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Hepatocyte KLF6 expression affects FXR signalling and the clinical course of primary sclerosing cholangitis

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Abstract

Background & Aims: Primary sclerosing cholangitis (PSC) is characterized by chronic cholestasis and inflammation, which promotes cirrhosis and an increased risk of cholangiocellular carcinoma (CCA). The transcription factor Krueppel-like-factor-6 (KLF6) is a mediator of liver regeneration, steatosis, and hepatocellular carcinoma (HCC), but no data are yet available on its potential role in cholestasis. Here, we aimed to identify the impact of hepatic KLF6 expression on cholestatic liver injury and PSC and identify potential effects on farnesoid-X-receptor (FXR) signalling.

Methods: Hepatocellular KLF6 expression was quantified by immunohistochemistry (IHC) in liver biopsies of PSC patients and correlated with serum parameters and clinical outcome. Liver injury was analysed in hepatocyte-specific *Klf6*-knockout mice following bile duct ligation (BDL). Chromatin-immunoprecipitation-assays (ChIP) and KLF6-overexpressing HepG2 cells were used to analyse the interaction of KLF6 and FXR target genes such as *NR0B2*.

Results: Based on IHC, PSC patients could be subdivided into two groups showing either low (<80%) or high (>80%) hepatocellular KLF6 expression. In patients with high KLF6 expression, we observed a superior survival in Kaplan-Meier analysis. *Klf6*-knockout mice showed reduced hepatic necrosis following BDL when compared to controls. KLF6 suppressed *NR0B2* expression in HepG2 cells mediated through binding of KLF6 to the *NR0B2* promoter region.

Abbreviations: ABCB11/BSEP, ATP-binding-cassette subfamily B member 11/bile salt exporting pump; AIH, Autoimmune hepatitis; ALT, Alanine aminotransferase; AP, Alkaline phosphatase; AST, Aspartate aminotransferase; BDL, Bile duct ligation; CCA, Cholangiocellular carcinoma; CDCA, Chenodeoxycholic acid; ChIP, Chromatin immunoprecipitation assay; Cyp7A1, CytochromeP450 family 7 subfamily A member 1; FXR, Farnesoid-X-receptor; FXRE, Farnesoid-X-receptor responsive elements; HCC, Hepatocellular carcinoma; IR-1, Inverted repeat; KLF6, Krüppel-like-factor-6; LTX, Liver transplantation; MMP9, Matrix metalloproteinase 9; NAFLD, Non-alcoholic fatty liver disease; NR0B2/SHP, Nuclear receptor-subfamily 0 group B member 2/small heterodimer partner; OCA, Obeticholic acid; pANCA, Perinuclear anti-neutrophil cytoplasmic antibody; PBC, Primary biliary cholangitis; PPAR α/γ , peroxisome proliferator-activated receptor alpha/gamma; PSC, Primary sclerosing cholangitis; RXR α , Retinoid-X-receptor alpha; SdhA, Succinate dehydrogenase complex flavoprotein subunit A; SLC10A1/NTCP, solute-carrier-family-10 member 1/ sodium-taurocholate-cotransporting-polypeptide; SLC51A/OST α , SLC51B/OST β , solute carrier family 51, alpha subunit/beta subunit; SP-1/-2, SP-1/-2 transcription factor; UC, Ulcerative colitis; UDCA, Urso-deoxycholic acid; WT, Wildtype; γ GT, Gamma glutamyltransferase.

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Conclusion: Here, we show an association between KLF6 expression and the clinical course and overall survival in PSC patients. Mechanistically, we identified a direct interaction of KLF6 with the FXR target gene *NROB2*.

KEYWORDS

cholangiocellular carcinoma, cholestasis, FXR, KLF6

1 | INTRODUCTION

The ubiquitously expressed transcription factor Krüppel-like-factor-6 (KLF6) has been established as a tumour suppressor gene and regulator of cell growth, differentiation, tumorigenesis, and signal transduction in numerous studies.¹ Members of the KLF family are zinc-finger-proteins and have a conserved zinc finger DNA-binding domain, which is able to bind to GC-rich motifs on diverse target genes.² KLF6 can act both as activator and repressor of transcription.^{3,4} Several studies indicated a general role for KLF6 in suppressing tumorigenesis by controlling key mechanisms involved in tumour formation, such as cell growth, differentiation, adhesion and endothelial motility.⁵⁻⁹ KLF6 expression or its functional loss have been associated with the progression and outcome of various tumours in patients, including hepatocellular carcinoma (HCC).¹⁰⁻¹²

Beside playing a role in HCC, KLF6 has been established as an important transcription factor in a variety of liver diseases, including hepatic fibrogenesis, acute liver injury, ischaemia reperfusion damage and non-alcoholic fatty liver diseases (NAFLD).¹³⁻¹⁵ In the previous studies, we characterized KLF6 as a regulator of autophagy in acute liver injury and as a mediator of hepatic glucose and lipid metabolism in NAFLD by binding directly to the promoter regions of autophagy-regulating factors and hepatic glucokinase, as well as a post-transcriptional regulation of peroxisome proliferator activated receptor alpha (PPAR α) expression.¹⁶⁻¹⁸ Interestingly, no data are yet available on a potential role of KLF6 in cholestatic liver diseases.

