Pathogenesis and reversal of liver fibrosis: Effects of genes and environment
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chapter 3
unexpected reduction of murine liver fibrosis by igfbp5
ABSTRACT

Background: The ATP-binding cassette, sub-family B member 4 knock-out mouse (Abcb4\(^{-/-}\)) is a relevant model for chronic cholangiopathy in man. Due to the lack of this P-glycoprotein in the canalicular membrane of hepatocytes, the secretion of phospholipids into bile is absent, resulting in increased bile toxicity. Expression of insulin like growth factor binding protein 5 (Igfbp5) increases in time in the livers of these mice. It is unclear whether this induction is a consequence of or plays a role in the progression of liver pathology. Aim of this study was therefore to investigate the effect of IGFBP5 induction on the progression of liver fibrosis caused by chronic cholangiopathy.

Methods: IGFBP5 and, as a control, green fluorescent protein were overexpressed in the hepatocytes of Abcb4\(^{-/-}\) mice, using an adeno-associated viral vector (AAV). Progression of liver fibrosis was studied 3, 6, and 12 weeks after vector injection by analyzing serum parameters, collagen deposition, expression of pro-fibrotic genes, inflammation and oxidative stress.

Results: A single administration of the AAV vectors provided prolonged expression of IGFBP5 and GFP in the livers of Abcb4\(^{-/-}\) mice. Compared to GFP control, fractional liver weight, ECM deposition and amount of activated HSCs significantly decreased in IGFBP5 overexpressing mice even 12 weeks after treatment. This effect was not due to a change in bile composition, but driven by reduced inflammation, oxidative stress, and proliferation.

Conclusion: Overexpression of IGFBP5 seems to have a protective effect on liver pathology in this model for chronic cholangiopathy.
INTRODUCTION

Abcb4−/− (ATP-binding cassette, subfamily b, member 4) mice are the model for progressive familial intrahepatic cholestasis type 3. In humans, ABCB4 deficiency results in spontaneous development of biliary fibrosis shortly after birth, due to the lack of biliary phospholipids essential for neutralizing the toxic effects of the high concentrations of bile acids (1). Since murine bile acids are less toxic, the phenotype is milder in mice than in humans, which also qualifies Abcb4−/− mice as a model for other forms of progressive cholangiopathies, such as progressive sclerosing cholangitis (2, 3). Recurrent chronic injury in this mouse model, caused by leakage of toxic bile into the portal area leads to progressive accumulation of extracellular matrix (ECM) components, mainly produced by activated hepatic stellate cells (HSCs) in an attempt to counter hepatic damage (4-6). This results in an imbalance between fibrogenesis and fibrolysis, leading to liver fibrosis, architectural distortion, cirrhosis and ultimately liver failure (7, 8).

Insulin like growth factor binding protein 5 (Igfbp5) is strongly induced in the livers of Abcb4−/− mice suggesting a potential role in the pathogenesis of chronic cholangiopathy (9). This seems to be supported by its high expression in patients with cholangiocarcinoma (10). We have shown previously that overexpression of IGFBP5 increases the survival of partially activated HSCs and myofibroblasts (MF) (11). This pointed to its profibrotic role in liver, as also reported for skin and lung where IGFBP5 overexpression stimulates the progression of fibrosis (12, 13).

To clarify the role of IGFBP5 in the pathogenesis of chronic cholangiopathies, we overexpressed it in the Abcb4−/− mice, using an adeno-associated viral (AAV) vector. This in vivo AAV delivery that efficiently provides expression in hepatocytes, should result in the supply of large amounts this excretory protein to the intracellular space and to the HSCs (14). To substantiate the experimental findings in this model, and to relate them to the physiological role of hepatic IGFBP5 expression, a systems genetics approach was applied to a mouse genetic reference population, using the open source GeneNetwork database. The results have pointed to a beneficial role of IGFBP5 in liver fibrosis caused by chronic cholangiopathy in Abcb4−/− mouse model.

MATERIALS AND METHODS

scAAV vectors

scAAV8-LP1-Igfbp5 and scAAV8-LP1-eGFP were generated by replacing the factor IX cDNA from scAAV-LP1-IX by the cDNA of mouse Igfbp5 or eGFP, using the EcoR1 and BspM1 sites (Figure 1A) (15). Correctness of constructs was checked by sequencing. scAAV8 pseudotyped vector was produced and titrated as described (16).

