Pathogenesis and reversal of liver fibrosis: Effects of genes and environment
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SCOPE OF THE THESIS

Hepatic fibrosis results from repeated liver tissue damage, caused by chronic viral hepatitis, parasitic diseases, inborn errors of metabolism, toxic damage and non-alcoholic fatty liver disease (NAFLD). Fibrosis is a generic wound healing response to chronic insults, regardless of their mechanism. In the liver, resulting progressive accumulation of fibrillar extracellular matrix (ECM) is associated with ECM degradation and remodeling that can result either in restoration of normal hepatic structure and function, or in cirrhosis, conversion of normal liver architecture into structurally abnormal nodules.

Efforts to elucidate the cellular and molecular basis of fibrogenesis have generated a comprehensive picture of progression and regression of liver fibrosis, essential for developing antifibrotic therapies. Due to the dynamic evolving nature of the disease, it seems that interventions even as late as in cirrhosis could improve clinical outcomes. Though translation of these findings into diagnostic tools and treatments does not seem too far away, there are still no effective antifibrotics. There is also a requirement for better markers of the stage and activity of fibrosis, predicting if patients will progress to cirrhosis or reverse.

The objective of this thesis was therefore to make use of multiple complementary experimental model systems, molecular techniques and metabolic challenges, to study liver fibrosis, with the final aim to create new opportunities for antifibrotic therapies.
INTRODUCTION

Fibrotic diseases account for up to 45% of deaths in the developed world (1). They are the most common non-neoplastic cause of death among hepatobiliary and digestive diseases in the USA and Europe, even though only 25-30% of patients affected by chronic liver diseases are likely to develop significant fibrosis and cirrhosis, with an inevitably poor prognosis, where 1-year mortality ranges from 1% in early cirrhosis to 57% in decompensated disease (2). The main causes of liver fibrosis in developed countries are chronic viral hepatitis, alcohol and drug abuse. The association of fibrosis/cirrhosis with primary liver cancer further increases the relative mortality rate (3, 4). The worldwide burden is equally compelling, with chronic liver diseases affecting hundreds of millions of individuals (5). In addition, the explosive growth in obesity worldwide has led to a dramatic increase in the prevalence of non-alcoholic steatohepatitis (NASH) (6), which also confers a risk of hepatocellular carcinoma (HCC), (fastest rising cancer incidence of any neoplasm in the USA and Western Europe) once cirrhosis develops (7, 8). The large number of people affected worldwide and the lack of effective anti-fibrotic treatment necessitates further studies of the pathophysiology in order to deliver novel therapies.

HEPATIC FIBROGENESIS

Liver fibrosis results from perpetuation of the normal wound healing response. Repeated cycles of hepatocyte injury and repair, accompanied with inflammation and excessive deposition of ECM proteins, result in an abnormal continuation of fibrogenesis. Its progression depends on the etiology of liver disease and is influenced by environmental and genetic factors (9-11).

During hepatic fibrogenesis, significant changes in quality, quantity and distribution of ECM components occur in the periportal and perisinusoidal space, increasing the content of collagens and non-collagenous components (12).

In normal liver, the subendothelial space of Disse that separates the epithelium (hepatocytes) from the sinusoidal endothelium, contains both an interstitial (high-density) and a basement membrane-like, perisinusoidal (low density) ECM (Figure 1a). During fibrosis, perisinusoidal ECM is replaced by an interstitial (scar-type) matrix with bundles of collagen fibrils and an electron-dense basement membrane (13). This is tied in with a loss of hepatocyte microvilli and a disappearance of endothelial fenestrations (Figure 1b). The dominant event in fibrogenic cascade is activation of hepatic stellate cells (HSCs) (14), remarkably adaptable mesenchymal cells that are vital to hepatocellular function and the liver’s response to injury. These cells resident in the space of Disse are the major storage of vitamin A in the body. They represent one-third of the nonparenchymal cells, and ~15% of the total number of resident cells in normal liver (Figure 1a). HSCs are primary effector cells that play a pivotal role in activating the immune response through secretion of cytokines and chemokines and interacting with immune cells, contributing thereby to angiogenesis and the regulation of oxidant stress, and orchestrating the deposition of ECM in normal and fibrotic liver (15). In the injured liver, numbers of activated hepatic stellate cells/myofibroblasts (HSCs/MFs) dramatically increase at sites of infection, inflammation and injury, promoting the
deposition of scar-tissue (Figure 1b). These cells are generated locally, by transdifferentiation of resident peri-sinusoidal HSCs and periportal fibroblasts (16, 17), or via the recruitment and differentiation of bone-marrow-derived mesenchymal stromal cells (18, 19). Activated HSCs secrete large amount of interstitial collagen, accompanied by matrix metalloproteinases (MMPs), which induce three-dimensional changes in the ECM. They degrade the normal basement membrane matrix, whilst having a mild effect on the neo-matrix rich in fibrillar collagen. On the other hand, the production of tissue inhibitor of metalloproteinase (TIMPs) by HSCs also increases, promoting ECM accumulation by inhibiting matrix degradation, improving survival of activated HSCs and preventing their clearance, all delaying the regression of liver fibrosis (20-22).