Primary sclerosing cholangitis (PSC) is a cholestatic liver disease characterized by chronic inflammation and consecutive constriction of

Key points

Chronic cholestasis can promote the development of chronic biliary inflammation, scarring of the liver and even hepatic cancer. So-called tumour suppressor genes, such as KLF6, have been identified to play crucial roles in the response to liver injury, far beyond their contribution to tumour formation. In this study, we investigate the effects of KLF6 expression on cholestasis and the development of biliary inflammation in patients with chronic cholestasis as well as in an animal model. We further aimed to identify the underlying mechanisms in a cell culture model. Our data indicate a novel mechanistic interaction of KLF6 with target genes of the FXR regulated bile acid metabolism.

the intra- and extra hepatic bile ducts resulting in progressive biliary fibrosis and cirrhosis.¹⁹ The underlying mechanisms are still insufficiently understood. The prevalence of PSC is notably increased in patients with chronic inflammatory bowel diseases, especially ulcerative colitis, predominantly in males.²⁰ Chronic cholestasis such as in PSC drives hepatocellular injury followed by inflammation promoting cell proliferation and tumorigenesis. Consecutively, PSC is associated with an increased risk of cholangiocellular carcinoma (CCA) and HCC.²¹ To reduce cholestasis in patients with so-called dominant strictures, dilatation therapy via endoscopic retrograde cholangiopancreatography may be performed as a therapeutic option. While there is no established

medical therapy, antibiotics are considered for treatment of bacterial cholangitis. As high-dose therapy appears harmful, some patients might benefit from low-dose ursodeoxycholic acid (UDCA) and early stage clinical studies suggest a beneficial effect of Farnesoid X Receptor (FXR) agonists in PSC.²² With established cirrhosis, liver transplantation is the only therapeutic option in progressive disease.²³⁻²⁵

Nuclear receptors, such as FXR, are ligand-activated transcription factors controlling crucial biological functions including cell growth, glucose and lipid metabolism, as well as bile acid synthesis and systemic bile acid accumulation and therefore represent promising therapeutic targets for various diseases.²⁶ Several FXR agonists are currently being tested in clinical trials as treatment options for cholestatic liver diseases. Among them, obeticholic acid (OCA) is already an established treatment option for primary biliary cholangitis (PBC), another autoimmune cholestatic liver disease.²⁷⁻²⁹ While we have established KLF6 as a regulator of the nuclear receptor PPAR α , others found KLF6 to repress the nuclear receptor PPAR γ and interact with its cofactor Retinoid-X-Receptor alpha (RXR α).^{18,30,31} However, no data are available yet on a potential interaction between KLF6 and the nuclear receptor FXR.

As FXR signalling is established as an important factor in cholestasis, we aimed to investigate the interaction of KLF6, FXR and its target genes in cholestatic liver diseases and potential effects of KLF6 expression on clinical features of PSC in a well-defined cohort.

2 | METHODS

2.1 | Patient material and ethical considerations

For this study we analysed retrospective data and stored sample biopsies from PSC and PBC patients that were treated in the Department of Gastroenterology and Hepatology at the University Hospital Essen. All PSC and PBC patients, biopsied in our institution between January 2008 and December 2016 with sufficient paraffin tissue as well as historical laboratory and clinical data from the time of biopsy available were included. For Kaplan-Meier analysis, the clinical course of patients was followed until May 2019.

All investigations in human material and the use of patients' data were approved by the Ethics Committee (Institutional Review Board) of the University Hospital Essen (reference number: 14-6065-BO) and the study protocol conformed to the ethical guidelines of the Declaration of Helsinki. As patient data and samples of the historic cohort of PSC and PBC patients were analysed retrospectively from stored samples that were obtained for routine clinical use, informed consent from these subjects was explicitly not required according to the local ethics committee.

2.2 | Statistical analysis

Statistical significance was determined using an unpaired (or paired, when applicable), two-tailed t test or by one-way ANOVA (with

TABLE 1 Comparison of demographic, clinical, histological and laboratory data of PSC patients with KLF6 low ($n = 9$) and KLF6 high ($n = 10$) hepatocyte expression levels; a comparison of the groups showed no significant differences for the different parameters. T = total number

	KLF6 low (<80%)	KLF6 high (>80%)
Gender	8 m/1 f	7 m/3 f
Age (years)	41.4 \pm 2.6	43.9 \pm 5.0
Therapeutic ERCP	9/9	8/10
Intrahepatic Manifestation	9/9	8/10
Extrahepatic Manifestation	3/9	2/10
ANCA (pos/T)	1/9	4/10
AIH overlap (pos/T)	3/9	2/10
UC (pos/T)	5/9	4/10
CCA (pos/T)	3/9	0/10
LTX (pos/T)	2/9	2/10
Deceased (pos/T)	4/9	1/10
ALT (U/L)	109.8 \pm 31.6	80.0 \pm 29.8
AST (U/L)	75.0 \pm 34.3	92.4 \pm 17.4
γ GT (U/L)	311.4 \pm 98.7	193.6 \pm 117.9
AP (U/L)	390.6 \pm 148.6	271.8 \pm 118.8
Bilirubin (mg/dl)	2.4 \pm 1.9	2.9 \pm 0.4
Mayo PSC Risk Score	0.33 \pm 0.5	-0.17 \pm 0.5
Fibrosis > 3 (pos/T)	5/9	7/10
Ductular Proliferation (pos/T)	8/9	6/10

Tukey's post-hoc test for individual experimental conditions) as well as Kaplan-Meier survival analysis were performed with GraphPad Prism 8 (GraphPad Software Inc). Significance was assumed at $P \leq .05$. If not stated otherwise all data are presented as mean \pm SEM.

All additional information on the individual experimental methods and materials are described in the Data S1 section.

3 | RESULTS

3.1 | Hepatocyte KLF6 expression is associated with clinical outcome in PSC

KLF6 expression levels were quantified in liver tissue obtained for different clinical indications in patients with established diagnosis of large duct PSC, as assessed via MRCP and later treated with dilation therapy via ERCP. We included a total number of 19, predominantly male PSC patients with large duct disease with a mean age of 42.7 \pm 10.6 years. Further demographic data and clinical features can be found in Table 1. Based on hepatocyte expression levels of KLF6 in patients' liver biopsies, as assessed by immunohistochemistry, we identified two clusters of either high (>80%) or low (<80%) KLF6 expression in hepatocytes. This pattern was even more pronounced when excluding patients,

who underwent liver transplantation (LTX), resulting in two clusters with either > 80% or < 60% of hepatocytes expressing KLF6 (Figure S1A,B). A significant number of KLF6 positive non-parenchymal cells (ie cholangiocytes, Kupffer cells etc) was not observed (Figure 1A).

