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Endoglin in human liver disease and murine models of liver fibrosis—A protective factor against liver fibrosis

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Abstract

Background & Aims: Liver fibrosis is the outcome of chronic liver injury. Transforming growth factor-β (TGF-β) is a major profibrogenic cytokine modulating hepatic stellate cell (HSC) activation and extracellular matrix homeostasis. This study analyses the effect of Endoglin (Eng), a TGF-β type III auxiliary receptor, on fibrogenesis in two models of liver injury by HSC-specific endoglin deletion.

Methods: Eng expression was measured in human and murine samples of liver injury. After generating $GFAP^{Cre(+)}Eng^{\Delta HSC}$ mice, the impact of Endoglin deletion on chronic liver fibrosis was analysed. For in vitro analysis, $Eng^{flox/flox}$ HSCs were infected with Cre-expressing virus to deplete Endoglin and fibrogenic responses were analysed.

Results: Endoglin is upregulated in human liver injury. The receptor is expressed in liver tissues and mesenchymal liver cells with much higher abundance of the L-Eng splice variant. Comparing $GFAP^{Cre(-)}Eng^{f/f}$ to $GFAP^{Cre(+)}Eng^{\Delta HSC}$ mice in toxic liver injury, livers of $GFAP^{Cre(+)}Eng^{\Delta HSC}$ mice showed 39.9% ($P < .01$) higher Hydroxyproline content compared to $GFAP^{Cre(-)}Eng^{f/f}$ littermates. Sirius Red staining underlined these findings, showing 58.8% ($P < .05$) more Collagen deposition in livers of $GFAP^{Cre(+)}Eng^{\Delta HSC}$ mice. Similar results were obtained in mice subjected to cholestatic injury.

Conclusion: Endoglin isoforms are differentially upregulated in liver samples of patients with chronic and acute liver injury. Endoglin deficiency in HSC significantly aggravates fibrosis in response to injury in two different murine models of liver fibrosis and increases α-SMA and fibronectin expression in vitro. This suggests that Endoglin protects against fibrotic injury, likely through modulation of TGF-β signalling.

KEYWORDS
Endoglin, hepatic stellate cells, liver fibrosis, liver injury, transforming growth factor-β

Abbreviations: ALF, acute liver failure; BDL, bile duct ligation; CTGF, connective tissue growth factor; ECM, extracellular matrix; Eng, Endoglin; GFAP, glial fibrillary acidic protein; HCV, hepatitis C virus; HHT, hereditary haemorrhagic telangiectasia; HSC, hepatic stellate cell(s); KC, Kupffer cell(s); LSEC, liver sinusoidal endothelial cell(s); MMP-14, matrix metalloprotease-14; NASH, non-alcoholic steatohepatitis; TGF-β1, transforming growth factor-β1; α-SMA, α-smooth muscle actin.

1 | INTRODUCTION

Chronic injury leads to scarring of the liver, causing loss of function and multiple complications such as oesophageal bleedings and cancer. Ongoing liver injury causes fibrosis of liver tissue by extracellular matrix (ECM) deposition.[1] Hepatic stellate cell(s) (HSC) are the major source of Collagen and ECM production. In response to liver injury, HSC undergo activation from quiescent HSC to activated myofibroblasts.[2] Because of their embryonic origin, HSC express neural markers (Glial fibrillary acidic protein [GFAP], Synemin and Synaptophysin) along with mesenchymal markers (Desmin, Vimentin). Activation of HSC is regulated by many pro- and antifibrotic cytokines.

Transforming growth factor-β (TGF-β) is a major profibrotic cytokine, signalling through a network of receptors and intracellular mediators. Endoglin is a type III TGF-β receptor which is highly expressed on proliferating vascular endothelial cells,[3,4] cardiac and scleroderma fibroblasts,[3] macrophages[5] and HSC.[3,6] In the presence of TGF-β receptors, type I and type II Endoglin binds different ligands of the TGF-β super-family mediating Smad-dependent and independent signalling.[3] Molecular cloning of human *endoglin* cDNA has revealed the existence of two transcript variants (L- and S-Endoglin), arising from alternative splicing.[7] Furthermore, a soluble form of Endoglin (sol-Eng) is created through shedding mediated by the matrix metalloprotease-14 (MMP-14).[3]

Because of its strong expression on vascular endothelial cells, Endoglin has been studied in diseases involving vascular dysfunction, including atherosclerosis[8] and haemorrhaghic hereditary telangiectasia (HHT1) but only sparsely in relation to fibrosis.[9] Homozygous Endoglin *knockout* animals die in utero at day 10-10.5 post-coitum because of defective angiogenesis and severe cardiovascular abnormalities[3] very similar in phenotype to the Osler-Weber-Rendu syndrome, an autosomal dominant vascular disorder that is characterized by focal telangiectasia and arteriovenous malformations.[3] Mutations in genes encoding *endoglin* and *activin-like kinase (ALK1)* are associated with HHT1 and HHT2 respectively.[10]

Therefore, most data rely on endothelial cell biology and claim that Endoglin modulates the balance between TGF-β1-ALK1 and TGF-β1-ALK5 signalling pathways.[11] L-Endoglin promotes cell proliferation via TGF-β1-ALK1 signalling, while interfering with the TGF-β1-ALK5 pathway whereas S-Endoglin activates the TGF-β1-ALK5 pathway.[12] In HSC, the TGF-β1/ALK5 signalling pathway generally regulates key profibrogenic responses.[13] However, it has been shown that the TGF-β1/ALK1/Id1 pathway is also involved in profibrogenic signalling.[14] Nevertheless, although Endolin is highly expressed in HSC, in vivo data analysing Endoglin function in fibrosis are missing.

Because of its interactions with TGF-β signalling, Endoglin expression can be linked to various pathological conditions such as cancer,[15] cancer angiogenesis[16] and fibrosis in different organs.[17] In the process of kidney fibrosis, Endoglin is upregulated in human patients and in animal disease models. However, the impact of Endoglin is not clear. Most in vitro studies imply an antifibrotic effect of Endoglin,[18] experiments using overexpression of full length Endoglin in HSC cell lines imply a profibrogenic role.[19,20]

KEY POINTS

- Endoglin is expressed and upregulated in human samples of acute and chronic liver disease.
- Hepatic stellate cells, the major extracellular matrix producing cell in liver injury show significant expression of Endoglin.
- Hepatic stellate cell-specific deletion in murine models of liver fibrosis leads to aggravation of liver fibrosis—suggesting a protective effect of Endoglin on TGF-β signalling in liver injury.
- Although depletion of Endoglin in HSC leads to a decrease in matrix accumulation in the affected liver in vivo, Endoglin promotes profibrogenic aspects in vitro.

