Pathogenesis and reversal of liver fibrosis: Effects of genes and environment

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igf1 enhances bile-duct proliferation and fibrosis in abcb4−/− mice
ABSTRACT

Background and Aims: Adamant progression of chronic cholangiopathies towards cirrhosis and limited therapeutic options leave a liver transplantation the only effective treatment. Insulin-like growth factor 1 (IGF1) effectively blocks fibrosis in acute models of liver damage in mice, and a phase I clinical trial suggested an improved liver function. IGF1 targets the biliary epithelium, but its potential benefit in chronic cholangiopathies has not been studied.

Methods: To investigate the possible therapeutic effect of increased IGF1 expression, we crossed Abcb4−/− mice (a model for chronic cholangiopathy), with transgenic animals that overexpress IGF1. The effect on disease progression was studied in the resulting IGF1-overexpressing Abcb4−/− mice, and compared to that of Abcb4−/− littermates. The specificity of this effect was further studied in an acute model of fibrosis.

Results: The overexpression of IGF1 in transgenic Abcb4−/− mice resulted in stimulation of fibrogenic processes - as shown by increased expression of Tgfβ, and collagens 1, 3 and 4, and confirmed by Sirius red staining and hydroxyproline measurements. Excessive extracellular matrix deposition was favoured by raise in Timp1 and Timp2, while a reduction of tPA expression indicated lower tissue remodelling. These effects were accompanied by an increase in expression of inflammation markers like Tnfα, and higher presence of infiltrating macrophages. Finally, increased number of Ck19-expressing cells indicated proliferation of biliary epithelium.

Conclusion: In contrast to liver fibrosis associated with hepatocellular damage, IGF1 overexpression does not inhibit liver fibrogenesis in chronic cholangiopathy.

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INTRODUCTION

Primary sclerosing cholangitis and primary biliary cirrhosis are chronic diseases of the bile duct characterized by progressive inflammation that leads to the development of biliary fibrosis. There is no effective therapy to halt the disease progression and most patients progress to cirrhosis and liver failure (1, 2). The lack of sufficient donor organs and surgical contraindications render development of therapeutic interventions that stop progression of liver fibrosis urgently needed.

Insulin-like growth factor 1 (IGF1) regulates the proliferation and differentiation of many cell types (3). The liver is responsible for the abundant presence of IGF1 in the circulation (4) where it is present attached to binding proteins (IGFBPs) that prolong its half-life and regulate its biological activity (3, 5). Once released from IGFBPs, IGF1 can bind and activate the receptor (IGF1R), and by activation of mitogen activated protein (MAP) kinase and PI3 kinase induce genes involved in cell survival, growth, and differentiation (6). Liver fibrosis reduces the IGF1 expression in the liver (7, 8). Administration of recombinant IGF1 or enhancing IGF1 expression using a viral vector significantly reduced liver fibrosis in rats with CCl4-induced liver cirrhosis (9, 10). In the follow-up phase I-II clinical trials, IGF1 administration increased the serum albumin level, suggesting an improved liver function in patients suffering from this devastating disease (11).

The potential use of IGF1 in chronic cholangiopathies has not been studied. Since the biliary epithelium expresses IGF1R (12), and IGF1 protects cholangiocytes against cholestatic injury in vitro (13), overexpression of IGF1 could be beneficial in these diseases. Therefore the aim of this study was to investigate if a prolonged increase of hepatic IGF1 expression in a model for chronic cholangiopathy, the Abcb4−/− mouse, was beneficial.

The Abcb4−/− mouse is a model for primary familial cholestasis type 3 (14). ABCB4 is a flippase essential for the excretion of phospholipids into bile, needed to neutralize the harmful effects of bile acids. Bile lacking phospholipids is toxic and causes hepatocyte and cholangiocyte injury (15). Since murine bile acids are less toxic, the damage in the Abcb4−/− is smaller compared to that in PFIC type 3 patients, making this mouse also a model for other chronic cholangiopathies, more specifically, primary sclerosing cholangitis (PSC) (16). To study the long term effect of IGF1 on chronic cholangiopathy we crossbred the Abcb4−/− with the transgenic SMP8-IGF1 mouse. This transgenic mouse expresses the rat IGF1 behind an α-SMA promoter (17). The increased α-SMA expression in Abcb4−/− livers (15) should make this cross-bred model well suited to study long-term effects of IGF1 on PSC.