The changes in ECM impair the flow of metabolites between the hepatocytes and the perfusing plasma, exposing hepatocytes to hypoxia (particularly in pericentral zones). In unopposed fibrosis this causes distortion of hepatic architecture, formation of septae (broad bands that divide the parenchyma into regenerative nodules), increase in intrahepatic vascular resistance, angiogenesis and sinusoidal remodeling, inducing portal hypertension (23). This ultimately leads to pathophysiological damage and cirrhosis as the final stage (24). Advanced fibrotic changes are strongly associated with hepatocellular carcinoma, which further reduces chances of survival (25).

Potential reversibility of hepatic fibrosis varies depending on the duration of injury, the degree of angiogenesis, and the composition, spatial distribution, and cellularity of scar. Cirrhotic areas resistant to degradation are fairly acellular and rich in elastin and highly cross-linked collagen, suggesting that ECM cross-linking might represent a “point of no return” in fibrosis (22). Fibrotic patients have a good short-term prognosis, as it usually requires several months to years of ongoing insult to reach cirrhosis (26). Cirrhosis, however, as present evidence indicates, is not completely reversible.

**SIGNALS FOR PATHOGENESIS IN LIVER FIBROSIS**

Similar to fibrotic disorders in other organs, chronic activation of the wound-healing process is the mechanism behind hepatic fibrogenesis. Upon hepatic injury, a fibrogenic response is provoked by activation of key signals in resident and infiltrating liver cells – reactive oxygen species (ROS), hypoxia, inflammation and apoptosis. As a consequence, ECM proteins accumulate in the liver due to imbalance between the fibrogenesis and fibrinolysis.

**Oxidative stress** in chronic liver diseases (CLD) results from an increased generation of ROS (and other reactive intermediates) and from a decreased efficiency of antioxidant defenses. This stress contributes to excessive tissue remodeling and fibrogenesis by stimulating the production of profibrogenic mediators by Kupffer cells and other resident and circulating inflammatory cells. It directly affects the behavior of HSCs/MFs by induction of critical profibrogenic genes, like procollagen type 1, monocyte chemoattractant protein 1, and tissue inhibitor of metalloproteinase 1 (TIMP1). This is likely enabled by the activation of transcription factors, such as c-jun N-terminal kinases (JNKs), transcription factor AP1 and nuclear factor zB (NFzB) (27).

**Hypoxia** is a critical, early fibrogenic stimulus, caused by capillarisation of sinusoids, intrahepatic shunting, vasoconstriction, compression and thrombosis (28). It impairs mitochondrial function, produces oxidative stress, induces vascular endothelial cell growth factor (VEGF) and its
receptors, and stimulates synthesis of collagen 1 in HSCs (29). Hypoxia also potentiates expression of transforming growth factor, beta 1 (TGFβ1) (30), contributing to both autocrine and paracrine loops that drive angiogenesis, chemotaxis and fibrogenesis.

Figure 1: Hepatic liver cells and the hepatic sinusoid in normal and injured liver

**Inflammation** initiates and regulates fibrosis by the release of soluble mediators and the secretion of products that remodel/degrade the normal basement membrane matrix within the liver (31). Inflammatory cells, resident (e.g. NK cells and macrophages) or recruited (e.g. T and B cells), are involved in pathogen elimination, cell removal (e.g. hepatocytes damage during anti-viral immune reaction), recruitment and activation of MFs, and spontaneous recovery of fibrosis (32, 33).
Apoptosis, a common feature of chronic liver disease, results in the generation of apoptotic bodies, which phagocytic clearance creates pro-inflammatory and fibrogenic stimuli. After engulfing the apoptotic bodies, Kupffer cells secrete death ligands and tumor necrosis factor alpha (TNFα). Similarly, the engulfment of apoptotic bodies by HSCs triggers a profibrogenic response with production of oxidative radicals, and upregulation of expression of TGFβ1 and collagen 1 (34-37).