The insight in the role of Endoglin in liver disease is limited. In hepatitis C infection or liver fibrosis/cirrhosis, high levels of sol-Eng were detected.[15] Endoglin expression is upregulated in murine liver disease models and in hepatic stellate cells.[20] In addition, overexpression of L-Endoglin in HSC cell lines of rat and mouse origin shows that Endoglin modulates TGF-β1 signalling.[19,20] The relation of Endoglin and TGF-β, the expression on HSCs and its expression in fibrotic diseases makes it an interesting target to study its impact on TGF-β signalling in chronic liver injury.

2 | METHODS

2.1 | Animal models

The mouse line expressing the floxed *endoglin* gene was generated by Arthur et al[21] at the Institute of Human Genetics, International Centre for Life, University of Newcastle upon Tyne, UK and has a C57BL/6 background. The $GFAP^{Cre}$ mouse used in this study was ordered at Jackson Labs and backcrossed to a C57Bl/6 background (FVB-Tg(GFAP-cre)25Mes/J, stock no. 004600).

2.2 | Human samples

Human liver samples (n = 6/group) were obtained from patients undergoing biopsy for diagnostic medical reasons after obtaining informed consent in accordance to the ethical guidelines of the 1975 Declaration of Helsinki. The study was approved by the "Ethic Regulations Committee of the University Hospital RWTH Aachen". The analysed material was excess biopsy material not used for further diagnostics, biopsies were not taken for study purposes only.

2.3 | Isolation of primary liver cells

Quiescent HSCs, KCs and liver sinusoidal endothelial cell(s) (LSECs) were isolated from untreated mice by the two step pronase-collagenase method.[22] HSCs were further purified by a single-step density gradient centrifugation and sorted by retinoid-dependent FACS analysis.[23]

2.4 | Induction of liver fibrosis

Wild-type C57BL/6 (n = 8), $GFAP^{Cre(-)}Eng^{f/f}$ (n = 8) and $GFAP^{Cre(+)}Eng^{\Delta HSC}$ (n = 8) mice were injected 3 times/week (i.p.) for 8 weeks with carbon tetrachloride (CCl_4; 0.6 mL/kg body weight)[23] or subjected to BDL surgery to induce liver fibrosis as described before.[24] Mice were sacrificed 3 days after the last CCl_4 injection[25] or 21 days after BDL.

For additional methods, see Appendix S1. For antibodies used in this study, see Table S1.

3 | RESULTS

3.1 | Endoglin splice variants show differentially increased expression in livers of patients with acute and chronic disease

Liver biopsies from patients with acute liver failure (ALF) and chronic liver injury (ie, non-alcoholic steatohepatitis [NASH] and HCV infection) were analysed for *Eng* mRNA expression. Figure 1A shows significantly ($P < .05$) increased L-Eng expression in ALF as well as in chronic liver injury compared to healthy control livers. However, the S-Eng levels were only increased in ALF patients and not in patients with chronic liver diseases suffering from NASH or HCV infection (Figure 1B).

3.2 | Endoglin splice variants are expressed in mouse tissues and isolated liver cells

L- and S-Endoglin expression was analysed in various mouse tissues and in isolated primary liver cells. L-Endoglin is the predominantly expressed isoform in all examined tissues as well as in KC, LSEC and HSCs. S-Endoglin is also present in significant levels in all tissues and liver cells tested (Figure 1C,D). Hepatocytes do not express Endoglin.[20]

Liver injury after BDL resulted in significant L-/S-Endoglin upregulation compared to sham-operated mice. Of interest, especially S-Endoglin expression is upregulated more pronounced early after injury, in the second and third week after BDL both isoforms were highly expressed compared to controls (Figure 1E). Similar effects were seen in CCl_4 treated animals (data not shown).

3.3 | $GFAP^{Cre}$ specifically targets HSCs

In order to analyse the contribution of Endoglin to liver fibrosis in vivo, mice with a floxed *endoglin* gene were crossed with $GFAP^{Cre}$ animals. Because of their developmental lineage, HSCs are the only GFAP-expressing cells in the liver.[2]

Genomic recombination in HSCs from $GFAP^{Cre(-)}Eng^{f/f}$ and $GFAP^{Cre(+)}Eng^{\Delta HSC}$ mice was investigated using FACS-sorted