Based on these results and in order to investigate a potential influence of hepatocyte KLF6 expression on clinical features of PSC, we then arbitrarily separated these patients in two groups with either high expression (>80%, n = 10) or low expression (<80%, n = 9) of KLF6 in hepatocytes for further analysis. There were no significant differences of pANCA levels, the histological presence of fibrosis or ductular proliferation comparing the groups (Table 1). Patients with high KLF6 expression showed a trend towards lower serum levels of ALT (alanine aminotransferase), γ GT (gamma glutamyltransferase) and AP (alkaline phosphatase), with higher AST (aspartate aminotransferase). However, these differences were not significant (Table 1). Interestingly, all three patients that developed CCA in the course of the disease as well as most who deceased during the observation period had low hepatocyte KLF6 expression (Figure 1B,C).

To compare the clinical outcome of the patients, we performed a Kaplan-Meier analysis to test the influence of KLF6 expression on patients' survival. Here, given the small sample size, it was remarkable that those patients who had higher hepatocyte KLF6 expression had significantly better survival rates than patients with low KLF6 expression (Figure 1D). This effect was still present, while attenuated, when we declared the time of surgery in the four individuals

who underwent LTX as negative endpoint in the Kaplan-Meier analysis (Figure 1E).

In contrast, in our PBC cohort (see demographics in Table S2), the distribution of hepatocyte KLF6 expression was less accentuated, based on a more even distribution between 1% and 92% (Figure S1C). When applying the 80% cutoff, in contrast to PSC, in PBC patients with high KLF6 expression we observed a trend towards higher transaminase levels and a significantly elevated γ GT, indicating potentially different roles of hepatic KLF6 in two distinct cholestatic diseases, PSC and PBC (Table S2). Our data indicate that higher hepatic KLF6 expression is associated with a better outcome in PSC.

3.2 | Attenuated liver injury with accentuated cholestasis in a model of hepatocyte specific *Klf6*-knockout mice following bile duct ligation

To further analyse the impact of hepatocyte KLF6 on cholestatic liver injury, we utilized an established conditional knockout model with hepatocellular deletion of *Klf6* (*DeltaKlf6*-hep) and performed BDL as a model of obstructive cholestasis and inflammation. Animals were sacrificed 2 weeks following BDL and we measured markers of liver injury in the serum. Although the results did not show significant changes, it seemed that BDL in *DeltaKlf6*-hep mice led to accentuated cholestasis as indicated by higher levels of bilirubin and AP yet decreased serum levels of ALT, AST, and triglycerides as compared to