HEPATIC STELLATE CELLS AND FIBROGENESIS

In response to liver injury, HSC undergo rapid activation, functional and morphological changes. Activation, which occurs in two phases, initiation and perpetuation, is, if liver injury has subsided, followed by resolution (Figure 2) (38). Initiation of HSCs activation comprises early changes in gene expression and phenotype, which make them responsive to cytokines and other stimuli released from affected cells, like damaged hepatocytes, bile duct cells, Kupffer cells and inflammatory cells (39). Perpetuation encompasses cellular series of events that amplify the activated phenotype through enhanced growth factor expression and responsiveness. It results from autocrine and paracrine stimulation, and accelerated ECM remodeling.

In response to acute and chronic liver injury, HSCs proliferation is promoted by strongly increased mitogens. They include platelet-derived growth factor (PDGF), VEGFA, thrombin and its receptor, epidermal growth factor (EGF), transforming growth factor α (TGFα), keratinocyte growth factor and fibroblast growth factors 2 (FGF2) and its receptors (FGFR1) (11). The most potent fibrogenic factor for HSCs is TGFβ1 (40, 41). Activated TGFβ1 released from the local ECM binds to an increased number of TGFβ receptors on HSCs. In response, signaling via the SMAD pathway enhances proliferation and survival of activated HSCs, increases collagen synthesis, upregulates TIMPs, and decreases MMPs expression (42). HSCs migrate and accumulate in zones of injury/repair (43) secreting large amounts of cytokines (amplifying the inflammatory and fibrogenic tissue responses), and MMPs (accelerating the replacement of normal with "scar" matrix). Activated HSCs accumulated in the collagenous bands impede portal blood flow by constricting individual sinusoids and entire organ (44, 45).

Resolution of fibrosis refers to the fate of activated HSCs when the primary insult is withdrawn or attenuated, and refers to pathways that cause apoptosis, senescence, or quiescence of HSCs (Figure 2) (46-48).

ANTIFIBROTIC THERAPIES

Current treatments of cirrhosis include withdrawal of the causative agent and treatment of complications, but the only curative treatment for advanced cirrhosis is liver transplantation. Its clinical applicability is, however, limited by shortage of donors, by the commitment of recipients to life-long toxic immunosuppression, and by poor condition of the potential recipient.
Since the progress of fibrosis is basically similar in all liver diseases regardless of the etiology, the development of antifibrotic therapies should benefit all patients with fibrosing liver injury. The liver's ability to regenerate and its first-pass metabolism allow lower doses of orally administered compounds to achieve a therapeutic response, minimizing systemic distribution and non-hepatic side effects, which makes liver fibrosis an attractive target for therapy. The progress in clinical research and basic science and improved diagnostic tools promise to halt the progression of liver fibrosis and/or promote its reversion. Potential therapeutic approaches for liver fibrosis include: treatment of the primary disease, suppression of hepatic inflammation, inhibition of HSCs activation, promotion of matrix degradation, stimulation of HSCs apoptosis, and targeted antifibrotic therapies (Figure 3). These approaches will be addressed individually.

**Treatment of the primary disease.** Clearing the primary cause of liver disease is still the most effective intervention in the treatment of liver fibrosis. Removal of excess iron or copper in haemochromatosis or Wilson’s disease, respectively, abstinence in alcoholic liver disease, anti-helminthic therapy in parasitic infections, clearance of HBV or HCV in chronic viral hepatitis, and biliary decompression in bile duct obstruction, or, most recently, weight loss in patients with NASH, lead to reduced fibrogenesis (49-53) This approach can be highly effective when applied early, resulting in a near-normal restitution of hepatic histology.
Figure 3: Targets for antifibrotic therapies.