Animals

The animal studies were reviewed and approved by the AMC committee for animal care and use. Three months after birth, a single dose of 5x10^{12} genomic copies (gc)/kg body weight of scAAV8-LP1-Igfbp5 or scAAV8-LP1-eGFP was injected in the tail vein of male FVB and Abcb4−/− mice (FVB background). Animals were sacrificed 3 (n=5), 6 (n=7) and 12 (n=7) weeks thereafter. In FVB controls (n=5) the effect was studied only after 3 weeks. Five animals per treatment group were used at 3 weeks, and 7 animals per group at weeks 6 and 12.
**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 (17). GSH/GSSG ratio in liver and bile was determined (18). ASAT, ALAT and alkaline phosphatase (ALP) concentrations in blood were determined using standard clinical chemistry methods.

**RNA isolation, reverse transcription and quantitative PCR**

These analyses were performed and analyzed as previously described (11). We used 5–7 animals for qRT-PCR, depending on the time point—5 animals per group at 3 weeks, and 7 animals per group at 6 and 12 weeks.

**Western blot analysis**

They were performed as described (11), using the following antibodies: IGFBP5 (Abcam, Cambridge, UK), GFP, PCNA and p21 (Santa Cruz biotechnology, CA, USA). Goat anti-mouse (Bio-Rad Laboratories, Veenendaal, The Netherlands) and goat anti-rabbit IgG horseradish peroxidase conjugated (Santa Cruz Biotechnology, CA, USA) were used as secondary antibodies. Chemiluminescence was quantified by Lumi-Imager F1, using CDP-Star (Roche, Mannheim, Germany) and β-actin as a reference. For detection of IGFBP5 in serum, the same amount of (10x diluted) serum was loaded for each sample.

**Histology and immunohistochemistry**

Formaldehyde-fixed liver sections were stained with Sirius red (morphometrical connective tissue assessment), or immunohistochemically, as described (19). Antibodies directed against PCNA (Santa Cruz biotechnology, CA, USA) and α-SMA (Sigma, Zwijndrecht, The Netherlands) were visualized with goat anti-mouse or goat anti-rabbit IgG coupled to horse-radish peroxidase (Sigma, Zwijndrecht, The Netherlands), or alkaline phosphatase, respectively. Anti-α-F4/80 (AbDSerotec, Dusseldorf, Germany) and anti-MAC-1 (R&D systems, Abingdon UK) were both visualized using rabbit-anti-rat-biotin as secondary antibody (DAKO, Glostrup, Denmark).

**Correlation analyses and QTL interval mapping in GeneNetwork**

These analyses were performed as described (20). Analysis of Igfbp5 expression in liver of the BxD genetic reference population. The BxD mouse genetic reference population (GRP) is a set of recombinant inbred (RI) mouse strains, derived by crossing F2 mice obtained from an intercross of C57BL/6J (B6) and DBA/2J (D2) mice, and inbreeding the resulting progeny for at least 20 generations to generate the BxD GRP (21, 22). RI panels, comprised of a number of lines with limited intra- and large inter-line phenotypic variation, are widely used to determine genotype-phenotype associations with quantitative trait locus (QTL) mapping techniques, or eQTL mapping, when mRNA levels are used as phenotypes (23-27). The relationships between the phenotypes and genotypes are calculated with a likelihood ratio statistic (LRS), a measure of the probability that a given genetic marker explains the variation in the phenotype. This genetic reference panel contains data for hundreds of phenotypic traits, including clinical and molecular ones (i.e. microarray expression studies in multiple tissues) acquired over 25-years. The data are publically available at GeneNetwork (www.genenetwork.org). The web-based software further allows extraction of sets of
transcripts or clinical phenotypes that tightly co-vary with a gene of interest across genetically different populations. Patterns of co-variations can be studied in multiple experimental crosses and multiple tissues.

For this study we used data from BxD published phenotypes database (www.genedata.com), and gene expression data from:
- liver (http://www.genedata.org/dbdoc/LV_G_0106_B.html),
- kidney (http://www.genedata.org/dbdoc/MA_M2_0806_R.html)

**Statistical analysis**

ANOVA analysis and Student’s *t*-test were used. The error bars represent the standard deviation, and significance threshold was set to *P*<0.05.