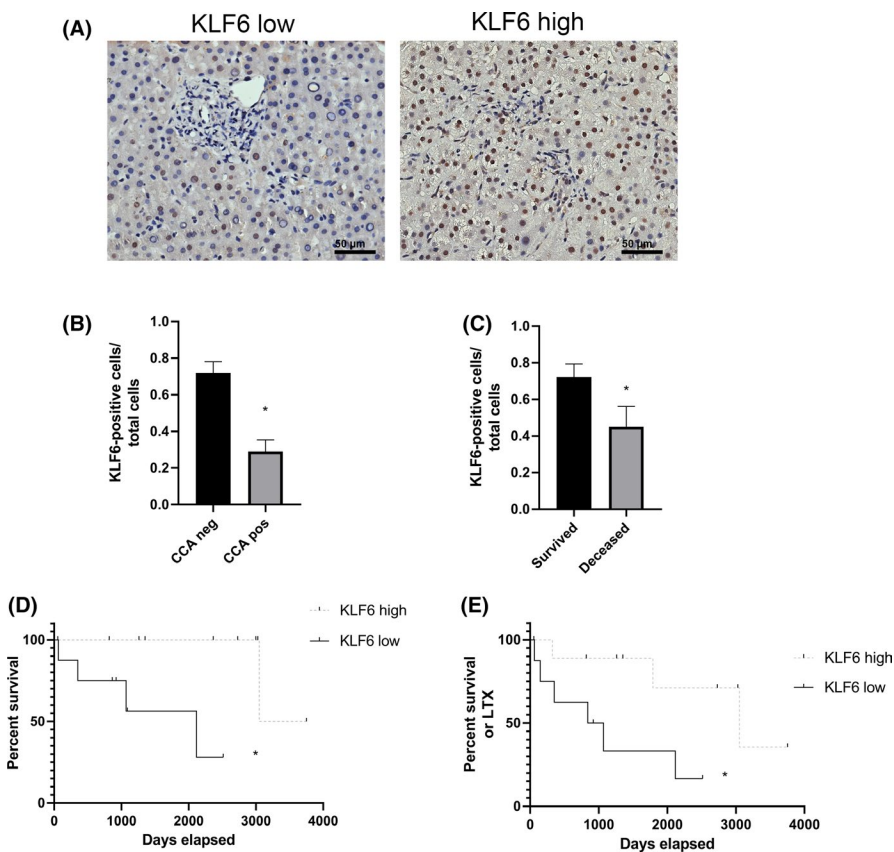
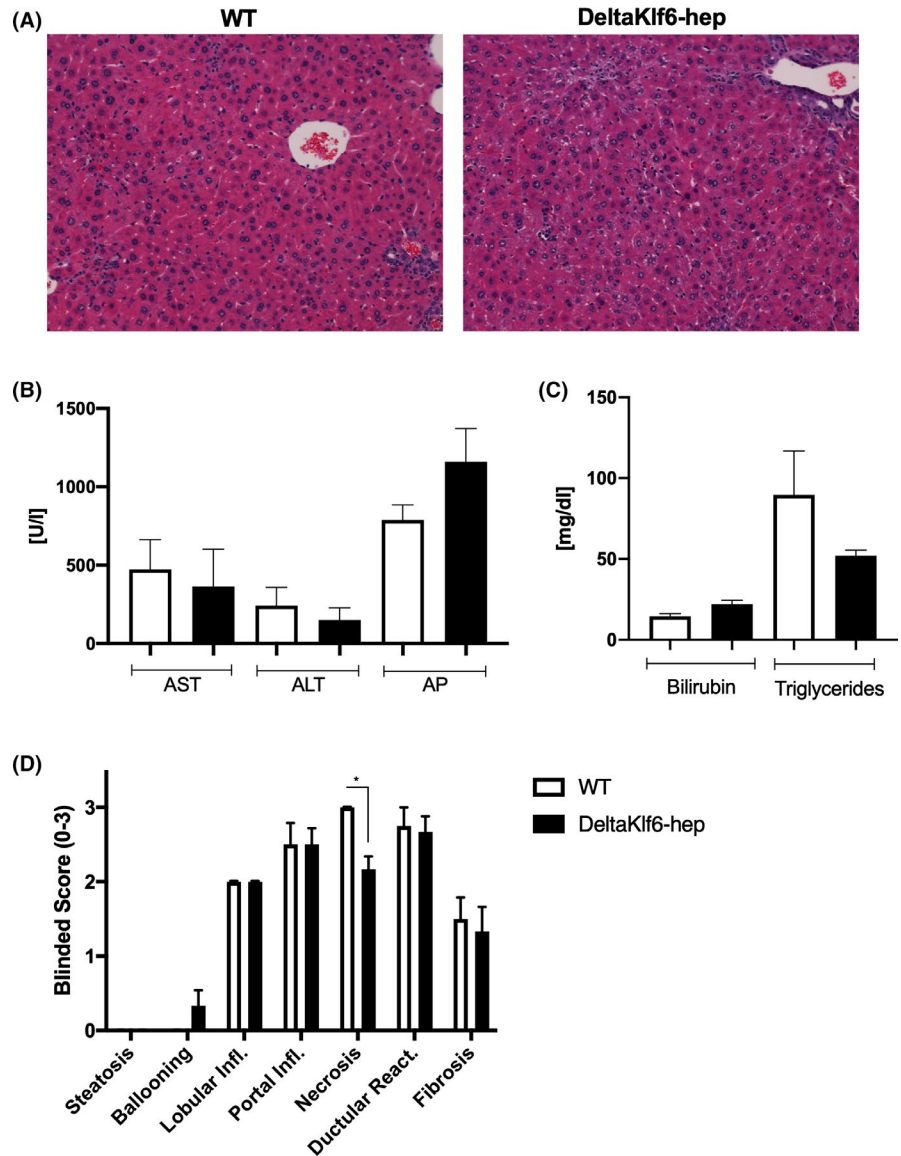


FIGURE 1 High hepatic KLF6 expression levels are related to a better outcome in PSC. Shown are representative images of liver sections from PSC patients with low (n = 9) or high (n = 10) hepatocellular KLF6 expression (A); 40-fold magnification. Patients who developed CCA (B) or deceased (C) during the observation period had lower hepatic KLF6 expression as shown by quantification of KLF6 positive cells per total cells in four different visual fields. Survival of PSC patients shows a significantly better outcome in those patients with high KLF6 expression levels as shown by Kaplan-Meier survival curve excluding those patients who underwent liver transplantation (LTX) (D) or a compound of deceased and transplanted PSC patients (E); * $P = .05$

FIGURE 2 Serum parameters of wildtype (WT) and conditional hepatocellular *Klf6* knockout (Delta*Klf6*-hep) mice 2 weeks following bile duct ligation (BDL). Representative tissue sections (20-fold magnification) from mouse liver of WT and Delta*Klf6*-hep animals following BDL (A) as well as serum parameters of ALT, AST and alkaline phosphatase (B) as well as bilirubin and triglycerides (C). Histopathologic evaluation of liver injury following a blinded scoring system (describing steatosis, ballooning, lobular inflammation, portal inflammation, necrosis, ductular reaction and fibrosis) was determined in WT and Delta*Klf6*-hep knockout animals 2 weeks following sham or BDL ($n = 5$ animals per group, data are shown as mean \pm SEM) (D); * $P = .05$



WT animals (Figure 2). Additionally, liver tissue sections were evaluated using a blinded scoring system to quantify steatosis, ballooning, lobular inflammation, portal inflammation, necrosis, ductular reaction and fibrosis by an expert hepatopathologist. Following BDL, Delta*Klf6*-hep animals showed slightly more cells with ballooning but significantly less necrotic cells were detected (Figure 2D). So in summary, a conditional *Klf6* knockout affects the phenotype of the liver injury following BDL.

