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 metallopeptidase 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; PPARα/γ, Peroxisome proliferator-activated receptor alpha/gamma; PCNA, Proliferating cell nuclear antigen; PSC, Primary sclerosing cholangitis; RXRα, Retinoid-X-receptor alpha; Sdhα, Succinate dehydrogenase complex flavoprotein subunit A; SLC10A1/NTCP, solute-carrier-family-10 member 1/sodium-taurocholate-cotransporting-polypeptide; SLC51A/OSTα, SLC51B/OSTβ; Statherin; Trangenic mice; WT, Wildtype; γ-GT, Gamma glutamyltransferase.

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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. 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. KLF6 can act both as activator and repressor of transcription. 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. KLF6 expression or its functional loss have been associated with the progression and outcome of various tumours in patients, including hepatocellular carcinoma (HCC). 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). 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 (PPARα) expression. 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 the intra- and extra hepatic bile ducts resulting in progressive biliary fibrosis and cirrhosis. 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. 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. 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

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 NR0B2.

KEYWORDS
cholangiocellular carcinoma, cholestasis, FXR, KLF6

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.
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. With established cirrhosis, liver transplantation is the only therapeutic option in progressive disease.22

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 PPARγ, others found KLF6 to repress the nuclear receptor PPARγ and interact with its cofactor Retinoid-X-Receptor alpha (RXRα).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 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 ≤ .05. If not stated otherwise all data are presented as mean ± 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 dilution 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 ± 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), γGT (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 γGT, 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 (ΔKlf6-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 ΔKlf6-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](image-url) 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
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 DeltaKlf6-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 DeltaKlf6-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 DeltaKlf6-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 DeltaKlf6-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 DeltaKlf6-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
reduction of bile acid uptake mechanisms. Especially in the absence of Klf6 expression, expression levels of Nr0b2 were increased.

KLF6 is a transcriptional repressor of the FXR target gene NR0B2.

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 3 Expression levels of Klf6 and FXR target gene Nr0b2/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 Nr0b2 (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/Nr0b2 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.
interaction closer, we measured the expression of Klf6 in liver tissue of Fxr−/− knockout mice 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 NR0B2, ABCB11, SLC51α and SLC51β. 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 pcIneo-KLF6 resulted in significant upregulation of KLF6 (Figure S2A).

KLF6 overexpression in HepG2-KLF6 cells significantly reduced NR0B2/SHP promoter activation. Even after CDCA treatment the activity of the NR0B2 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 NR0B2 and attenuates the activation of ABCB11 following CDCA stimulation.

3.4 KLF6 binds directly to the promoter region of NR0B2 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. Interestingly, FXR-binding elements (IR-1 sequences) in the promoter regions of FXR target genes overlap with potential binding sites for KLF6 (CACC motif 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 NR0B2, and then confirmed transcriptional interaction by luciferase reporter assays. To specify the direct interaction of KLF6 and the NR0B2 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 NR0B2 (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 NR0B2. 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 NR0B2 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. 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 NR0B2 (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 NR0B2 interacts with a SP2/KLF6 complex during regulation of matrix metallopeptidase 9/MMP9 expression via FXR. 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. FXR interacts with RXRα 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 NR0B2, 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 dyslipidemia. 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. 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. PSC differs from PBC in that the hepatocellular damage is caused by ductular cholestasis of the interlobular bile ducts within the parenchyma. 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. 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. 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. 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 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. 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.

ACKNOWLEDGEMENTS
The authors thank Lena Wingert, Sami Jafoui, Alina Schlinkheider, Claudia Arndt and Nadine Köhler for excellent technical assistance. The authors thank Dorothe Möllmann for her excellent technical assistance in the preparation of tissue sections from patient material. Furthermore, the authors thank Rebekka Hannivoort for interpretation and discussion of data and sharing important intellectual content.

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, statistical analysis. SSc contributed to analysis and technical assistance IHC. SSh 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 the technical support, data interpretation with cell culture and luciferase experiments. MS contributed to acquisition, analysis and interpretation of data, statistical analysis, study supervision, drafting of the manuscript and obtained funding.

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

How to cite this article: Sydor S, Manka P, van Buren L, et al. Hepatocyte KLF6 expression affects FXR signalling and the clinical course of primary sclerosing cholangitis. Liver Int. 2020;40:2172–2181. https://doi.org/10.1111/liv.14542