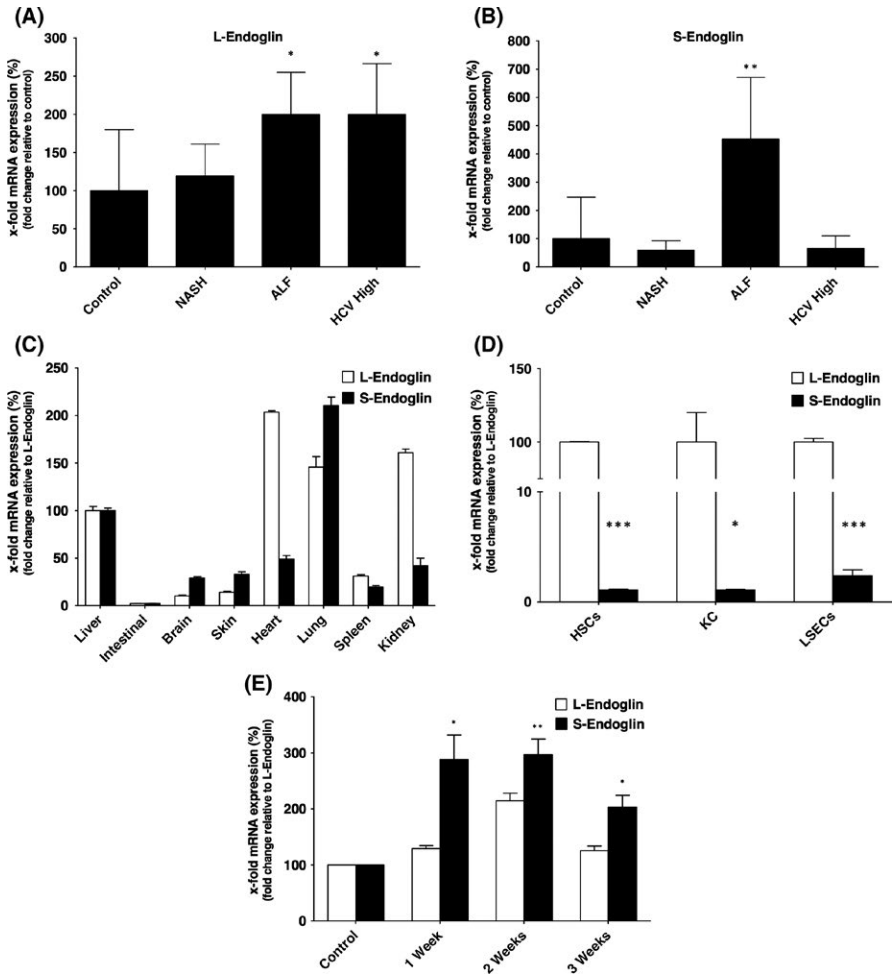


FIGURE 1 Expression of S- and L-endoglin. (A,B), human L- and S-endoglin: Expression of L- and S- endoglin mRNA in different patient cohorts (n = 6). (C), Expression of L- and S- endoglin mRNA in different C57Bl6 *wild-type* mouse tissues, normalized to the expression in liver. Data are expressed as means ± SEM of three mice per group (*$P < .05$, **$P < .01$, ***$P < .001$). (D), Change of endoglin isoform expression in isolated FACS sorted (HSCs, KC) or MACS purified liver sinusoidal endothelial cell(s) (LSECs) primary cells of untreated C57BL/6 *wild-type* mice and after CCl_4 treatment for 8 wk, bars show relative changes in percent compared to expression levels of untreated animals. Results are means ± SEM of three mice per group (*$P < .05$, ***$P < .001$). (E), Expression of endoglin mRNA in whole liver tissue from C57BL/6 wild-type mice which underwent bile duct ligation (BDL) for 3 wk. Especially, S-endoglin expression is upregulated in cholestatic liver injury. Data are expressed as means ± SEM of 3 mice per group (*$P < .05$, **$P < .01$)

isolated primary cells.[26] PCR analysis of *wild-type*, $Eng^{f/f}$ and $Eng^{\Delta HSC}$ mice was performed with specific primers,[21] Figure 2A. *Wild-type* mice showed only the shorter DNA product for the *wild-type* allele, missing the lox-P sites. The floxed and the deleted Eng alleles were only detected by their specific primers in $Eng^{f/f}$- and $Eng^{\Delta HSC}$ mice respectively. However $Eng^{\Delta HSC}$ mice showed an $Eng^{f/f}$ allele as well, suggesting incomplete genetic recombination in HSCs.

Next, Endoglin protein expression was analysed in $GFAP^{Cre(+)}Eng^{\Delta HSC}$ mice, primary HSCs from $GFAP^{Cre(-)}Eng^{f/f}$ and $GFAP^{Cre(+)}Eng^{\Delta HSC}$ mice after isolation as described before.[22] Western blots revealed up to 70% decrease of Endoglin protein expression in HSCs of Cre^{+} mice (Figure 2B). Since genetic recombination is not achieved in 100% of the targeted HSCs, a faint Endoglin signal is detected in activated $Eng^{\Delta HSC}$ cells.

Furthermore using $Rosa26^{f/f\text{-}mT/mGFP}$ reporter mice specific Cre recombination in HSCs was investigated in mouse liver. $GFAP^{Cre}$ mice were crossed to $Rosa26^{f/f\text{-}mT/mGFP}$ mice (Figure 2C). After genetic recombination, Cre^{+} cells in these animals lose mTRed expression and express mGFP. As shown by confocal microscopy, $GFAP^{Cre}$ labels Desmin-expressing HSCs specifically in mouse liver (Figure 2C). However more cells were GFP^{+} than $Desmin^{+}$, most likely because of permanent genetic labelling of the Cre recombinase in contrast to the transient protein expression detected by immunofluorescence. For generating cell-specific *knockouts*, this is advantageous because recombination occurs in a higher number of cells.