3.3 | *Klf6* knockout affects expression of FXR target genes following BDL

The mRNA levels of *Klf6* showed a robust (>20-fold) increase after BDL in WT mice, but not in Delta*Klf6*-hep animals indicating that the sharp increase in *Klf6* in BDL-treated WT mice is hepatocyte-specific (Figure 3A). As FXR activation is involved in cholestasis and PSC, we next measured mRNA expression in the whole liver tissue

of different FXR target genes and factors that are involved in bile acid transport and metabolism. In comparison to sham-operated mice, the expression of the FXR target gene *Nr0b2* (encoding the small heterodimer partner *SHP*) increased following BDL with a slightly higher increase in Delta*Klf6*-hep animals as compared to WT animals (Figure 3B). Additionally, protein levels of *Nr0b2*/*SHP*, as determined by Western blot analysis, were significantly increased in liver tissue of Delta*Klf6*-hep animals in comparison to WT after BDL (Figure 3C/D). Bile acid de novo synthesis is regulated by *SHP*/*NR0B2* as it represses the transcription of *Cyp7A1* (cytochrome *p450* family 7 subfamily A member 1), but here *Cyp7A1* expression was increased after BDL. However, *Cyp7A1* expression did not differ between WT and Delta*Klf6*-hep (Figure 4A). Expression levels of the bile acid exporters *Abcb11*, *Slc51b* and *Abcc3* were slightly increased after BDL, whereas this effect was more prominent in Delta*Klf6*-hep (Figure 4B-D). Taken together, a conditional knockout of *Klf6* in hepatocytes induces a modest attenuation of cholestatic liver injury and is characterized by induction of hepatic bile acid export and

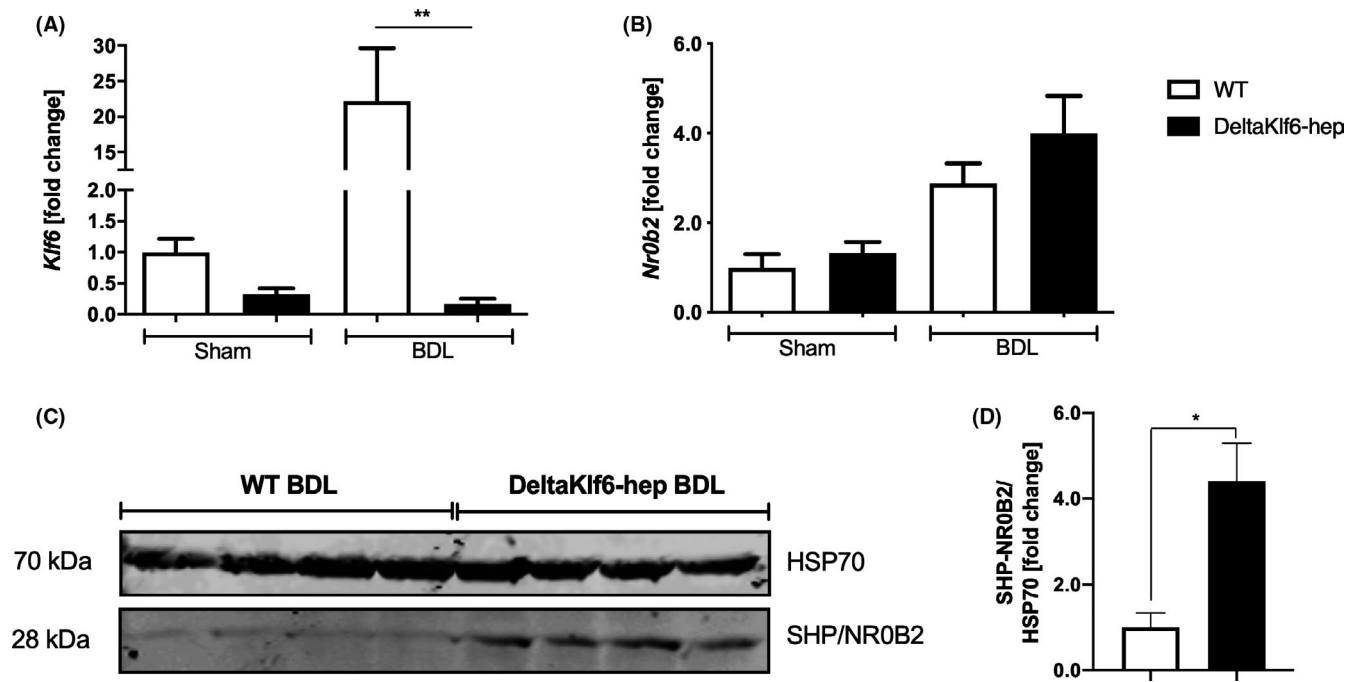


FIGURE 3 Expression levels of *Klf6* and FXR target gene *NrOb2/Shp* in liver tissue of wildtype (WT) and conditional hepatocellular *Klf6* knockout (*DeltaKlf6-hep*) mice 2 weeks after bile duct ligation (BDL). mRNA expression levels of *Klf6* (A), and *NrOb2* (B) in liver tissue of WT and *DeltaKlf6-hep* knockout mice 2 weeks after sham or BDL (n = 5 animals per group) were measured via quantitative RT-PCR. Protein expression of *Shp/NrOb2* in whole mouse liver of WT and *DeltaKlf6-hep* animals was determined via western blot analysis (C), quantified via densitometric analysis and normalized to the housekeeping gene *Hsp70* (D). Data are shown as mean ± SEM; *P = .05, **P = .01