**RESULTS**

Figure 1: Overexpression of IGFBP5 in liver using AAV. A) Time course and genome structure of scAAV-LP1-Igfbp5 and scAAV-LP1-GFP. B) Western blot of GFP (left panel) and IGFBP5 (30kD; right panel) present in liver homogenates obtained 3, 6 and 12 weeks after injection, and of IGFBP5 in plasma after 3 weeks (right panel). In both panels, the GFP-overexpressing animals are on the left (“g”) and IGFBP5-overexpressing mice (“i”) on the right side. For detection, the Western blots in the left panel were incubated with anti-GFP, while those in the right panel were incubated with an anti-IGFBP5 antibody. Every lane represents an individual animal. In total, 4–6 animals of each treatment group were analyzed.

C–D) Relative gene expression of Igfbp5 in Abcb4<sup>−/−</sup> mice 3, 6 and 12 weeks after administration of scAAV-LP1-Igfbp5 (right panel) or scAAV-LP1-GFP as a control (left panel) (*n*=5–7). Asterisks denote significance (*p*<0.05) in comparison with control treatment.

**IGFBP5 overexpression reduces hepatocyte damage and proliferation in Abcb4<sup>−/−</sup> mice**

To investigate the effect of IGFBP5 on the liver pathogenesis in Abcb4<sup>−/−</sup> mice, its expression was increased in hepatocytes using the liver specific scAAV8-LP1-IGFBP5 vector and scAAV8-LP1-GFP as a control (Figure 1A). qPCR and Western blot confirmed the hepatic overexpression of both
transgenes 3, 6 and 12 weeks after injection (Figure 1B). During this period, the expression declined, but was still significantly increased for Igfbp5 at 12 weeks (~15-fold) compared to mock transduction (Figure 1C). Because of the ongoing hepatocyte proliferation in the Abcb4⁻/⁻ mice this loss of episomal AAV vectors was expected (28, 29). The lower expression of GFP seen at 3 weeks after injection while the vector dose was equal, suggested a more rapid decline of GFP expression during this period, which could be due to a higher hepatocyte proliferation rate in these control mice (Figure 1D).

Therefore, the expression of proliferation markers PCNA and Ki67 was determined (Figure 2A-C). The 2-3 fold lower PCNA expression level after three weeks, and the lower expression level of Ki67 at both later time points, confirmed that proliferation was reduced in mice overexpressing IGFBP5.

This may be due to enhanced expression of p21, involved in repair of DNA damage by arresting cell cycle and proliferation. Both p21 mRNA and protein expression level were significantly upregulated in Igfbp5 injected animals (Figure 3A-B). This was accompanied by an increased presence of senescence-associated β-galactosidase (a widely used biomarker for senescent and aging cells), especially in fibrotic regions (Figure 3C). Lower ALP (alkaline phosphatase) and ASAT (aspartate transaminase) levels in these mice 6, 9 and 12 weeks after injection, suggested that IGFBP5 alleviates hepatocyte damage (Figure 3D–E).
Figure 3: IGFBP5 overexpression reduces hepatocyte damage in Abcb4−/− mice. A) Gene expression of p21 in liver after 3, 6 and 12 weeks after vector administration. B) p21 level 12 weeks after vector administration as detected by Western blot, and quantified as percentage of the control (GFP). C) Representative SA-β-gal staining of control (GFP) and IGFBP5-treated animals 12 weeks after vector injection. D–E) Plasma concentrations of ALP and ASAT in GFP- and IGFBP5-treated Abcb4−/− mice 1, 3, 6, 9, and 12 weeks upon vector administration.