MATERIALS AND METHODS

Animals.

This study was approved by the Ethical Committee for Animal Research of the University of Navarra with the number: 008/08.

Abcb4−/− IGF1 model

Homozygous Abcb4−/− mice (FVB background; (18)) were cross-bred with transgenic SMP8-IGF1 mice, with a germline integration of a transgene composed of the murine α-SMA promoter linked to a
full length rat Igf1 cDNA (17). The resulting heterozygotes for Abcb4 were back-crossed with Abcb4/- mice to obtain Abcb4/- SMP8-Igf1+/ mice and SMP8-Igf1+/ transgenic mice (TG) and their nontransgenic (NT) littermates as a control. After weaning, the mice WT (FVB; n=4), Abcb4/- (n=7) and Abcb4/- SMP8-Igf1+/ (n=7) were fed a 0.03% cholate diet to augment fibrosis, and were sacrificed after 3, 6 and 12 weeks (WT control for 3 weeks).

**Bile duct ligation (BDL) model**

SMP8-IGF1 (n=5) and WT (FVB; n=5) mice were bile duct ligated. After a 1 cm abdominal midline incision, the common bile duct was isolated, double ligated close to the liver hilus (immediately below the bifurcation) with a silk suture, and transected between ligatures. In sham operated mice (SMP8-IGF1 and WT; n=5), the same operation was performed, but neither ligatures nor bile duct section were carried out. All the mice used in the protocol were 12 week old at the beginning of the experiment. The mice were sacrificed a week after BDL.

**Tissue collection and biochemical analyses**

Upon sacrificing, blood was collected, livers were snap-frozen in liquid nitrogen and stored at -80°C, or fixed in 4% formaldehyde in PBS. Total liver collagen was determined by measuring hydroxyproline content as described (19) Serum transaminases (ALT and AST), alkaline phosphatase and bilirubin were determined in retro-orbital collected blood, using standard clinical chemistry methods in a Cobas C311 autoanalyzer (Roche Diagnostics, Mannheim, Germany).

**Reverse transcription, quantitative PCR and data analyses**

The qPCR analysis was performed and the data analyzed as previously described (20). Shortly, total liver RNA was extracted from snap-frozen livers using TRIzol reagent (Invitrogen, Breda, The Netherlands) following the manufacturer’s instructions. 4M LiCl was used to specifically precipitate RNA. All RNA isolates had A260/A280 ratio around 2, as assessed by NanoDrop ND-1000 spectrophotometer. The RNA integrity was confirmed by denaturing agarose gel electrophoresis. The absence of DNA contamination was established by including RT- controls in cDNA synthesis reactions. Gene specific primers (for Igf1, Hgf, aSma, Tgfβ, Col1, Col3, Col4, Timp1, Timp2, tPa, Tnfa, Ki67 and Ck19) were designed using Primer3Plus program and their sequences are available upon request. qPCR was performed using a Light Cyler 480 (Roche) as described (21). The expression level for each of the studied genes in liver was calculated using the LinRegPCR software (22, 23), thereby avoiding the assumption of equal amplification efficiency between the reactions. Gene expression was normalized by dividing the expression levels of tested genes by those of a reference gene (36B4), and calculating the average of normalized values per condition. 36B4 was used only after having validated it against three reference genes in our previous studies (24). In BDL model, qPCR was performed as previously described (25). To assess the significance of the data, ANOVA analysis and Student’s t test were employed. The error bars in the Figures represent the standard deviation. Significance threshold was set to P<0.05 (denoted by the asterisks in the Figures and Tables).

**Histology and immunohistochemistry**

Formaldehyde-fixed liver sections were stained with Sirius red (morphometrical connective tissue assessment), or immunohistochemically, as described (26). Liver collagen content was quantified by
Sirius red staining, using an Axioplan 2 Imaging microscope (Zeiss, Jena, Germany); random acquisition of images was performed with Metamorph software (Molecular Devices, Sunnyvale, CA, USA), and scoring of areas was carried out with Matlab software and the imaging processing libraries of DIPlib (MathWorks, Natick, MA, USA). Ck19 (Eurogentec, Koln, Germany) was detected using GAR-AP (DAKO, Glostrup, Denmark) as a secondary antibody. F4/80 (AbD Serotec, Dusseldorf, Germany) was visualized using rabbit anti Rat-Ig (DAKO, Glostrup, Denmark) as a secondary antibody.