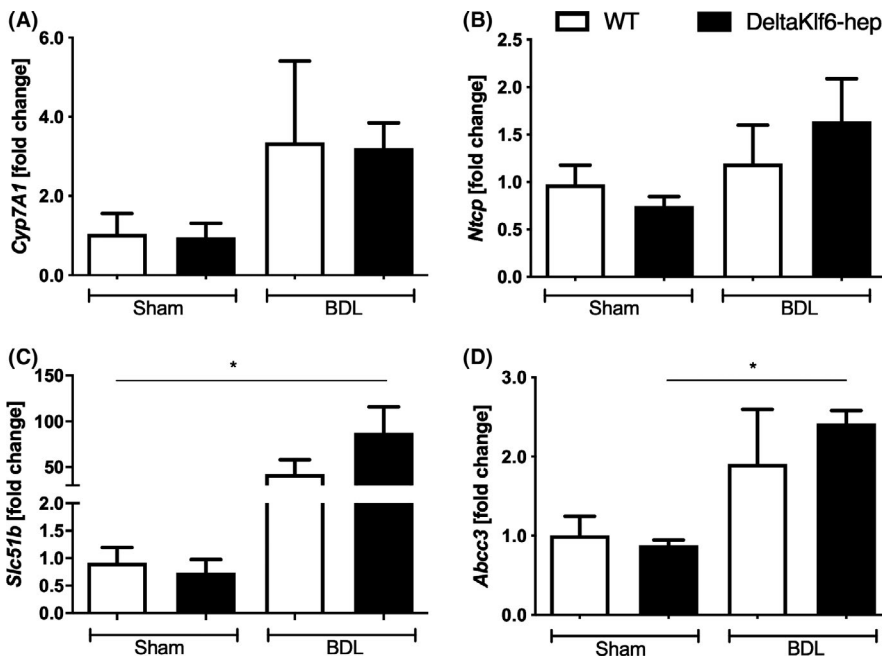


FIGURE 4 Expression levels of FXR target genes in liver tissue of wildtype (WT) and conditional hepatocellular *Klf6* knockout (*DeltaKlf6-hep*) mice 2 weeks after bile duct ligation (BDL). mRNA expression levels *Cyp7A1* (A), *Abcb11* (B), *Slc51b* (C) and *Abcc3* (D) in liver tissue of WT and *DeltaKlf6-hep* knockout mice 2 weeks after sham or BDL (n = 5 animals per group) were measured via quantitative RT-PCR. Data are shown as mean ± SEM; *P = .05

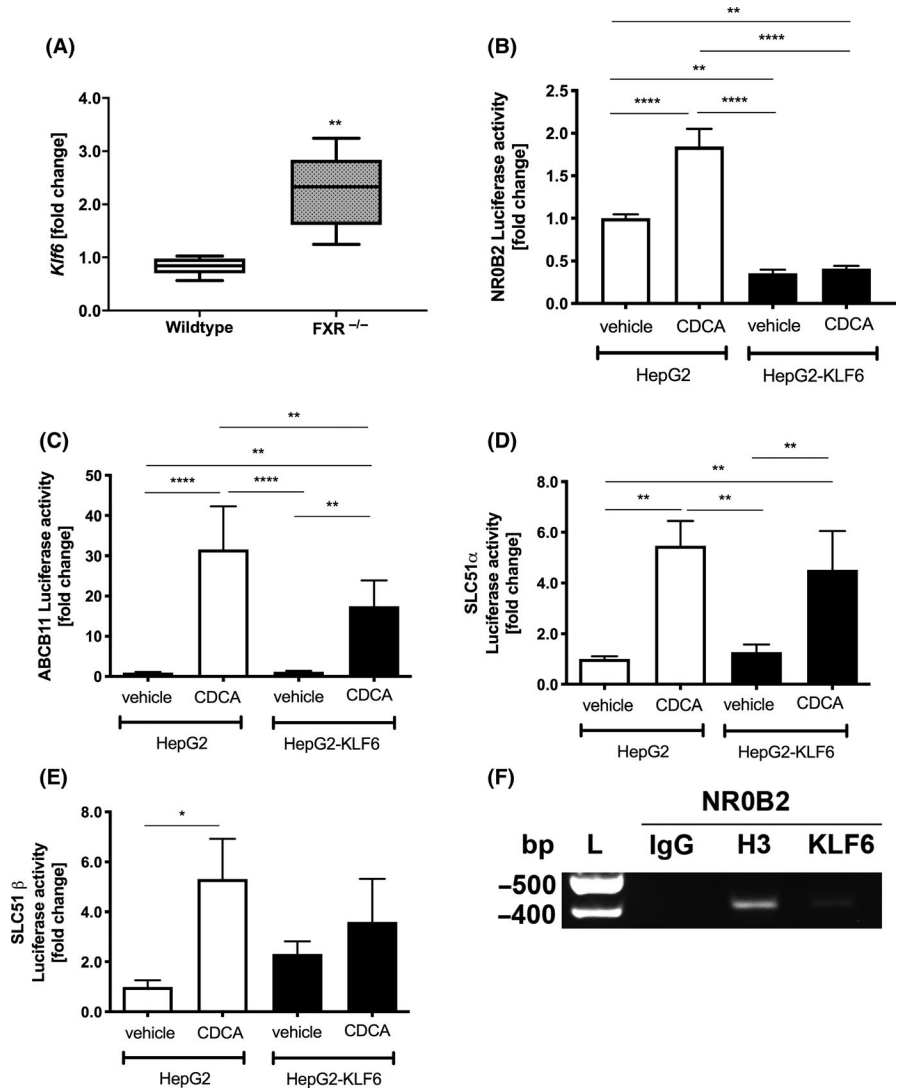
reduction of bile acid uptake mechanisms. Especially in the absence of *Klf6* expression, expression levels of *NrOb2* were increased.

KLF6 is a transcriptional repressor of the FXR target gene *NROB2*.

In different rat models, treatment with FXR agonists lowers serum levels of ALT and AST, decreases necrosis, inflammation, and bile duct proliferation following BDL.^{32,33} Our studies indicate that FXR

signalling is over-activated in BDL-treated *DeltaKlf6-hep* knockout mice but not in sham-treated animals. Furthermore, the expression of *Klf6* was increased in WT mice following BDL. The results suggest a close interaction of both factors, although interestingly in our PSC cohort we did not see significant differences of FXR expression in relation to *KLF6* expression (Figure S1D). However, to examine the mutual