**Suppression of hepatic inflammation.** In sustained liver injury, persistent inflammation perpetuates the fibrogenic HSCs phenotype and leads to fibrosis. Therefore, a number of anti-inflammatory agents have been evaluated in vitro and in vivo. Treatment with corticosteroids in patients with severe autoimmune hepatitis leads to hepatic fibrosis only in a minority of patients (54). Blocking interleukin 1 by expressing the interleukin-1 receptor antagonist, also reduced liver damage and pro-inflammatory cytokine levels in an in vivo model of ischaemia-reperfusion injury (55). Recombinant interleukin 10 reduces inflammation and fibrosis in patients with chronic hepatitis C, but results in an increased viral load (56). Local expression of interferon alpha (IFNα) improved liver fibrosis in a dimethylnitrosamine-induced model of cirrhosis (57). Finally, the beneficial effects of ursodeoxycholic acid in the treatment of primary biliary cirrhosis (PBC) are thought to be in part via anti-inflammatory mechanisms (58).

**Inhibition of HSCs activation.** HSCs activation, proliferation and fibrogenesis are pivotal steps in the development of liver fibrosis, making the mechanisms that mediate them attractive targets for therapeutic intervention. Oxidative stress, which stimulates HSCs activation, is one such target. Several therapeutic agents have been tested in animal models to counteract the activation of HSCs by oxidative stress, like vitamin E and C (59), and S-adenosyl-L-methionine (60) but the effects were minor. A number of other agents have been shown to inhibit the HSC activation. They include interferon gamma (IFNγ) (61) and ligands for peroxisomal proliferator activated receptor (PPAR) gamma.(62). TGFβ antagonists are extensively tested, since neutralizing of this potent cytokine would have the dual effect: inhibition of matrix production and acceleration of its degradation. Adenoviral expression of Tgfβ1 antisense mRNA, and gene transfer of Smad7, which blocks TGFβ intracellular signaling, also had positive effect. Gene transfer of soluble TGFβR2 that
inhibits liver fibrogenesis in rats is shown to attenuate apoptosis, injury, and fibrosis in bleomycin-induced lung fibrosis in mice. Blocking TGFβ activity by synthetic peptide reduces fibrosis in mice in carbon tetrachloride (CCL₄)-induced liver fibrosis, and bleomycin-induced skin and lung fibrosis (41, 63). Although inhibitors of TGFβ1 are effective in short-term animal models, they are not suitable for long term therapy because of the significant role of TGFβ1 in homeostasis and repair (47).

Promotion of matrix degradation. Remodeling of ECM occurs through the action of MMPs that efficiently cleave collagens and other matrix components, and the TIMPs, critical determinants of fibrosis reversal, which inhibit their activity. During the regression, decreased TIMP-1 levels are associated with clearance of activated HSCs through apoptosis (64). Fibrinolysis and matrix rearrangement can be induced by a number of agents, as shown by studies in animal models of liver fibrosis. For example, direct adenoviral administration of Mmp1 mRNA attenuated established liver fibrosis in thioacetamide treated rats, while administration of an anti-TIMP1 antibody decreased HSCs activation and attenuated fibrosis in a CCl₄ rat model (65, 66). Downregulation of TIMPs by the reproductive hormone relaxin also inhibits liver fibrosis in vitro (67). Furthermore, adenoviral delivery of urokinase-type plasminogen activator (uPA), which initiates the matrix proteolysis and upregulates hepatocyte growth factor (HGF) (68), results in enhanced collagenase activity, fibrosis regression and hepatocyte regeneration (69). MMPs gene delivery in advanced liver fibrosis shows that even transient shifts in the imbalance between MMPs and TIMPs are sufficient for the resolution - if the underlying causative stimuli have been successfully removed (70). Dietary supplementation with zinc, an essential co-factor for MMP activity, has also been shown to promote collagen degradation in CCl₄-injured rats (71). Collagen synthesis is also sensitive to changes in food intake, and malnutrition may have profound effects on its production (72).