Double staining for Ck19 and Ki67 was performed on frozen sections, fixed for 5 minutes in ice cold acetone and air dried for 30 minutes. Slides were rinsed in PBS, blocked for 30 minutes in TengT and incubated o/n at 4°C with the primary anti-Ki67 Ab (Abcam, Cambridge, UK; 1:500). Washing was followed by 30 minutes incubation at RT with the secondary Alexa Fluor488 goat anti rabbit IgG (Invitrogen, Paisley, UK; 1:1000). Sections were then rinsed and incubated with the primary anti-Ck19 Ab (Eurogentec, Koln, Germany; 1:1000) for 1 hour at RT, washed again, and incubated with Alexa Fluor594 goat anti rabbit IgG (Invitrogen, Paisley, UK; 1:1000) for 30 minutes at RT. After washing, the slides were covered with vectashield + DAPI and visualized by confocal microscopy. Quantification of Ki67-positive cholangiocytes after 12 weeks of cholate diet was performed in 5 animals per phenotype, 5 images per mouse. The results are expressed as a percentage of Ki67-positive in the total number of cholangiocytes.

In BDL model, bromodeoxyuridine (BrdU) method was used for counting proliferating cholangiocytes as previously described (27). Shortly, formalin-fixed and paraffin-embedded liver sections were stained with mouse monoclonal anti-BrdU antibody (1:1000 GE Healthcare, Barcelona, Spain), at 4°C, overnight. Secondary peroxidase-labeled goat anti-mouse antibody (Dako Envision System, Carpinteria, CA) was incubated at room temperature for 30 min. Two persons unaware of the study groups scored BrdU positive cholangiocytes in SMP8-IGF1 BDL (n=5) and WT BDL (n=5), analyzing 6 panels per animal.

RESULTS

Overexpression of IGF1 in Abcb4+/− mice

To study the long-term effect of IGF1 on the pathology of chronic cholangiopathy, Abcb4+/− mice were crossbred with a transgenic mouse expressing rat Igf1 behind an a-Sma promoter (17). This restricts rat Igf1 hepatic expression to the α-SMA expressing, activated hepatic stellate cells (HSC) (27). To aggravate the pathology, after weaning the animals were given a cholate-supplemented diet (0.03%) (15, 18). The expression of rat Igf1 was confirmed by qPCR in livers of the transgenic (TG) Abcb4+/−IGF1 mice (Fig.1A). Since rat Igf1 expression in TG mice is controlled by the a-Sma promoter, its stable expression reflects the α-Sma expression, that remained comparable at all three time points (Fig.1B). IGF1 induces HGF expression in HSC (28). The significant increase of Hgf expression at 6 and 12 weeks in TG animals compared to non-transgenic (NT) Abcb4+/− littermates confirmed that IGF1 signaling was indeed enhanced (Fig.1C).
Fig. 1: Functional expression of IGF-1 in livers of Abcb4⁻/⁻ mice. After weaning, WT, Abcb4⁺/⁺ and Abcb4⁻/⁻ IGF1 mice - WT (n=4; for 3 weeks), Abcb4⁺/⁻ (n=7) and Abcb4⁻/⁻ IGF1 (n=7) - were fed a 0.03% cholate diet. (A-C) mRNA levels of transgenic Igf1, α-Sma and Hgf in livers of WT, Abcb4⁺/⁺ and Abcb4⁻/⁻ IGF1 mice fed a cholate supplemented diet for 3, 6 and 12 weeks. (D) Average fractional liver weight of Abcb4⁻/⁻ and Abcb4⁻/⁻ IGF1 mice, after 3, 6 and 12 weeks of cholate diet. Asterisks denote significance (P<0.05).

