibrotic liver disease through stimulating sustained HSC activation and myofibroblast differentiation. This would open a promising door for therapeutic intervention, because pharmacologic small-molecule PHD enzyme inhibitors are already available, and putative clinical applications for these drugs are currently highly investigated by translational researchers and pharmaceutical companies.¹⁴ In fact, it could recently be shown that pharmacologic pan-hydroxylase inhibition significantly attenuated the development of intestinal fibrosis in mice exposed to experimental colitis.⁴⁷ However, because most of these drugs are neither specific for the oxygen-sensing PHD enzymes (PHD1, PHD2, and PHD3) nor for distinct cell populations, it remains to be investigated whether pharmacologic PHD enzyme inhibitors can be applied to rather specifically target PHD1 in HSCs.

Conclusion

This study unravels the promotion and maintenance of profibrotic HSC functions as a novel function of the PHD1 oxygen sensor and indicates that interference with PHD1 enzyme function may be of therapeutic value to counteract the pathogenesis and progression of biliary liver fibrosis.

Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2018.08.003>.

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