Overexpression of IGFBP5 reduces liver fibrosis in Abcb4−/− mice

A protective role of IGFBP5, as suggested by decreased hepatocyte proliferation and lowered liver enzymes in serum, may also slow down the progression of fibrosis. The 20% lowered fractional liver weight, the reduced presence of ECM shown by hydroxyproline content (Figure 4A-B) and Sirius red staining (Figure 4C) all confirmed that IGFBP5 overexpression impairs the progression of liver fibrosis in this model.
Figure 5: IGFBP5 overexpression decreases expression of fibrotic markers in Abcb4⁻/⁻ mice. A) Representative α-SMA staining in control (GFP; left and middle panel; 5X and 20X magnified respectively) and IGFBP5 overexpressing livers (right panel) 12 weeks after vector administration. B) Number of α-SMA expressing cells per mm² in liver 12 weeks after vector administration as determined by a custom written counting macro in Scion Image (Scion Corp, Frederick, MD). C–F) Relative mRNA level for procollagen, collagens 1, 3, 4 and Timp1, after 3, 6 and 12 weeks. All results are given as a percentage of the control. Asterisks denote significance (p < 0.05).

This was supported by 70% reduction of α-SMA (marker for activated HSCs/MFs) expressing HSCs/MFs 12 weeks after vector administration (Figure 5A-B), and, as a consequence, reduced expression of procollagen, collagens 1, 3, 4 and Timp1 (Figure 5C-G).

Since the pathogenesis in the Abcb4⁻/⁻ mouse results from toxic bile, an effect of IGFBP5 on bile composition could provide an alternative explanation of its protective role in this model. The absence of significant differences in bile composition 12 weeks after vector administration however renders this explanation highly unlikely (Table 1).

Table 1: Bile salt composition of GFP control and IGFBP5-treated Abcb4⁻/⁻ mice and FVB.

<table>
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<tr>
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<th>FVB</th>
<th>Abcb4⁻/⁻ ctrl</th>
<th>Abcb4⁻/⁻ BP5</th>
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<tbody>
<tr>
<td>tauro β-muricholate</td>
<td>223.52 ± 67.90</td>
<td>277.96 ± 142.05</td>
<td>253.98 ± 172.18</td>
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<td>taurocholate</td>
<td>94.97 ± 22.84</td>
<td>145.85 ± 68.17</td>
<td>180.53 ± 162.50</td>
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<td>tauro α-muricholate</td>
<td>1.92 ± 0.84</td>
<td>6.73 ± 4.86</td>
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<td>tauroursodeoxycholate</td>
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<td>0.74 ± 0.36</td>
<td>1.18 ± 0.89</td>
</tr>
<tr>
<td>taurchenodeoxycholate</td>
<td>1.56 ± 0.59</td>
<td>4.36 ± 1.66</td>
<td>5.55 ± 3.14</td>
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<tr>
<td>Σ (bile salts)</td>
<td>322.32 ± 85.73</td>
<td>435.65 ± 209.62</td>
<td>450.58 ± 334.74</td>
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**IGFBP5 overexpression reduces inflammation and oxidative stress in the liver of Abcb4⁻/⁻ mice**

IGFBP5 has been reported to impair the influx of proinflammatory cells (30). Influx of these cells into the liver will result in the release of inflammatory mediators that enhance fibrosis and cause oxidative stress (31). Inhibition of this influx by IGFBP5 may therefore explain the protective effect observed in this model. To investigate this, markers for inflammatory cells were studied. Presence of F4/80, a marker for infiltrating macrophages and liver-resident Kupffer cells, was significantly lower
6 and 12 weeks (mRNA) and 12 weeks (protein) after vector administration (Figure 6A-C). Expression of *Mpo*, a marker for infiltrating neutrophils, was reduced at all three time points (Figure 6B). Furthermore, the presence of MAC1-expressing activated macrophages was clearly reduced in IGFBP5 overexpressing animals (Figure 6D). In addition, the expression of *Mcp1*, which plays a role in the recruitment of monocytes to sites of injury and infection, was reduced 3 and 6 weeks upon IGFBP5 administration (Figure 6E). Finally, *Tgfβ1* mRNA level was also reduced in Abcb4−/− mice overexpressing IGFBP5 (Figure 6F).

Figure 6: IGFBP5 reduces expression of inflammatory markers. A, B, E and F) Relative mRNA levels of F4/80, Mpo, Mcp1 and Tgfβ1, respectively, in livers of control (GFP) and IGFBP5 overexpressing mice, 3, 6 and 12 weeks upon vector administration. All results are given as a percentage of the control (GFP). Asterisks denote significance (P < 0.05). C and D) Representative pictures of immunostainings for F4/80 and MAC1, respectively, 12 weeks after vector administration.