Stimulation of HSCs apoptosis. Apoptosis is a key event in the spontaneous recovery from liver fibrosis (73) and can be induced by two general mechanisms (34). The first, "extrinsic" pathway, works via stimulation of specific cell surface death receptors, which trigger an intracellular cascade of caspases, ensuing cellular apoptosis. Second, "intrinsic" pathway, operates at the level of the mitochondria. Stimuli like toxins, UV radiation, and reactive oxygen species stimulate the proapoptotic factors in the mitochondrial membrane, resulting in leakage of cytochrome C into the cytosol, caspase activation, and apoptosis. In addition, upon activation of HSCs, the expression of a cell surface death receptors CD95 (APO-1/Fas) and CD95L (APO-1/Fas-ligand) increases, which may explain the increased apoptosis seen upon their activation in vitro and in vivo (74). Also, activation of TNFα receptor, and low-affinity nerve growth factor (NGF) receptor (p75) increases HSCs apoptosis in vitro (75). Transformed HSCs also express peripheral benzodiazepine receptors, which render the cells sensitive to peripheral benzodiazepine receptor-ligand-induced apoptosis (76). NFκB, on the other hand, protects HSCs from apoptosis, and its inhibition with gliotoxin accelerates recovery from drug-induced liver fibrosis in rats (77), proving that HSCs pro-apoptotic agents could abrogate liver fibrosis. The approaches mentioned in this section may sound attractive for in vitro work, but the selectivity may be too low to use this approach in vivo.

Targeted antifibrotic therapy. Wound healing is an important universal process, hence liver-specific targeting of antifibrotic therapies minimizes the potential harmful effects on other tissues. Furthermore the extensive first pass metabolism makes liver an attractive target for directed therapy with orally administered drugs. Still, hepatic delivery needs to be optimized via cell-specific
targeting (of hepatocytes, Kupffer cells, or HSCs). It has been shown, for instance, that administration of recombinant insulin-like growth factor 1 (rIGF1) or HSCs-targeted induction of its expression using a viral vector in CCl₄-treated mice and rats, significantly reduced liver fibrosis (78-80). Treatment of cirrhotic rats with rIGF1 promotes weight gain, nitrogen retention, and intestinal absorption of nutrients and was able to exert hepatoprotective effect (78, 81). Concerns regarding the safety of gene therapy with regard to genotoxicity and effects on the immune system remain. However, with improved delivery techniques and the conditional expression systems, the anti-fibrotic trials in humans are no longer a matter of (distant) future.

**IGF AXIS IN LIVER FIBROSIS**

*Insulin-like growth factor 1* (IGF1) is a potent cytoprotective and anabolic hormone, synthesized mainly in the liver. It circulates bound to a set of binding proteins (IGFBPs) that prolong its half-life and, by modulating its interaction with the IGF1 receptor (IGF1R), control its biological activity (82). Detachment from IGFBPs releases free IGF1, which can bind to and activate the IGF1R. Interaction with IGF1R leads to activation of mitogen activated protein (MAP) kinase and phosphatidylinositol 3-kinases (PI3) cascades that regulate genes involved in cell survival, growth, and differentiation (83). In advanced liver fibrosis, the IGF1 axis is severely impaired mostly due to a reduced number of healthy, IGF1 producing hepatocytes. The decrease in IGF1 signalling can disturb systemic metabolism (e.g. in liver cirrhosis), and provide a pro-fibrotic environment, since the progression of liver fibrosis could be delayed by IGF1 administration (79, 84).

As mentioned, the action of IGF1 depends on six IGFBPs of multifunctional nature, which can act both via IGF-dependent and independent mechanisms. Among them, *insulin-like growth factor binding protein 5* (IGFBP5) that binds to IGFs with high affinity (85) is strongly induced in the fibrotic livers of Abcb4⁻/⁻ mice (86), suggesting a potential role in the pathogenesis of chronic cholangiopathy. Its expression is also high in skin and lung fibrosis (SSc and IPF; (87, 88)). IGFBP5 induces collagen and fibronectin production in fibroblasts, and their transdifferentiation to myofibroblast *in vitro* and *in vivo* (89, 90). In IGFBP5-induced dermal fibrosis, an increase in the number of fibroblasts expressing proliferating cell nuclear antigen PCNA is accompanied by increased vimentin and α-SMA expression (suggesting an IGFBP5-induced myofibroblast differentiation). This leads to an excessive production of ECM and the development of fibrosis.