**Overexpression of IGF1 induced liver fibrosis in Abcb4⁻/⁻ mice**

Liver function tests were performed to investigate if IGF1 could protect this mouse model against the toxicity of bile salts, thereby reducing chronic bile duct proliferation. Serum levels of ALT, AST, ALP and bilirubin significantly increased in Abcb4⁻/⁻IGF1 and Abcb4⁻/⁻ mice compared to WT, indicating liver damage in both models (Table 1). The initially high ALP serum levels, a result of osteoblast activity in growing bones (29), decreased to normal after weaning (66-260 U/L). After 12 weeks of cholate diet, however, ALP and bilirubin concentration significantly increased in the IGF1 transgenics compared to their Abcb4⁻/⁻ littermates, suggesting cholestasis and possibly an enhanced progression of liver fibrosis (Table 1).

Table 1: The influence of IGF1 overexpression on liver function shown by clinical parameters in plasma.

<table>
<thead>
<tr>
<th></th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
<th>ALP (U/L)</th>
<th>tBil (mg/dl)</th>
<th>TP (g/dl)</th>
<th>Chol (mg/dl)</th>
<th>TG (mg/dl)</th>
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<tr>
<td><strong>WT</strong></td>
<td></td>
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<tr>
<td>3 weeks</td>
<td>76.1±16.9</td>
<td>126.9±29.5</td>
<td>188.2±36.6</td>
<td>0.08±0.01</td>
<td>5.2±0.2</td>
<td>183.5±9.9</td>
<td>83.50±17.4</td>
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<tr>
<td><strong>Abcb4⁻/⁻</strong></td>
<td></td>
<td></td>
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<tr>
<td>3 weeks</td>
<td>355.4±125.8</td>
<td>502.5±186.9</td>
<td>1992.6±1257.6</td>
<td>0.2±0.2</td>
<td>5.2±0.3</td>
<td>107.2±25.9</td>
<td>137.3±24.7</td>
</tr>
<tr>
<td>6 weeks</td>
<td>185.3±95.4</td>
<td>238.7±74.8</td>
<td>448.8±319.5</td>
<td>0.2±0.1</td>
<td>5.3±0.8</td>
<td>103.6±28.7</td>
<td>144.9±12.9</td>
</tr>
<tr>
<td>12 weeks</td>
<td>356.6±220.3</td>
<td>591.1±460.4</td>
<td>285±136.4</td>
<td>0.3±0.3</td>
<td>5.9±0.5</td>
<td>80.1±16.6</td>
<td>89.14±34.2</td>
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<tr>
<td><strong>Abcb4⁻/⁻IGF1</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>3 weeks</td>
<td>320.7±86.2</td>
<td>502.2±231.2</td>
<td>1020.5±657.4</td>
<td>0.2±0.1</td>
<td>5.9±0.5</td>
<td>112.9±15.9</td>
<td>116.6±29.1</td>
</tr>
<tr>
<td>6 weeks</td>
<td>238.5±127.7</td>
<td>314.6±152.9</td>
<td>533.4±323.2</td>
<td>0.2±0.1</td>
<td>5.4±1.4</td>
<td>101.5±29.8</td>
<td>132.1±56.7</td>
</tr>
<tr>
<td>12 weeks</td>
<td>388.8±191.2</td>
<td>600.9±322.7</td>
<td>1712.9±1095.5*</td>
<td>1.9±1.6*</td>
<td>5.0±1.2</td>
<td>103.3±24.6</td>
<td>128.3±42.9</td>
</tr>
</tbody>
</table>

The table shows mean values and standard deviation of serum parameters measured in plasma of WT (n=4), Abcb4⁻/⁻ (n=7) and Abcb4⁻/⁻IGF1 (n=7) mice. The differences between Abcb4⁻/⁻ and Abcb4⁻/⁻IGF1 mice were tested by Student's t-test, with asterisks denoting significant difference (P<0.05). The values for WT are shown as a reference. ALT (alanine transaminase), AST (aspartate transaminase), ALP (alkaline phosphatase), tBil (total bilirubin), TP (total protein), Chol (cholesterol), Tg (triglycerides).