FIGURE 5 *KLF6* overexpression in HepG2 cells affects luciferase activity of FXR target genes and *KLF6* binds to promoter region of *NROB2*. Expression levels of *Klf6* in liver tissue of female *Fxr*^{-/-} knockout mice or wildtype controls (both n = 7 animals per group) was measured via quantitative RT-PCR (A) and did show a significant up-regulation in *Fxr*^{-/-}. Luciferase activity was measured in HepG2 and *KLF6* overexpressing HepG2 cells (HepG2-KLF6) following transient transfection with specific reporter plasmid for *NROB2* (B), *ABCB11* (C), *SLC51α* (D) and *SLC51β* (E) with and without CDCA stimulation (n = 3 individual cell culture experiments shown as mean ± SEM). The interaction of *KLF6* with the *NROB2* promoter containing putative *KLF6*-binding motifs was confirmed by chromatin immunoprecipitation (ChIP) in HepG2 cells (n = 3 individual experiments) using a specific *KLF6* antibody (F); IgG was used as a negative control, Histone-H3 antibody was used as a positive control for ChIP; **P* = .05, ***P* = .01, ****P* = .001, *****P* > .0001



interaction closer, we measured the expression of *Klf6* in liver tissue of *Fxr*^{-/-} knockout animals and observed an increase of *Klf6* expression in *Fxr*^{-/-} mice as compared to WT animals (Figure 5A).

To study the interaction of *KLF6* and FXR signalling more closely we performed cell culture experiments using HepG2 and *KLF6*-overexpressing HepG2 cells (HepG2-KLF6). *KLF6* expression was induced by transient transfection with a *KLF6* expression vector, which resulted in significant upregulation of *KLF6* mRNA levels (Supplementary Figure S2A), which was also described previously.¹⁶ To analyse the transcriptional effects of *KLF6* on FXR-controlled genes/promoter elements we performed luciferase reporter assays with control and HepG2-KLF6 cells and different luciferase reporter vectors monitoring the promoter activation of the FXR target genes *NROB2*, *ABCB11*, *SLC51A* and *SLC51B*. In addition, to stimulate FXR activation, we treated the cells with the bile acid CDCA. CDCA treatment did show significant activation of all the analysed FXR target genes as shown via reporter assay and on mRNA levels measured by qRT-PCR in HepG2 cells (Figure 5, Figure S2B-D). Treatment with CDCA did not influence mRNA levels of *KLF6* while transfection with pcneo-*KLF6* resulted in significant upregulation of *KLF6* (Figure S2A).

KLF6 overexpression in HepG2-KLF6 cells significantly reduced *NROB2*/SHP promoter activation. Even after CDCA treatment the activity of the *NROB2* promoter element could not be stimulated in HepG2-KLF6 cells (Figure 5B). The effect of reduced basal promoter activation was not seen for the other FXR target genes *ABCB11*, *SLC51A* or *SLC51B* in untreated HepG2-KLF6 cells. However, after treatment with CDCA, the activity of the *ABCB11* promoter was significantly lower than in control HepG2 cells (Figure 5C-E). While CDCA treatment for 24 hours activates FXR target gene promoters, here *KLF6* overexpression had no effect on the mRNA expression of FXR target genes (Figure S2). In summary, *KLF6* represses the induction of *NROB2* and attenuates the activation of *ABCB11* following CDCA stimulation.

3.4 | *KLF6* binds directly to the promoter region of *NROB2* and represses its expression

Members of the KLF family bind with varying affinity to DNA-binding sites of target gene promoters and therefore function as

transcriptional activators or repressors.³⁴ Interestingly, FXR-binding elements (IR-1 sequences) in the promoter regions of FXR target genes overlap with potential binding sites for KLF6 (CACCC represents a prominent binding motif), which might explain inhibition of FXR-induced promoter activation in the presence of high levels of KLF6. We identified potential KLF6-binding motifs within the promoter regions of *NROB2*, and then confirmed transcriptional interaction by luciferase reporter assays. To specify the direct interaction of KLF6 and the *NROB2* promoter region we performed ChIP assays in HepG2-KLF6 cells using a KLF6-specific antibody, which indeed showed that KLF6 binds directly to the promoter region of *NROB2* (Figure 5F).

Our findings indicate that KLF6 influences FXR signalling by potentially binding to FXREs and therefore KLF6 may work as an agonist or antagonist. ChIP assay confirmed a physical interaction between KLF6 and the promoter region of the FXR target gene *NROB2*. These experiments underline a potential role of KLF6 as a novel mediator in FXR signalling and give new options to examine the clinical context of cholestatic liver diseases.

4 | DISCUSSION

The clinical course of PSC is primarily characterized by cholestasis promoting fibrogenesis and the occurrence of cirrhosis. For advanced stage PSC, liver transplantation is the only curative option.

Here, we describe a novel interrelation between hepatic KLF6 expression and overall-survival as well as a potential role in the formation of CCA in a small, but well-characterized cohort of PSC-patients. Mechanistically, we propose a novel interaction of KLF6 with FXR signalling via its functional target gene *NROB2* while the expression of FXR itself is unaffected by KLF6. In previous experiments, we have shown that KLF6 contributes to different models of acute liver injury as an important regulator of autophagy.¹⁶ With experiments using the *DeltaKlf6*-hep animal model in cholestasis following BDL, we observed an effect of hepatocyte KLF6 on cholestasis mediated cell-death while laboratory parameters of cholestasis appeared to be increased. In WT animals, as a response to cholestatic injury, the expression of *Klf6* was strongly induced following BDL. Similarly, in PBC patients, hepatocyte KLF6 expression was associated with a more cholestatic laboratory profile.

The clinical data and experiments with *DeltaKlf6*-hep animals point towards a mechanistic relation between *Klf6* and cholestasis-associated factors. Further in vitro studies using KLF6-overexpressing HepG2 cells point towards a direct interaction of KLF6 with the FXR target gene *NROB2* (*SHP*), an orphan receptor, which influences bile acid metabolism via repression of several target genes including *Cyp7A1*, the key mediator of hepatic bile acid synthesis. A previous study found that *NROB2* interacts with a SP2/KLF6 complex during regulation of matrix metalloproteinase 9/MMP9 expression via FXR.³⁵ Here, FXR induced *SHP* displaces the complex of SP2 and KLF6 by binding to a SP1 motif. However, a direct binding of *SHP* to KLF6 has not been described yet.