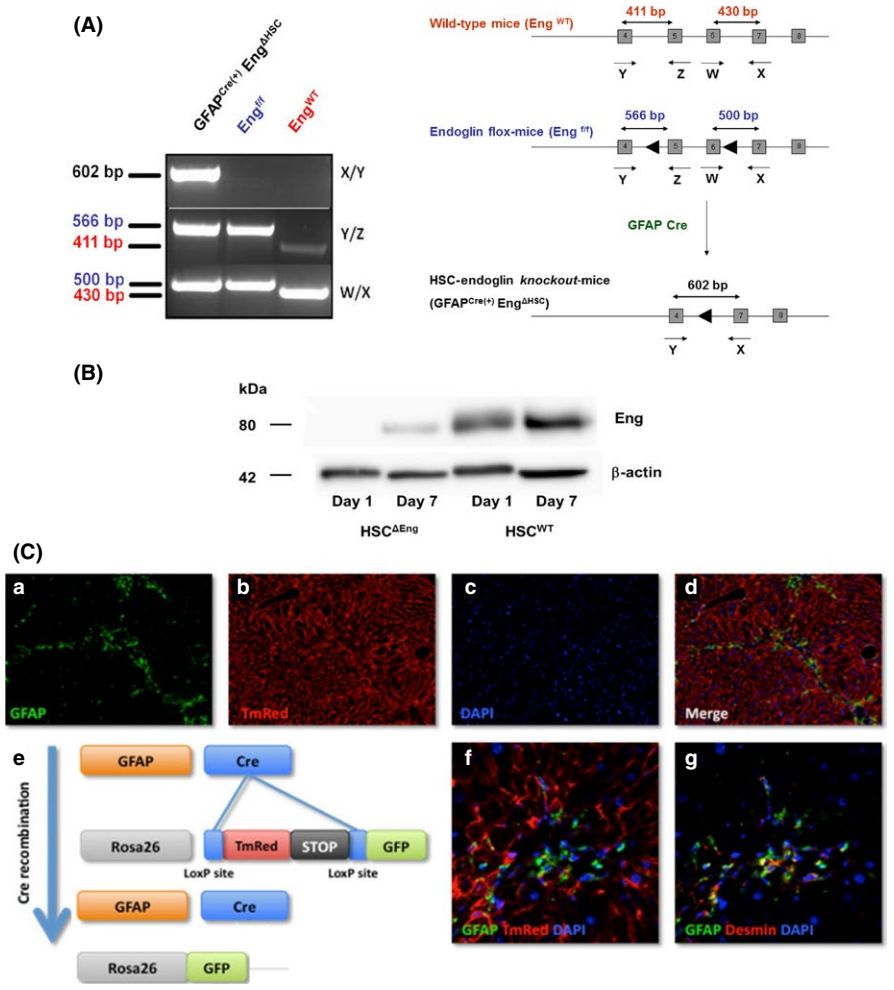


FIGURE 2 $GFAP^{Cre}$ targets hepatic stellate cells (HSCs) specifically. (A), Genomic recombination in $GFAP^{Cre(+)}Eng^{\Delta HSC}$ HSCs. Primer pair w/x amplifies a product of 430 bp, primer pair y/z amplifies a product of 411 bp for the wild-type endoglin allele (Eng^{wt}). In Eng-floxed mice ($Eng^{f/f}$) exons 5 and 6 of the *endoglin* gene are flanked by lox-P sites, so that the above mentioned primer pairs amplify a product of 500 and 566 bp, respectively, in cells which do not express the Cre transgene. Upon Cre-mediated recombination in HSC, the floxed exons 5 and 6 of *endoglin* are deleted which results in an amplification product of 602 bp using primers x/y (n = 3). Since the deletion is not quantitative, the products of the floxed gene without deletion are also amplified to a lower content from Cre positive HSC. (B), Western blot shows $GFAP^{Cre}$ deletion of floxed *endoglin* in isolated and FACS-sorted primary hepatic stellate cells after 1 and 7 d of primary culture. Three animals ($GFAP^{Cre(-)}Eng^{f/f}$ and $GFAP^{Cre(+)}Eng^{\Delta HSC}$) were used in each experiment. (C), Specificity of $GFAP^{Cre}$ recombination in a double fluorescence reporter mouse. $GFAP^{Cre}$ labels specifically hepatic stellate cells in fibrotic septa (8 wk CCl_4 treatment). (a-d) fluorescence microscopy of CCl_4 treated C57BL/6 mice, 200×, no staining. GFAP labelled cells express mGFP protein, but no mT RED. (e) Genetic recombination in this reporter mouse after crossing to $GFAP^{Cre}$. (f,g) Double staining with anti-Desmin and anti-GFAP antibodies. Pseudo colour red, merge with GFAP labelled stellate cells yellow (magnification: 600×)

3.4 | Decreased soluble Endoglin expression in GFAP$^{Cre(+)}$Eng$^{\Delta HSC}$ mice

As a membrane bound auxiliary TGF-β receptor, Eng is shedded by MMP-14[27,28] and can be measured in serum as sol-Eng. Besides decreased Endoglin expression in HSCs, the serum levels of sol-Eng were significantly reduced in GFAP$^{Cre(+)}$Eng$^{\Delta HSC}$ mice during chronic injury (Figure 3A). After BDL or CCl_4 treatment, MMP14 levels did not change in response to *endoglin* deletion (Figure 3B,C). Therefore reduced soluble Endoglin serum levels in GFAP$^{Cre(+)}$Eng$^{\Delta HSC}$ mice are the result of reduced membrane bound Endoglin expression in HSCs. Furthermore, these results suggest that HSCs contribute to systemic sol-End levels in liver injury.

3.5 | HSC-specific endoglin deletion in liver injury leads to aggravation of liver fibrosis

Next, the contribution of *endoglin* expression in HSCs during liver fibrogenesis in BDL- and CCl_4-induced injury was analysed. CCl_4 injections for 8 weeks i.p. or BDL for 3 weeks were performed in GFAP$^{Cre(-)}$Eng$^{f/f}$ and GFAP$^{Cre(+)}$Eng$^{\Delta HSC}$ mice respectively. Liver fibrosis was investigated by measuring Hydroxyproline concentration and Sirius Red stainings for collagen fibres. Additionally, α-smooth muscle actin (α-SMA) staining was performed. GFAP$^{Cre(+)}$Eng$^{\Delta HSC}$ mice showed significantly higher Hydroxyproline levels (39.9%) and more Collagen deposition (58.8%) compared to Eng$^{f/f}$ mice in CCl_4-treated animals. Staining for α-SMA confirmed these results (Figure 4).

Moreover, GFAP$^{Cre(+)}$Eng$^{\Delta HSC}$ mice showed more pronounced fibrosis compared to control animals GFAP$^{Cre(-)}$Eng$^{f/f}$ after BDL. Hydroxyproline levels in livers of GFAP$^{Cre(+)}$Eng$^{\Delta HSC}$ mice were higher (20.4%) and more Collagen deposition (98.6%) as well as α-SMA expression was detected by Sirius Red staining and immunohistochemistry respectively (Figure 5). mRNA expression of fibrotic markers such as Collagen α1(I) and α-SMA showed higher expression levels in GFAP$^{Cre(+)}$Eng$^{\Delta HSC}$ livers in both injury models. Even though mRNA expression results slightly missed significance levels (P = .057-.073) in synopsis with above mentioned histological long-term findings, these results confirm increased fibrogenesis after *endoglin* deletion in HSCs.