The reduced influx caused by IGFBP5 overexpression will also lower the release of proinflammatory cytokines in liver and as such may explain the lower the oxidative stress in these
livers as indicated by the significant increased ratio of glutathione (GSH) and its oxidized form glutathione-disulfide (GSSG; Figure 7A). In support, mRNA expression level of heme oxygenase 1 was significantly upregulated upon injection of IGFBP5 (Figure 7B).

Figure 7: IGFBP5 overexpression lower the oxidative stress. A) The GSH/GSSG ratio determined at 6 weeks (liver) and 12 weeks (liver and bile). B) Relative mRNA level for HO-1 at 6 and 12 weeks. All results are given as a percentage of the control. Asterisks denote significance (P < 0.05). C) Absence of anti-GFP antibodies in plasma of GFP- and IGFBP5-expressing mice demonstrated by an ELISA.

Specific expression of GFP in hepatocytes did not induce an immune response, as shown by the lack of anti-GFP antibodies in serum of control mice and the comparable degree of liver fibrosis in non-treated and GFP-treated Abcb4−/− mice (Figure 7C). This confirms previous findings of our group and others that hepatocyte specific expression provided by an AAV vector does not result in an immune response towards the transgene (16).

Altogether these data support an anti-inflammatory effect of IGFBP5 in the liver of Abcb4−/− mice.

Co-variation of Igfbp5 expression with phenotypic traits in a mouse genetic reference population supports its anti-inflammatory role

To gain insight into the physiological role of Igfbp5 expression in adult liver, its co-variation with published phenotypic traits in the BxD genetic reference population (GRP) was analyzed. This experimental model that incorporates the natural range of genetic variation is obtained by 20 generation inbreeding in the F2 mice from an intercross between C57BL/6J and DBA/2J strains (www.genenetork.org) (24, 26). The analysis revealed a significant negative correlation between liver Igfbp5 expression and plasma concentrations of ALAT and ASAT (Figure 8A and B) (32). These relationships underline a hepato-protective role of IGFBP5. The negative correlation between Timp1 and Igfbp5 expression in the BxD GRP livers (5) provides further support for a possible physiological role of IGFBP5 in ECM remodeling (Figure 8C). In the reference population, the expression of pro-fibrotic cytokine Tgfβ1 positively correlated with that of F4/80 (Figure 8D), which corroborated the reduction of inflammatory response by IGFBP5.

Furthermore, the correlation of IGFBP5 expression in liver of BxD GRP with clinical phenotypes was calculated. A number of phenotypes were related to immune function (Supplementary file 2 in a published manuscript), which was consistent with the anti-inflammatory effect of IGFBP5 in the liver of Abcb4−/− mice. Igfbp5 expression in the livers of BxD mice negatively
correlated with inflammation in spleen, bone marrow and with a general inflammatory response, the exception being a positive correlation with TBC infection in the lung (Figure 8E).

Figure 8: Igfbp5 expression in liver correlates with suppressed immune response in the BxD reference population. Correlation between Igfbp5 mRNA in liver and ASAT A) and ALAT B) serum levels (n=7) as found in (GeneNetwork database). C) Correlations between Igfbp5 and Timp1 mRNA levels liver of BxD recombinant inbred strains (as determined in GeneNetwork database). D) Expression of Tgfβ in liver correlates with that of F4/80 in BxD genetic reference population. E) A correlation network between Igfbp5 expression level (blue rectangle) in livers of BxD mice and immune-linked phenotypic features measured in the same animals. The phenotypic features (depicted in green rectangles) are as follows: “IrradSpleen” (10407) - Antigenic activity of irradiated BXD spleen cells for Thy-1+CD3+CD4+CD8- T-cell clone E4.3 [Thymidine uptake cpm]; (33); “CpvInDMCs” (12678) - Infectious disease, immune function: Cowpox virus measure of disease onset measured by the day of maximum clinical score over two weeks after 10^6 pfu intranasal inoculation (Rice AD, Moyer RW, 2010, unpublished data); “ProgCellMob” (10206) - Immune function: Granulocyte colony stimulating factor (G-CSF) induced progenitor cell mobilization from bone marrow to blood [PB-CFC/ul]; (34); “BCGPulmGranInflm” (10695) - Immune function: Lung inflammatory response, pulmonary granulomatous inflammation induced by BCG [units] (35).