After 3 weeks of cholate diet, the fractional liver weight in Abcb4⁻/⁻ mice was significantly higher than in Abcb4⁻/⁻ IGF1 group. At 6 weeks this difference had disappeared, while at 12 weeks there was even a tendency toward an increased liver/body weight ratio in Abcb4⁻/⁻ IGF1 mice (Fig.1D). Sirius red staining and hydroxyproline measurement revealed a significant increase in the
amount of connective tissue in the Abcb4+/− IGF1 group, indicating increased liver fibrosis in transgenic mice after 12 weeks of cholate diet (Fig.2A-C). The increased expression of the pro-fibrotic transforming growth factor beta 1 (Tgfβ1), which stimulates trans-differentiation of HSC into myofibroblasts and enhances TIMP and collagen production (30), may also support the increased fibrosis in IGF1 expression Abcb4+/− mice at twelve weeks (Fig.3A). Also the mRNA levels of Col1, 3 and 4 at 12 weeks were significantly increased in these mice (Fig.3B-D), providing additional support that IGF1 enhanced the progression of liver fibrosis in the Abcb4+/− mouse model.

In addition to enhanced synthesis, the increased ECM deposition in mice overexpressing IGF1 may also in part be due to reduced matrix remodeling. Inhibition of matrix metalloproteinases (MMP) by their tissue inhibitors (TIMPs) plays an important role in this process (31, 32). The significantly increased expression of Timp1 and 2 after 12 weeks suggested that tissue remodeling was reduced in the Abcb4+/− IGF1 mice (Fig.3E, F). Additional support for this was provided by the decreased expression of tPa, an activator of plasminogen that plays an essential role in the activation of MMP needed for tissue remodeling (33).
Fig. 3: IGF1 overexpression hinders matrix remodeling in Abcb4⁻/⁻ livers. (A-G) Relative mRNA level of Tgfβ1, Col1, 3, 4, tPA, Timp1, 2 and Tnfα in livers of WT, Abcb4⁻/⁻IGF1 and Abcb4⁻/⁻ mice after 3, 6 and 12 weeks of cholate diet. Asterisks denote significant difference (P<0.05).

**Overexpression of IGF1 enhances inflammation in Abcb4⁻/⁻ liver**

Inflammation due to regurgitation of bile acids plays an important role in the development of pathology in the Abcb4⁻/⁻ mice. The increased pathology in Abcb4⁻/⁻IGF1 animals was accompanied by an increased inflammation, as shown by the enhanced expression of Tnfa (Fig. 4A). Immunostaining for F4/80, a marker for infiltrating macrophages and liver-resident Kupffer cells, was higher at 12 weeks in Abcb4⁻/⁻IGF1 animals, which suggested an increased influx of macrophages compared with non-transgenic controls (Fig. 4B).

Fig. 4: IGF1 overexpression induces expression of inflammatory markers in Abcb4⁻/⁻ mice. Relative mRNA level of Tnfa in livers of WT, Abcb4⁻/⁻IGF1 and Abcb4⁻/⁻ mice after 3, 6 and 12 weeks of cholate diet. Asterisks denote significant difference (P<0.05). (B) The representative pictures of F4/80 immunostaining in non-transgenic (left panel) and Abcb4⁻/⁻IGF1 mice (right panel) after 12 weeks of cholate diet are shown. (C) Representative CK19 immunostaining of the same livers.
**Overexpression of IGF1 increases cholangiocyte proliferation in Abcb4−/− mice**

Since bile duct proliferation is one of the major characteristics of the pathology in Abcb4−/− mice, and cholangiocytes are target of IGF1 signaling (12), the increased pathology could be due to an enhanced cholangiocyte proliferation in response to the IGF1 overexpression. Therefore, the level of the cholangiocyte marker Ck19 was determined in these livers. A significant increase in mRNA expression level and an increased presence of Ck19-expressing cells detected by immunohistochemistry confirmed an enhanced presence of cholangiocytes in the transgenic animals (Fig.5C and Fig.4C).

Subsequently, the expression of the proliferation marker Ki67 was determined to investigate if this effect was caused by a higher proliferation. Although a higher Ki67 level was observed, the difference did not reach significance (Fig.5B). This lack of significance could be due to the fact that the proliferation was not limited to cholangiocytes, hence the proliferation of biliary epithelium was determined by fluorescent cell labelling and co-localisation for Ck19 and Ki67. This indicated an ongoing proliferation of Ck19 positive cells in the transgenic animals (Fig.5A). Quantification of Ki67-positive cholangiocytes with respect to the total number of cholangiocytes demonstrated a significant increase in bile duct proliferation in IGF1 overexpressing animals after 12 weeks of cholate diet (Fig.5D). This enhanced proliferation of biliary epithelium could have resulted in the formation of numerous large bile duct structures seen in the IGF1 overexpressing mice (Fig.2A).