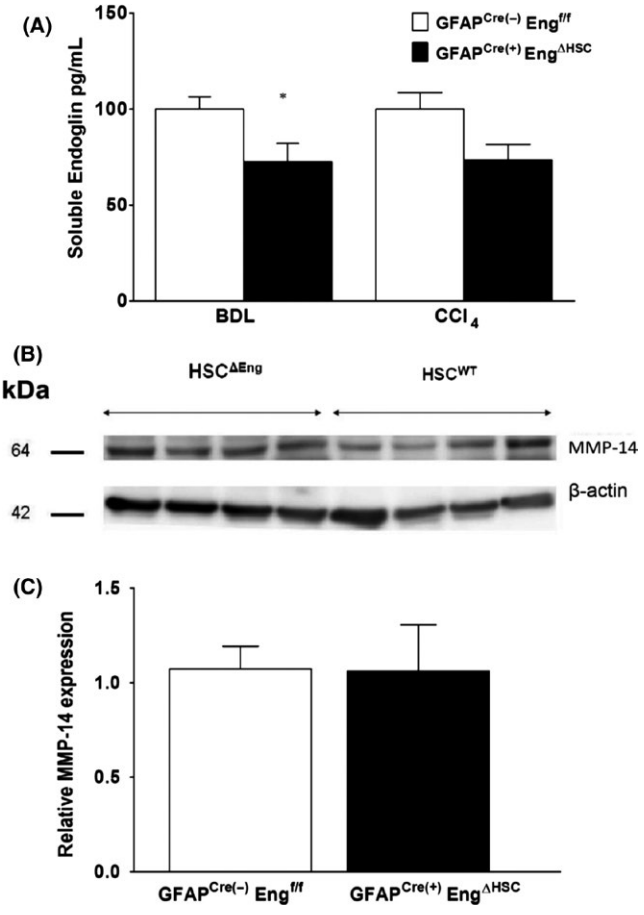


FIGURE 3 Decreased soluble endoglin expression in Eng$^{\Delta HSC}$ mice. (A), ELISA of sol-Eng in serum from CCl_4- and BDL-treated GFAP$^{Cre(-)}$Eng$^{f/f}$ and GFAP$^{Cre(+)}$Eng$^{\Delta HSC}$ mice (*P < .05). (B), Western blot of MMP-14 from whole GFAP$^{Cre(-)}$Eng$^{f/f}$ and GFAP$^{Cre(+)}$Eng$^{\Delta HSC}$ mouse liver. (C), Quantification of MMP-14 expression levels compared to β-actin expression. Data are expressed as means ± SEM

3.6 | Endoglin-deficient primary HSCs express more fibrogenic proteins

After in vivo experiments showed a protective role of Endoglin in HSC during fibrogenesis, TGF-β1 signalling in isolated primary HSC was analysed in vitro. Primary HSC were isolated from Eng$^{f/f}$ mouse livers and infected with adenoviral Cre or a control virus expressing luciferase. Cre-induced deletion of floxed Eng resulted in a strong reduction of Eng mRNA and protein expression (Figure 6A,B, Table S1), which is more potent than the partial deletion caused by endogenous Cre-expression (cf. Figure 2A,B). Although there is an increase in matrix deposition in injured livers as a consequence of Endoglin reduction in HSC, there is no significant change in expression and secretion seen for Fibronectin and expression of α-SMA. This applies to Cre-infected primary HSC (Figure 6B, Table S1) and the siRNA-treated HSC Col-GFP cell line (Figure 6C, Table S1). However, TGF-β1-mediated expression and the secretion of the profibrogenic matricellular protein connective tissue growth factor (CTGF) is reduced in all settings, for example, endogenous (not shown) or viral mediated Cre-expression in primary cells and siRNA treatment of the cell line HSC Col-GFP (Figure 6B,C). In addition, *knock down* of endoglin leads to a reduced activation of Smad1/5/8 and expression of its target gene Id1. The dependency of the aforementioned expressions/activations on ALK5 activity is underscored by the impact of SB431542 (ALK5 specific inhibitor) on these responses.

4 | DISCUSSION

There are many reviews discussing TGF-β signalling and its impact on fibrosis development.[29,30] Especially TGF-β1 is known to play a pivotal role in fibrosis development. Already Kanzler and colleagues generated transgenic mice overexpressing a fusion gene consisting of the cDNA coding for an activated form of TGF-β1 controlled by the regulatory