We identified a novel interaction of KLF6 with the regulation of bile acid metabolism. The composition of the bile acid pool consisting of different primary and secondary bile acids and its regulation is dependent on different biological processes in different parts of the liver and the intestine. FXR is an important nuclear receptor regulating bile acid homeostasis and keeps the balance of circulating and de novo synthesis of bile acids. In the liver, FXR regulates the expression of multiple genes that are involved in cellular bile salt import and export as well as in bile salt synthesis.³⁶ FXR interacts with RXR α via heterodimer formation to induce transcriptional activity. The heterodimer typically binds to a specific DNA sequence consisting of two inverted repeat sequences separated by one nucleotide (IR-1) within the promoter region of its target genes. FXR is mainly activated by steroids such as bile acids to regulate intracellular bile acid levels and synthesis by activation of FXR target genes. An important downstream target gene of FXR signalling represents *NROB2*, which controls bile acid influx and synthesis by regulating the expression of the bile acid importer *SLC10A11* encoding the Na⁺-taurocholate co-transporting polypeptide (NTCP). Modulation of FXR signalling influences metabolic homeostasis and genetic FXR variances are associated with insulin resistance and dyslipidaemia.^{35,37} In chronic cholestasis-related diseases such as PSC, FXR and its target genes have been described to reduce cholestasis-induced liver injury. Intestinal FXR expression influences transcription of bile acid transporters and the expression of the fibroblast-growth-factor-19 in the intestines and via the close interaction of liver and gut within the enterohepatic circulation intestinal FXR regulation affects liver injury.³⁸ However, we did not observe alterations in FXR-IHC in liver tissue associated with KLF6 expression levels in PSC patients.

PSC is characterized by recurrent ascending cholangitis due to obstruction of the large intra- and extrahepatic bile ducts.³⁹ PSC differs from PBC in that the hepatocellular damage is caused by ductular cholestasis of the interlobular bile ducts within the parenchyma.⁴⁰ Clinical data from patients with PBC, an autoimmune cholestatic disease with a unique pathophysiology, we observed a cholestatic pattern associated with hepatic KLF6 expression, comparable to our observations in the BDL model. In contrast, in PSC, KLF6 expression was associated with a trend toward a milder cholestasis and more strikingly, a better clinical outcome. Therefore, we propose a distinct effect of hepatocyte KLF6 in PSC, independent of cholestasis.

Besides acute-on-chronic organ failure, the insufficient bile flow or bile blockage causes necrosis, which promotes proliferation and thus the formation of hepatic tumours such as CCA and with a much lower incidence HCC. Tumorigenesis of CCA is complex and its timely detection and therapy is complicated due to limited detection rate, surgical and medical therapy options, tumour resistance and tumour-own survival strategies. Thus, CCA is associated with poor prognosis.⁴¹⁻⁴³ Therapeutic agents such as FXR agonists show promising effects in cholestatic diseases in order to prevent bile acid overload causing liver injury and the use of FXR agonists has been proposed in the context of CCA treatment.^{44,45} It has been shown that the tumour suppressor gene KLF6 can inhibit the formation of numerous cancer types while the expression of the alternative splice

variant KLF6-SV1 is associated with enhanced tumour growth and a worse outcome in HCC.^{5-7,10,46} However, so far no data are available on potential effects of KLF6 on bile acid signalling, cholestatic injury or CCA tumorigenesis. While our cohort is clearly too small to draw conclusions on potential effects of KLF6 on CCA, we were able to show that all PSC patients developing CCA during the observation period had a low hepatocyte KLF6 expression. In tumorigenesis as well as PSC progression, different cell types have been proposed to play pivotal roles.⁴⁷ While we mainly focused this work on hepatocyte KLF6 expression, we did not assess potential effects of KLF6 in non-parenchymal and progenitor cells in the context of cholestasis and CCA. However, these effects might explain in parts the contradictory observations we made between PSC, PBC and the knockout model. This is a clear limitation of this study and has to be addressed in future projects.

Our data indicate that the hepatocellular expression of KLF6 is associated with the clinical course of PSC. Here, we show in a small, but well-defined cohort of PSC patients, that KLF6 expression is associated with the clinical outcome of PSC and may as well affect the development of CCA. We further mechanistically link KLF6 expression with FXR target genes that play pivotal roles in bile acid metabolism. Using in vitro cell culture experiments we show that KLF6 and the FXR target gene *NR0B2* interact with each other via a direct binding of KLF6 to promoter regions of *NR0B2*. However, to confirm our results and especially validate a potential effect of KLF6 on tumorigenesis in CCA, further studies need to be conducted.

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AUTHOR CONTRIBUTIONS

SS contributed to acquisition, analysis and interpretation of data, study design, statistical analysis, drafting of the manuscript, study supervision. PM contributed to acquisition, analysis and interpretation of patient data, statistical analysis. LvB contributed to data acquisition, analysis and interpretation of patient data. ST contributed to analysis and technical assistance IHC. SSch contributed to analysis and technical assistance IHC. JB contributed to acquisition and analysis of data. JH contributed to technical support, data interpretation with cell culture and luciferase experiments. AS contributed to acquisition, analysis, interpretation and statistical analysis of *Fxr*^{-/-} mouse data. DV contributed to performance of BDL. MS contributed to analysis and technical assistance for IHC. AD contributed to performance, interpretation and analysis of Western blot. MIF contributed to histopathological evaluation of mouse liver tissue. HAB contributed to obtained material and data of CCA patients. AD contributed to acquisition of data and patient material. FJC contributed to critical revision of the manuscript for important intellectual

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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