Tissue specific regulation of Igfbp5 expression

Given the unanticipated effect of IGFBP5 on liver fibrosis, we addressed the tissue-specificity of its expression. Notably, a direct analysis of variation in Igfbp5 mRNA levels in different organs - liver, kidney, (whole) brain, hippocampus and cartilage - did not reveal any correlation (not shown), underscoring organ specific regulation of Igfbp5 expression. The correlations between Igfbp5 expression in different organs and phenotypic features in the BxD GRP were distinct for liver, kidney and brain, (as published in Supplementary file 2). Unsurprisingly, the variation of Igfbp5 expression in other two organs did not correlate with ALAT and ASAT levels in serum.

The systems genetic approach was further employed to identify the quantitative trait loci (QTLs) that specifically modulate the expression of Igfbp5 in mouse liver. No cisQTLs were determined on Chr.1, where Igfbp5 resides (Figure 9A). A putative trans-regulatory region that modulates Igfbp5 expression in liver was identified on Chr.7. The QTL peak (135-142Mb) comprised
83 positional candidates that may modulate hepatic Igfbp5 expression (Figure 9B, as published in Supplementary table 1).

Figure 9: Interval mapping, a WebQTL analysis of upstream controllers of liver Igfbp5 expression. A) The X-axis represents 19 mouse autosomes and the X chromosome. The small purple triangle marks the location of the Igfbp5 gene. The thick blue line summarizes the strength of association between variation in Igfbp5 expression and the two genotypes of all markers and the intervals between markers. The height of the peak (primary Y-axis) provides the likelihood ratio statistic (LRS; which can be read as a chi-square-like statistic). The additive coefficient (green line, secondary Y-axis) indicates that DBA/2J alleles increase trait values. In contrast, a negative additive coefficient (red line) indicates that C57BL/6J alleles increase trait values. The yellow histogram bars summarize the results of a whole-genome bootstrap of the trait performed 1000 times, supporting thereby the robustness of the results. The horizontal dashed lines at 10.5 and 17.3 are the LRS values associated with the suggestive and significant genome-wide probabilities that were established by permutations of phenotypes across genotypes. B) Physical map of variation in Igfbp5 expression in liver on distal Chr 7 (a blow up of the whole-genome map in panel A). A physical scale in megabases (Mb) is shown on the X-axis. The small irregular colored blocks and marks at the top of the map indicate the locations of genes superimposed on the physical map. The orange hash marks along the X-axis represent the number of single nucleotide polymorphisms that distinguish the two parental strains (C57BL/6J and DBA/2J) from each other.

To narrow them down to a biologically relevant subset, genes were scrutinized by analyzing their co-expression, by QTL mapping (to establish if they were cisQTLs per se), by examining the number of SNPs, and by studying the literature. Among the four most promising candidates (Tacc2, Nkx1-2, Znmb1 and C1hp) Nkx1-2 appeared the most likely one, since it was a cisQTL, it was modulated by the same transQTL on Chr.14 that possibly also regulates the expression of Igfbp5, and
its expression positively correlated with that of Igfbp5 (Figure 10A-C). The almost linear relationship between Nkx1-2 expression in the liver and the survival coefficient in infectious diseases suggests its potential important role in orchestrating inflammatory responses, possibly including Igfbp5 expression (Figure 10D; Kotb M., 2010, unpublished data). Although this analysis reveals Nkx1 as an interesting candidate, the role of other candidate genes cannot be excluded.

Figure 10: NKX1-2 is a likely modulator of Igfbp5 expression in liver. A) Interval mapping for Nkx1-2. (For explanation see Supplementary figure 6.) B) QTL heatmap for Chr. 1, 7 and 14 for Igfbp5 (left column) and Nkx1-2 (right column). The yellow triangles indicate the location of the two genes (Igfbp5 on Chr.1 and Nkx1-2 on Chr.7). The intensity of color indicates the likelihood ratio statistic (LRS) values associated with the suggestive and significant genome-wide probabilities established by permutations of phenotypes across genotypes for both of the parental strains. C) Correlation of Igfbp5 and Nkx1-2 expression in BxD GRP. D) Correlation between the expression level of Nkx1-2 and the coefficient of survival in infectious diseases (M. Kotb, 2010, unpublished data).