IGFBP5 seems to play an important role in other aspects of fibrosis, such as *epithelial injury*. IGFBP5 expression is dramatically increased in the involuting mammary gland and prostate during apoptosis, and in the brain after ischaemia (91, 92). IGFBP5 induces *epithelial–mesenchymal transition* in pulmonary epithelial cells *in vitro* (90). *Senescence*, a process in which cells lose the ability to proliferate, is also associated with increased IGFBP5 expression in human dermal fibroblasts and endothelial cells (93, 94). A knockdown of IGFBP5 partially reverses, whereas its induction causes a premature senescence in human umbilical-vein endothelial cells (95).
LIVER FIBROSIS AND DIET

As the prevalence of obesity is rapidly increasing, the prevalence of NASH is also on the rise. NASH has been recognized as a major cause of liver fibrosis (96), ranging from steatosis to cirrhosis, eventually leading to HCC. NASH is a component of metabolic syndrome and type 2 diabetes, characterized by obesity, dyslipidemia and insulin resistance (97). Insulin resistance is a prominent mechanism that links steatosis and fibrogenesis (98), hence adipokines that play a role in insulin resistance but also steatosis and inflammation are considered to be involved in liver fibrosis. Fibrogenic action of insulin itself works via the release of TGFβ1, which stimulates type 1 collagen production in HSC (99). Leptin promotes stellate cell mitogenesis and cell survival, two seminal events that promote liver fibrosis (100). In contrast, administration of recombinant adiponectin and its agonists reduced steatosis, attenuated inflammation and the elevated levels of serum alanine aminotransferase (ALT) in (non)alcoholic fatty liver diseases in mice, indicating potential clinical application (101, 102).

Treatment options in obesity-linked liver fibrosis are limited so lifestyle changes like introducing exercise and healthy diet are advised. Weight loss in morbidly obese patients is associated with a reduction in the prevalence of hepatic fibrosis (103). In a mouse model of metabolic syndrome, stimulation of weight loss by blocking the endocannabinoid receptor CB1 prevents progression of fibrosis (104). Still, beneficial effects of insulin sensitizers on progression of liver fibrosis need to be documented in therapeutic trials.

MODELS OF LIVER FIBROSIS

Although fibrosis occurs as a part of normal wound healing, excessive (dysregulated) deposition of ECM leads to severe organ dysfunction and is a feature of a variety of diseases. Due to its insidious onset, fibrosis tends to go undetected in its early stages. This is, in part, why these diseases remain so poorly understood. To improve our understanding of development and reversal of liver fibrosis, a number of experimental models have been established.

The first are in vitro models - purified primary cells from normal or experimentally injured livers, or stable cell lines representative of specific hepatic cell types. These models enhance the understanding of the molecular pathogenesis of liver fibrosis (15), and permit high-throughput testing and improvement of potential antifibrotic agents, thanks to low cost and high reproducibility. However, they cannot recapitulate the in vivo situation that involves the complex interplay of resident and incoming cells in a dynamic microenvironment.

The alternative are the ex vivo models - human hepatic tissue obtained from biopsies, resections or explants. This type of models is vital for validating observations made in animal and cell culture models (105). However, ethical reasons prevent multiple liver biopsies in humans, resulting in limited amount of data, usually at the advanced end of the disease.

In vivo experimental animal models of fibrosis are used to address the limitations of the other two types, and to facilitate the study of a highly dynamic process (106, 107). In spite their questionable relevance to human disease, they allow consecutive sampling of tissue in the volumes required for detailed studies of cellular and molecular pathogenesis. These models provide a chance
to examine the early stages of fibrosis and changes in signaling pathways, chemokines, and cytokines. The two most frequently used models of experimental fibrosis are repetitive toxic damage by carbon tetrachloride (CCL\textsubscript{4}) and bile duct ligation (BDL) \cite{107}. CCL\textsubscript{4} induces zone III necrosis, hepatocyte apoptosis and, with iterative dosing, can be used to generate bridging hepatic fibrosis, early cirrhosis and advanced micronodular cirrhosis (4, 8 and 12 weeks of twice weekly dosing, respectively) \cite{73, 108}. BDL stimulates the proliferation of biliary epithelial cells and oval cells (hepatocyte progenitors), resulting in proliferating bile ductules and an accompanying portal inflammation and fibrosis. Fibrosis can also be established using CCL\textsubscript{4} or BDL in specific transgenic mice (over-expressing pro- or anti-fibrotic factors) and knock-out mice (in which the expression of a pro or anti fibrotic factor is abolished) \cite{79, 109}. This allows addressing the molecular mechanisms and/or importance of these factors in hepatic functions and diseases.