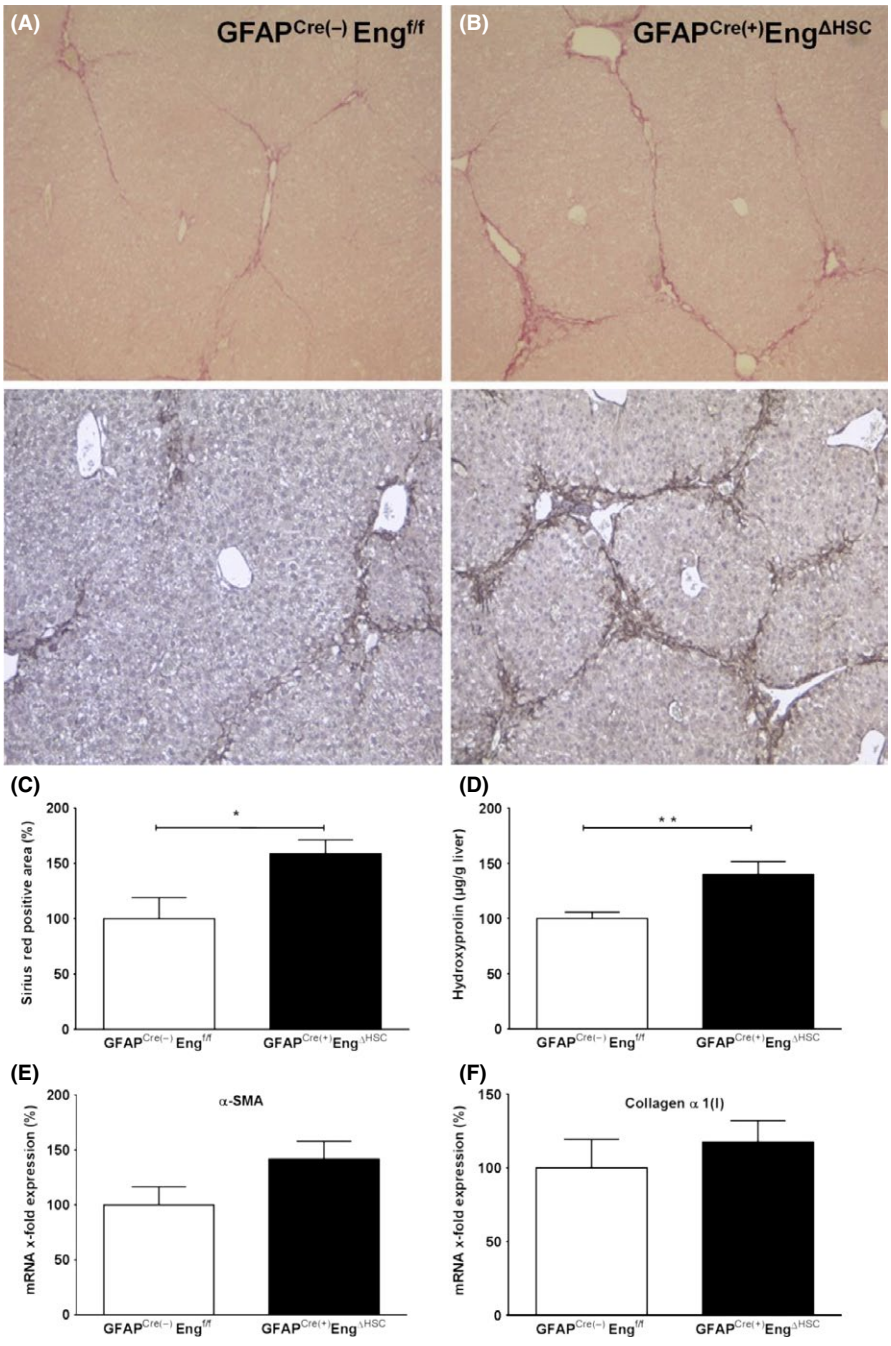


FIGURE 4 Hepatic stellate cell (HSC) specific *endoglin* deletion in toxic liver injury leads to aggravation of liver fibrosis. (A,B), Sirius Red staining of Collagen fibres (*upper*) and α-SMA staining (*lower*) in paraffin embedded liver sections (×100 magnification) show increased Collagen and α-SMA deposition in CCl_4-treated GFAP$^{Cre(+)}$Eng$^{\Delta HSC}$ mice. (C), Increased fibrosis in GFAP$^{Cre(+)}$Eng$^{\Delta HSC}$ mice was evident by a significantly higher Sirius Red-positive area (*P < .05). (D), Hydroxyproline levels in GFAP$^{Cre(-)}$Eng$^{f/f}$ and GFAP$^{Cre(+)}$Eng$^{\Delta HSC}$ mice (**P < .01). E-F, GFAP$^{Cre(+)}$Eng$^{\Delta HSC}$ mice show higher expression of fibrosis-related genes like *Col1α1* and α-*SMA* compared to GFAP$^{Cre(-)}$Eng$^{f/f}$ mice. Data are expressed as means ± SEM of 8 mice per group

elements of the inducible human CRP (C-reactive protein) gene promoter.[13] After induction with lipopolysaccharide, mice showed highly elevated plasma levels of TGF-β1, directly resulting in increased HSC activation and liver fibrosis. In other organs such as kidney, Sato et al[31] showed less tubulo-interstitial fibrosis induced by unilateral ureteral obstruction after disruption of TGF-β1/Smad3 signalling. Modulation of TGF-β signalling therefore is a valid option to ameliorate liver fibrosis. Although previously thought to be mainly expressed in endothelial tissue, Quintanilla et al[32] suggested the effect of Endoglin on TGF-β also in other organs. Even more important is the finding of up to 30% of liver involvement in patients suffering from HHT1.[33] A study by Ianora et al[34] shows vascular abnormalities in livers of 74% of patients, however only 8% were symptomatic.

Two different isoforms of Endoglin (L-Endoglin, S-Endoglin) and the soluble shedded sol-Endoglin must be taken into account when analysing Endoglin function. In human liver disease, several groups have shown elevated serum and tissue Endoglin levels.[15,35] In the present study, we found an elevation of the L-Endoglin isoform mRNA in human liver biopsy samples of ALF and chronic HCV infection. In contrast, transcripts of S-Endoglin were increased in ALF but not in HCV-infected patients. These findings show a differential regulation of both isoforms. This is pivotal for evaluating Endoglin function since both isoforms show opposing effects in vitro and in vivo and underscore the critical role of the intracellular domain which differs in both splice variants.[11,36] RT-PCR analysis shows that both splice variants are co-expressed in all tissues tested and that the L-variant is the predominantly expressed