Table 2: Plasma liver function parameters, hepatic mRNA expression levels, and percentage of BrdU-positive cholangiocyte in WT and SMP8-IGF1 mice in response to BDL

<table>
<thead>
<tr>
<th></th>
<th>WT ctrl</th>
<th>WT BDL</th>
<th>SMP8-IGF1 ctrl</th>
<th>SMP8-IGF1 BDL</th>
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<tr>
<td>AST</td>
<td>133.30 ± 59.90</td>
<td>730.70 ± 186*</td>
<td>113.30 ± 47.72</td>
<td>507.20 ± 158.70*</td>
</tr>
<tr>
<td>ALT</td>
<td>77 ± 48.08</td>
<td>777 ± 262*#</td>
<td>73.67 ± 38.84</td>
<td>386.20 ± 105.40*</td>
</tr>
<tr>
<td>ALP</td>
<td>83 ± 10.17</td>
<td>1543 ± 332.90*</td>
<td>112.30 ± 14.47</td>
<td>1333 ± 523.60*</td>
</tr>
<tr>
<td>bilirubin</td>
<td>0.08 ± 0.01</td>
<td>10.07 ± 0.65*</td>
<td>0.10 ± 0.02</td>
<td>11.23 ± 2.09*</td>
</tr>
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<table>
<thead>
<tr>
<th>mRNA expression</th>
<th>transgen Igf1</th>
<th>no expression</th>
<th>no expression</th>
<th>2.28 ± 0.63</th>
<th>5.03 ± 2.13*</th>
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<tbody>
<tr>
<td>αSma</td>
<td>1.56 ± 0.69</td>
<td>23.55 ± 7.69*#</td>
<td>2.53 ± 0.33</td>
<td>11.65 ± 5.44*</td>
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<tr>
<td>Ck19</td>
<td>1.22 ± 0.54</td>
<td>7.04 ± 3.09*</td>
<td>1.47 ± 0.83</td>
<td>10.63 ± 4.70*</td>
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<tr>
<td>Tnfa</td>
<td>6.83 ± 3.63</td>
<td>214.35 ± 88.56*</td>
<td>9.22 ± 7.08</td>
<td>115.68 ± 56.46*</td>
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<tr>
<td>Coll</td>
<td>0.73 ± 0.82</td>
<td>1.18 ± 0.79</td>
<td>0.31 ± 0.19</td>
<td>0.78 ± 0.17*</td>
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<tr>
<td>Tgfβ</td>
<td>1.34 ± 0.89</td>
<td>1.73 ± 1.07</td>
<td>1.35 ± 0.01</td>
<td>1.34 ± 0.61</td>
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<tr>
<td>Asbt</td>
<td>19.16 ± 13.64</td>
<td>25.01 ± 15.68</td>
<td>20.89 ± 4.72</td>
<td>49.07 ± 20.57*</td>
<td></td>
</tr>
<tr>
<td>BrdU+ cholangiocytes (%)</td>
<td>11.69 ± 4.30</td>
<td>12.70 ± 5.89</td>
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</table>

The table shows mean values and standard deviations of liver function parameters and hepatic mRNA expression levels (relative to housekeeping 36B4) in WT and SMP8-IGF1 mice and one week after BDL treatment and in sham-operated controls (n=5 per group). Percentage of BrdU-positive cholangiocytes was assessed in WT and SMP8-IGF1 mice one week after BDL. Asterisks denote significant difference between sham-operated animals and BDL counterparts; hashtags indicate significant difference between BDL groups (P<0.05).
Fig. 5: IGF1 overexpression in Abcb4⁻/⁻ mice enhances proliferation of cholangiocytes: (A) Representative pictures of double staining for Ck19, Ki67 in Abcb4⁻/⁻ (left panels) and Abcb4⁻/⁻IGF1 (right panels) after 12 weeks of cholate diet. (B-C) Relative gene expression of Ki67 and Ck19 in control, Abcb4⁻/⁻ and Abcb4⁻/⁻IGF1 mice. (D) Percent of Ki67-positive cholangiocytes with respect to the total number of cholangiocytes, as detected by immunohistochemistry.