DISCUSSION

The aim of this study was to investigate the role of IGFBP5 in the liver pathogenesis in the Abcb4−/− mice, a model for chronic cholangiopathy. Prolonged overexpression of IGFBP5 in hepatocytes decreased their proliferation, reduced inflammation and oxidative stress, and lowered number of activated HSCs, all leading to reduced development of liver fibrosis.

IGFBP5 has cell- and tissue-type specific effects. It induces apoptosis in the involuting mammary gland (36) and inhibits cell proliferation in the involuting prostate gland (37), but supports the survival of skeletal myoblast (38). To clarify the differential role and expression of Igfbp5 in liver and other organs, its steady-state expression was analyzed in the BxD genetic reference population.
The correlations with clinical features not only indicated an organ specific regulation of IGFBP5, but also a role that differed between organs. This seems to explain why IGFBP5 was found a profibrotic factor in lung and skin (12, 13), while the current study revealed its protective effect in liver fibrosis. The latter is underscored by the negative correlation between Igfbp5 expression and serum parameters of liver damage in IGFBP5 overexpressing Abcb4-/- mice, and in the BxD genetic reference population. The systems genetic approach made it clear that the relation between these liver enzyme levels and IGFBP5 goes beyond the issue of damage in the Abcb4-/- model. In addition, a protective effect of IGFBP5 on hepatocytes in vivo is consistent with its prosurvival effect seen in activated HSCs in vitro (11). Reduced liver fibrosis after 12 weeks of IGFBP5 expression, as demonstrated by considerably reduced portal bridging compared to that seen in control animals at the time of vector administration and compared to GFP-expressing controls (Figure 4C), indicated involvement of IGFBP5 not only in impediment of ongoing fibrogenesis, but in reduction of the existing scar tissue.

The most likely mechanism of the anti-fibrotic effect of IGFBP5 expression in the Abcb4-/- mice is a reduction of inflammation. The portal inflammation, caused by leakage of toxic bile is one of the most prominent pathological features in this model for chronic cholangiopathy (3). Overexpression of IGFBP5 lowers the influx of inflammatory cells into this area, possibly by impairing their migration (39). This would also lower the production of inflammatory cytokines, known to stimulate collagen synthesis and to enhance hepatocyte damage. The negative correlation of Igfbp5 expression with inflammation in the BxD genetic reference population further supports such an anti-inflammatory role. In addition, the systems genetics approach indicated Nkx1-2 as the putative modulator of liver Igfbp5 expression, possibly in response to an inflammatory assault, which would then explain the Igfbp5 upregulation in Abcb4-/-mice upon cholate feeding (9). Damaged hepatocytes and activated inflammatory cells release reactive oxygen species (ROS) and other reactive intermediates (40, 41), which enhance the expression of profibrotic genes in human HSCs and MFs (42-44). The reduced oxidative stress in IGFBP5 overexpressing livers is in agreement with the observed decrease in inflammation, and may additionally alleviate liver fibrosis. However, the lower influx may also reduce the immune surveillance in these livers and as such may provide protection of cancer cells. Such a mechanism could explain the high IGFBP5 expression reported in patients with cholangiocarcinoma (10).

IGFBP5, however, may have a more direct effect. The increased amount of p21 in IGFBP5 overexpressing livers may be instrumental for the reduced proliferation. p21 regulates PCNA binding to damaged DNA (45), reducing its nuclear presence as seen in this study (Figure 2A-B). In addition, the increased presence of p21 may play a role in senescence (46), particularly that in the fibrotic regions of IGFBP5 treated livers. Changes in gene expression profiles of senescent HSCs point to the cell cycle standstill, decreased synthesis of ECM, increased release of ECM remodeling enzymes (47). In this case, the increased expression of IGFBP5 seems aimed at reducing the pathology in this model of chronic cholangiopathy.

In conclusion, this study reveals that expression of IGFBP5 in the Abcb4-/-mice reduces inflammation, oxidative stress, ECM deposition and hepatocyte proliferation, thereby evidently reducing liver fibrosis. Furthermore, the systems genetics approach reveals correlation between
endogenous hepatic Igfbp5 expression and decrease of inflammation in a reference population, underscore its role in ameliorating pathology in the model for chronic cholangiopathy.

References