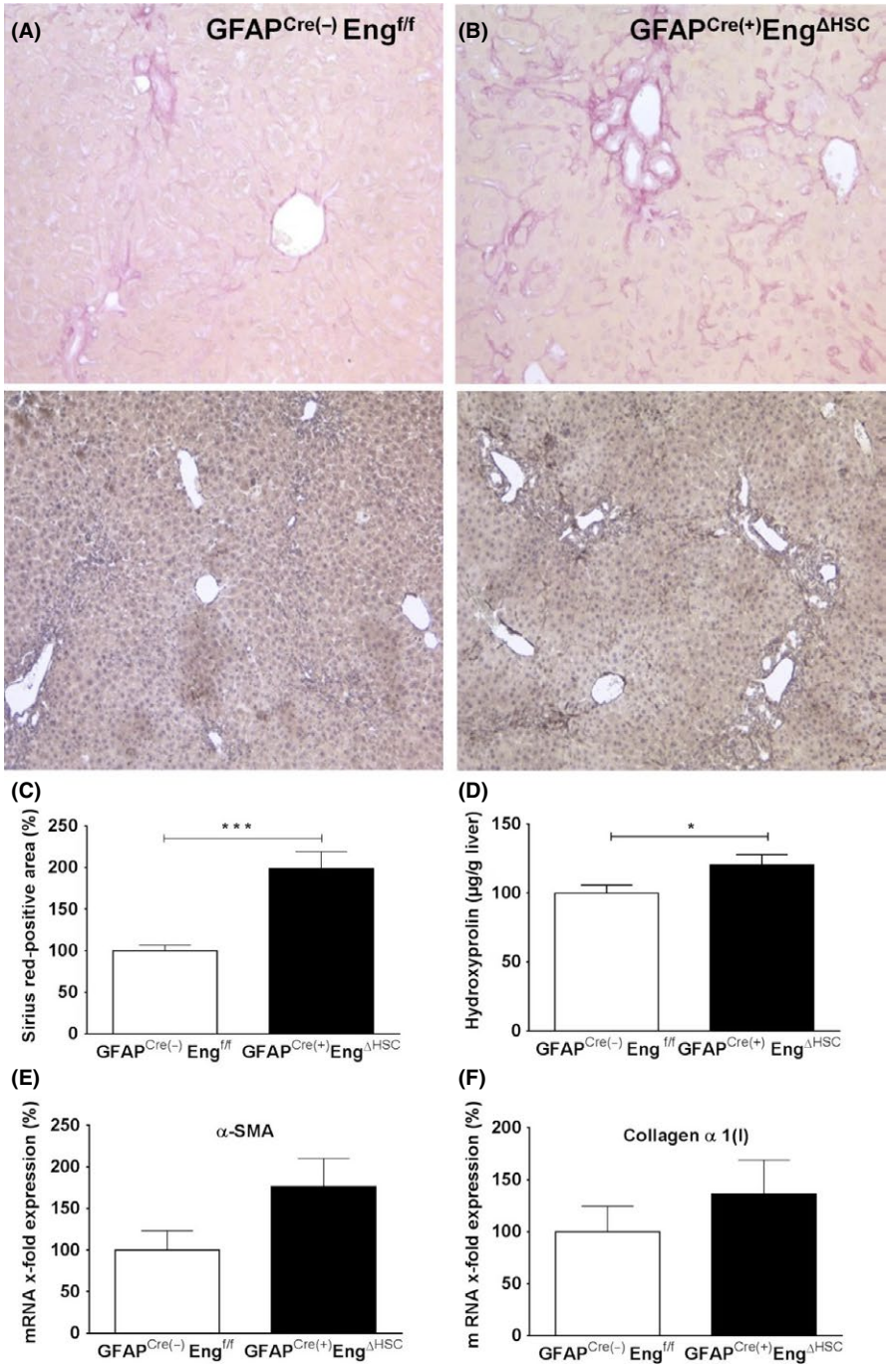


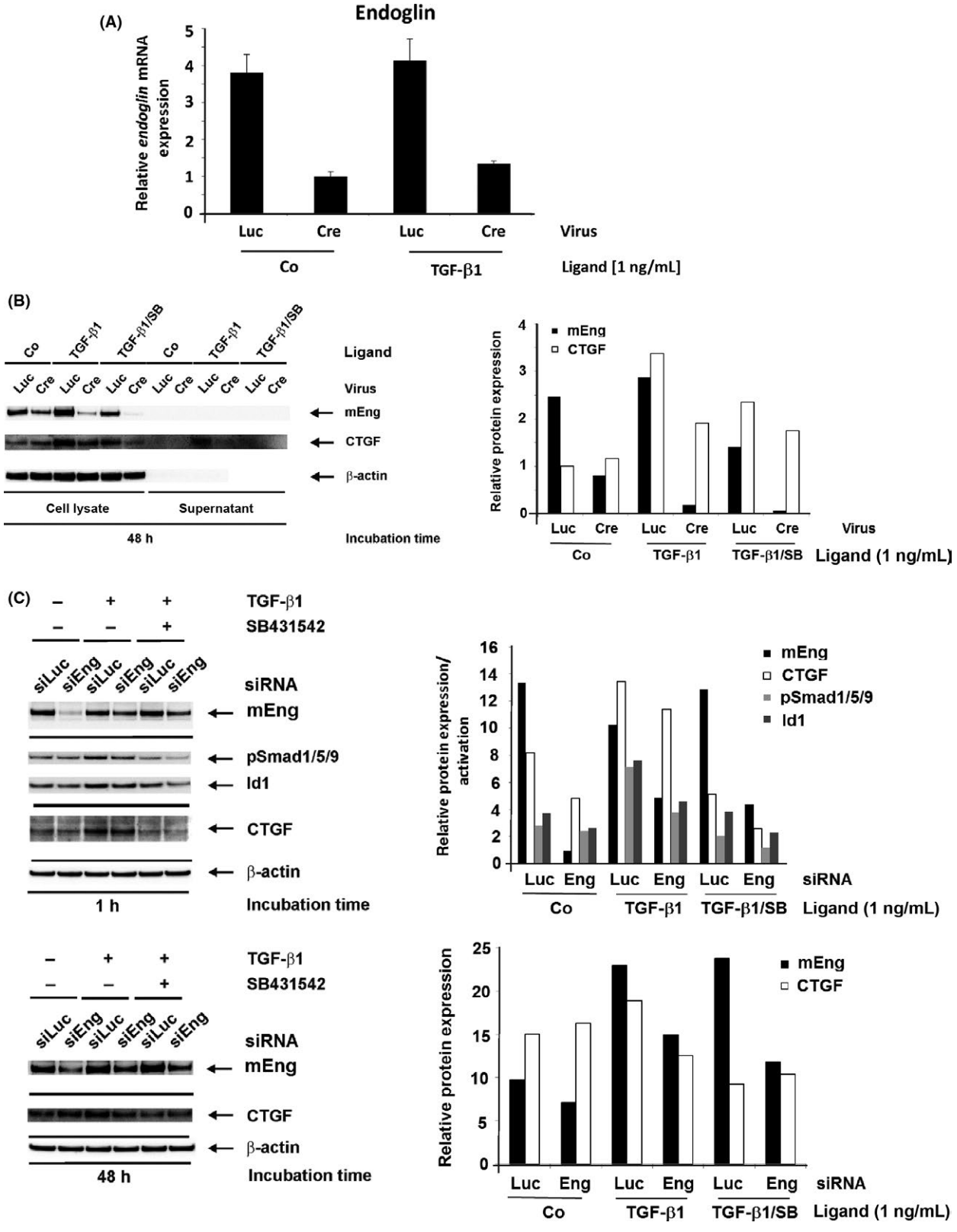
FIGURE 5 Hepatic stellate cell (HSC)-specific *endoglin* deletion in cholestatic liver injury leads to aggravation of liver fibrosis. (A,B), Sirius Red staining of Collagen fibres (*upper*) and α-SMA staining (*lower*) in paraffin embedded liver sections (×100 magnification) show increased Collagen and α-SMA deposition in GFAP$^{Cre(+)}$Eng$^{\Delta HSC}$ mice. Liver injury was induced by ligation of the common bile duct ligation (BDL for 3 wk). (C), Increased fibrosis in GFAP$^{Cre(+)}$Eng$^{\Delta HSC}$ mice was evident by significantly higher Sirius Red-positive area (***P < .001). (D), Hydroxyproline levels in GFAP$^{Cre(-)}$Eng$^{f/f}$ and GFAP$^{Cre(+)}$Eng$^{\Delta HSC}$ mice (*P < .05). (E,F), GFAP$^{Cre(+)}$Eng$^{\Delta HSC}$ mice show higher expression of fibrosis-related genes like *Col1α1* and α-*SMA* compared to GFAP$^{Cre(-)}$Eng$^{f/f}$ mice. Data are expressed as means ± SEM of 8 mice per group

form. This finding is in line with a previous report confirming the high organ expression of the L-form compared to the varying but low expression of the S-form.[37] This ratio has been of vital importance for cells since a change in endothelial cells has caused cellular senescence.[12]

To further analyse the impact of Endoglin on TGF-β signalling in fibrotic liver disease, we investigated Endoglin isoform expression in isolated liver cells. As expected HSC, liver resident macrophages (Kupffer cells) and liver sinusoidal endothelial cells express both Endoglin isoforms. Similar to the organ analysis, the ratio is highly in favour of the L-variant.