The number of a-Sma expressing activated HSC increased, and was accompanied by an increased scar formation reflected by higher Coll1 levels, by increased cholangiocyte proliferation demonstrated by higher Ck19 levels, and more inflammation based on Tnfα expression.

**Overexpression of IGF1 in BDL model**

Though Abcb4⁻/⁻ is a relevant model for chronic cholangiopathy, it does not reproduce all features of acute models of cholestasis, e.g. CCl₄- and BDL-induced. To compare the effect of IGF1 in Abcb4⁻/⁻ mice with that in an acute model, we used BDL to induced acute cholestasis in SMP8-IGF1 mice. Sirius red staining of livers in BDL-treated SMP8-IGF1 and WT animals demonstrated fibrosis in peribiliary areas (data not shown). Upon BDL, the expression of IGF1 was significantly higher in transgenic SMP8-IGF1 mice in comparison to sham-operated animals indicating transgenic upregulation by cholestatic injury. Changes in gene expression profiles in BDL treated animals and
liver function test (ALT, AST, ALP and bilirubin) confirmed the development of liver fibrosis in both SMP8-IGF1 and WT mice (Table 2).

The gene expression data one week after BDL did not reveal an increased development of fibrosis in SMP8-IGF1 mice compared to WT mice. In fact, the only significant difference was a decreased expression of α-Sma in transgenic compared to WT (Table 2), while that of Tnfα, Tgfβ, and Col1 did not differ between the models. In contrast to the Abcb4−/− model, IGF1 overexpression did not cause an increased Ck19 level in BDL-treated mice (Table 2). This absence of an effect on proliferation was confirmed by the similar percentage of BrdU-positive cholangiocytes (Table 2). In conclusion, in contrast to the chronic model for bile duct damage, increased IGF expression did not affect pathology in the acute BDL model, but did not display a protective effect either.

**DISCUSSION**

Prolonged overexpression of IGF1 increased fibrosis in the Abcb4−/− mouse, a model for primary sclerosing cholangitis. The enhanced IGF1 signaling resulted in stimulated cholangiocyte proliferation, one of the main pathological features of the liver damage in these mice.

The expression of transgenic Igf1, controlled by the α-Sma promoter, was stable during the entire experiment. It was previously reported that in Abcb4−/− mice expression of α-Sma (also of pro-fibrotic Tgfβ and Pdgfr) reaches maximum at 4 weeks of age and then gradually decreases (34). This was due to the generation of fibrous septa that shield the healthy liver from the toxic bile, thereby limiting the increase in pro-fibrotic factors. In our study, the expression of pro-fibrotic factors in the Abcb4−/− mice remained high, resulting in a hydroxyproline level about 2-fold higher than previously reported. The difference between the studies is most likely due to the cholate diet, which enhances the pathology in this model, resulting in prolonged high expression of pro-fibrotic factors (15).

IGF1 induces the expression of hepato-protective HGF in HSC (28). Confirming our previous observation (27), Hgf mRNA levels also increased in the IGF1 overexpressing Abcb4−/− mice compared to the non-transgenic litter-mates. The extent of damage in this model is much less severe than in the previously used acute CCl₄ model (100-fold lower levels of liver enzymes in serum; (9, 10)). The increase of AST and ALT in serum of the Abcb4−/− mice was comparable to the data reported before for this model (15), and indicated an ongoing liver damage. The elevation of serum ALP and bilirubin after 12 weeks of cholate diet points to an increase in progression of bile duct damage in mice that overexpress IGF1. This worsening of pathology was subsequently confirmed by the increased level of hydroxyproline and collagen, indicating an enhanced deposition of ECM. Thus, in contrast to our previous study in animals with CCl₄-induced parenchymal damage, in the present work IGF1 overexpression enhanced the damage and increased portal fibrosis in the cholangiopathic livers of Abcb4−/− mice.

Fibrogenesis depends on tissue remodeling, composed of strictly controlled processes. A subtle balance of MMPs (mainly involved in the ECM degradation) and TIMPs (their endogenous inhibitors) modulates liver fibrogenesis (35). MMPs are regulated by uPA/tPA/plasmin proteases, which directly contribute to degradation of collagens, and so play a pivotal role in the maintenance of ECM and tissue homeostasis (36-38). IGF1 overexpression increased the expression of Timp1, which could reduce MMP activity and ECM degradation in Abcb4−/−IGF1 animals. Moreover, lower tPA
expression points to a less active tissue remodeling resulting in a more prominent fibrosis in IGF1 overexpressing mice, which may further promote liver fibrosis.

Several studies have shown that bile-duct epithelium cells, in contrast to hepatocytes, express the IGF1R and directly respond to IGF1. IGF1 enhances proliferation and survival of cholangiocytes via IRS1/2 and ERK and PI3-kinase pathways (12). The proliferative effect of IGF1 has also been reported for smooth muscle cells in IGF1-overexpressing mice (17). Activation of the IGF1 system improves the survival of cholangiocytes in PBC patients suffering from chronic bile duct damage (39). In the acute CCl4 model, IGF1 did not reduce the initial damage, but caused a more rapid DNA synthesis, resulting in liver regeneration (27). Depletion of IGF1R reduces ductular and fibrogenic responses and increased cholestasis tolerance in BDL mice (40). The damage in the Abcb4−/− mouse is chronic, less severe, and localized to the portal areas (16). This results in a persistent increase in IGF1 signaling in the portal area, enhancing cholangiocyte proliferation (12). In addition, IGF1 enhances the proliferation of liver cystic epithelium and reduction of its expression reduces the size of liver cysts in a mouse model (41). A similar action may explain the formation of extremely large, but ill-developed bile-duct structures observed in those mice, leading to the formation of strictures. Presence of strictures may explain the cholestasis that, according to serum bilirubin and ALP level, developed eventually in our transgenic mice. Alternatively, the increased proliferation of bile ducts could promote the cholehepatic shunt (42), thereby increasing the amount of bile salts reaching the hepatocytes.

Submitting SMP8-IGF1 and WT mice to BDL revealed an absence of protective effect of overexpression of IGF1 also in this model. However, no difference in bile duct proliferation was seen between the WT and the SMP8-IGF1 mice. BDL did induce a-Sma mRNA expression in both models, suggesting that one week was enough to enhance IGF1 signaling. Nevertheless, 1 week BDL protocol seemed insufficient to induce the extent of liver damage needed to reveal an effect of enhanced IGF1 signaling.

Bile duct proliferation increased in IGF1 overexpressing Abcb4−/− and showed a tendency to increase in the SMP8-IGF1 mice submitted to BDL based on Ck-19 expression and BrdU staining. Increased Asbt expression in SMP8-IGF1 BDL mice suggests an increased cholehepatic shunt of bile salts, probably in an attempt to reduce the presence of damaging bile salts in biliary ducts. Likewise, Abcb4−/− mice showed an increased expression of Asbt (43). The augmented expression of IGF1 in Abcb4−/−/IGF1 mice could result in an even higher increase of Asbt that would enhance the excessive return of bile salts to the hepatocyte. Thus although these responses are aimed at the protection and repair of the bile duct epithelium, the additional increase of IGF1 to supra-normal levels induced extensive proliferation resulting in more bile duct abnormalities.

A difference in pathogenesis between the models may explain the discrepancy of the effect of IGF1 seen between Abcb4−/− mice and other mouse models of liver fibrosis (e.g. BDL- and CCl4-induced). Ligation of the common bile duct results in an acute interstitial (biliary) fibrosis, associated with massive proliferation of bile ducts that is only rarely observed in man. On the other hand, progression of fibrosis in Abcb4−/− mice is spontaneous due to cholangiocyte proliferation and massive up-regulation of profibrogenic genes, and bears more resemblance to human biliary fibrosis (34).
Our data demonstrate that rise in IGF1 expression in Abcb4−/− mouse, model for primary sclerosing cholangitis, worsens the pathology by increasing cholangiocyte proliferation leading to the formation of bile duct strictures, comparable to those in patients (44). This indicates that, in contrast to chronic parenchymal live damage, IGF1 administration does not seem an option for treating fibrosis caused by chronic cholangiopathies, and should be taken into account in the design of IGF1 replacement therapy for cirrhotic patients.

References