Endoglin splice variant expression was analysed in detail in the process of liver fibrosis. L- and S-Endoglin transcripts were measured in normal and fibrotic mouse livers and both isoforms were upregulated in response to liver injury. Nevertheless, upregulation of the S-form was more pronounced compared to the L-form.

During fibrogenesis, TGF-β signalling has been reported to act through the ALK5 receptor, activating the Smad2/3 pathway, leading to activation of HSC, as evidenced by the upregulation of the activation marker α-SMA, and induction of genes coding for ECM components like Fibronectin.[38] Other studies showed that most profibrotic activities of TGF-β in the kidney are mediated by Smad3[39] whereas the Smad1/5/8 pathway showed antifibrotic effects.[40] In general, possibly influenced by the cell type analysed and the concentration of TGF-β



itself, the Smad1/5/8 axis can act pro- as well as antifibrotic. However, in the liver the signalling pathway, TGF-β1/ALK-1/Smad1/Id1 favours profibrogenic responses.[14,41,42] Obviously, the ALK1/ALK5 ratio plays a role in Smad signal transduction and regulation in ECM protein expression as seen in human chondrocytes[43] and other in vivo studies.[44]

Conflicting data have been reported on the role of Endoglin regulating TGF-β-mediated ALK1-Smad1/5/8 and ALK5-Smad2/3 signalling in several cell types when analysed either in homozygous Endoglin *knockout* mice or isolated cells treated with Endoglin specific siRNA or L-Endoglin expression vector.[45]

FIGURE 6 Endoglin-deficient primary hepatic stellate cell (HSC) show increased fibrotic markers. (A), Quantitative analysis of Endoglin transcripts in primary HSC containing floxed Endoglin alleles infected with a Cre or luciferase (Luc) expressing virus and stimulated with transforming growth factor-β1 (TGF-β1) (1 ng/mL) for 48 h using the primers x/w. Note that the Endoglin mRNA is strongly reduced in Cre-expressing cells compared to the Luc-expressing controls (n = 3). (B), *left* Western Blot analysis of proteins from isolated HSC with floxed Endoglin alleles, infected or not with adenovirus expressing Cre (see (A)). Cells were treated with a luciferase control adenovirus (Luc) or a Cre-expressing vector. Thereafter, cells were treated or not with TGF-β1 (1 ng/mL) in the presence or absence of the ALK5 inhibitor SB421543 (SB, 5 μmol/L) for 48 h and analysed by Western blot. Respective proteins were detected by the indicated specific antibodies. To demonstrate equal protein loading, the membrane was re-probed with a β-actin-specific antibody (n = 2). (B), *right* Representative densitometric analysis of the shown Western blot. The relative expression of the analysed proteins was normalized to β-actin. (C), *left panels* Western blot analysis of proteins from HSC Col-GFP treated with control siRNA (Luc) or endoglin-specific siRNA (Eng). The cells were treated with TGF-β1 (1 ng/mL) in the presence or absence of the ALK5 inhibitor SB421543 (SB, 5 μmol/L) for 1 h (*upper left*) or 48 h (*lower left*) and corresponding protein extracts were analysed by Western blot. Cells that were not stimulated with TGF-β1 served as controls. Respective proteins were detected by the indicated specific antibodies. To demonstrate equal protein loading, the membrane was re-probed with a β-actin-specific antibody (n = 3). (C), *right panels* Representative densitometric analysis of the shown Western blots. The relative expression of the analysed proteins was normalized to β-actin

In endothelial cells, myoblasts and chondrocytes, overexpression of L-Endoglin favours signalling through the TGF-β-ALK1-Smad1/5/8 pathway.[45,46] However, depending on cell type and cellular environment Endoglin can modulate TGF-β signalling differently, resulting in multiple effects on proliferation, migration and ECM production. To further analyse these effects in liver fibrosis in vivo, we generated a HSC-specific Eng *knockout* mouse. Subjecting these mice to liver injury, we show that Endoglin deficiency aggravates liver fibrosis in response to CCl_4-treatment or BDL surgery. As previously shown by our group,[19] in an HSC cell line the presence of Endoglin shifts TGF-β signalling from ALK5-Smad2/3 towards the ALK1-Smad1/5/8 pathway. Overexpression of L-Endoglin in this cell line decreased Collagen expression upon TGF-β stimulation. The present study now verifies these results in vivo, suggesting a protective effect of Endoglin in the development of liver fibrosis. Upon deletion of Endoglin in HSCs, liver injury leads to increased Collagen expression as demonstrated by Sirius Red staining and elevated Hydroxyproline levels in injured liver most likely because of promoting TGF-β signalling through Smad2/3. Contrasting to the antifibrotic effects, the *knockout* of Endoglin in primary cells or siRNA-mediated *knock down* in a HSC cell line causes a decrease in the profibrogenic protein CTGF. In addition, the activation of Smad1/5/8 and its target gene Id1 is reduced in response to TGF-β1 upon lowering the Endoglin concentration. These results are compatible with the results that we previously published for HSC Col-GFP and which show exactly the opposite when Endoglin is transiently overexpressed.[19] Therefore, although depletion of Endoglin in HSC leads to a decrease in matrix accumulation in the affected liver in vivo, Endoglin promotes profibrogenic aspects in vitro. If CTGF expression is directly linked to Smad1/5/8 activation is speculative so far. Since previous data implicated Endoglin in ERK activation,[19] CTGF in HSC might be regulated via a MAP kinase pathway.[46] In addition to the direct effects on HSC, depletion of Endoglin leads to a reduced abundance of soluble Endoglin, and therefore to a modulation of the paracrine effects on hepatocytes for example which might also impact the outcome of fibrosis. In a follow-up study of our group performed with an ubiquitous *endoglin knockout* using an inducible CAG-Cre mouse *endoglin* deficiency showed no significant effects on inflammation, or the amount of liver injury in CCl_4-exposed mice, suggesting that ENG-mediated TGF-β signalling is mainly involved in fibrosis and scarring (data not shown).

In summary, our present study shows a differential regulation of Endoglin isoforms in liver samples of patients with chronic and acute liver injury. In response to injury, liver fibrosis is aggravated by Endoglin deficiency in HSC significantly. Therefore, Endoglin protects against fibrotic injury by modulating TGF-β signalling.

CONFLICTS OF INTEREST

The authors do not have any disclosures to report.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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