\textit{In vitro} and \textit{in vivo} models used in this thesis will be described in more detail in the coming paragraphs. The LX2 cell and MFs were used to study the mechanism of action of IGFBP5 in (activated) HSCs. LX2 human HSCs lines are generated by spontaneous immortalisation in low serum culture conditions. They retain key features of HSCs, like expression of \(\alpha\)-SMA, vimentin, and glial fibrillary acid protein. Similarly to primary HSCs, LX2 cells express key receptors regulating liver fibrosis, like platelet derived growth factor receptor \(\beta\) (PDGF\(\beta\)-R), as well as proteins involved in matrix remodelling; MMP2, TIMP1 and 2 \cite{110}.

\textit{Abcb4\textsuperscript{-/-}} mice used in this thesis are a well characterized non-surgical mouse model for chronic cholangiopathies. These mice deficient in the canaliculard phospholipid flippase are created on FVB background (FVB.129P2-Abcb4tm1Bor; \cite{111}). They are characterized by a lack of Mdr2 P-glycoprotein (multidrug resistance transporter 2, Abcb4), a phosphatidylcholine transporter. This results in absent phospholipid secretion into bile and spontaneous bile duct injury, with macroscopic and microscopic features closely resembling human sclerosing cholangitis (PSC) \cite{112}. These animals are an animal model for human MDR3 deficiency (progressive familial intrahepatic cholestasis type 3) which progresses to liver cirrhosis \cite{113}. The hepatic pathogenesis in \textit{Abcb4\textsuperscript{-/-}} mice include disruption of tight junctions and basement membranes of bile ducts, and bile leakage to the portal tract. This triggers a multistep process that upregulates fibrogenesis and downregulates fibrinolysis, leading to the formation of peripoortal biliary fibrosis \cite{114}, which makes these mice an attractive model for liver fibrosis. As a consequence of chronic inflammation and progressing fibrosis, \textit{Abcb4\textsuperscript{-/-}} mice develop HCC within 12 to 15 months of age \cite{115}, and, at later age, they serve as a model for studying HCC. The effect of genetic and environmental factors on the development of liver fibrosis was studied in this thesis by exposing these mice either to liver specific overexpression of genes (belonging to IGF axis), or to metabolic challenges (like food deprivation).
OUTLINE OF THE THESIS

The aim of the research described in this thesis was to identify novel approaches and therapeutic targets for the treatment of liver fibrosis. The initial experiments were based on our previous finding that IGFBP5 was strongly upregulated upon HSC activation and transdifferentiation into MFs in vitro and in vivo (86). In Chapter 2, we therefore investigated the role of IGFBP5 in HSCs using gain- and loss-of-function approaches. We used the human LX2 cell line (110) that recapitulates many features of the activated HSCs phenotype. To assess its role in more advanced stages of fibrosis, the response to IGFBP5 was also dissected in human primary liver MFs. To clarify the role of IGFBP5 in the pathogenesis of liver fibrosis caused by chronic cholangiopathies (Chapter 3), we overexpressed this gene in hepatocytes of Abcb4−/− mice using an adeno-associated viral (AAV) vector large amounts of IGFBP5 were delivered to the hepatic tissue including HSCs. To substantiate the findings in the Abcb4−/− model and relate them to the physiological role of hepatic IGFBP5 expression, a systems genetics approach was applied to a mouse genetic reference population, using an open source database. Since the biliary epithelium expresses a receptor for IGF1 (116), and insulin-like growth factor 1 (IGF1) protects cholangiocytes against cholestatic injury in vitro (117), overexpression of IGF1 could be beneficial in cholestatic liver diseases. Therefore, in Chapter 4 we aimed to study if a prolonged increase of hepatic IGF1 expression in a model for chronic cholangiopathy, the Abcb4−/− mouse, was beneficial. Using a rat IGF1 gene behind an α-Sma promoter the expression of this growth factor was enhanced in activated HSCs.

Intermittent fasting and caloric restriction have beneficial effect on health status of laboratory animals (118). In NASH and NAFLD patients, histological improvements of liver pathology positively correlate with the size of weight loss (119-121), but the mechanism is not fully understood. Weight loss has beneficial effects on fibrosis induced by nutrient overload in men and mice (103, 104). We therefore postulated that the complex adaptive response to nutrient deprivation would also benefit the fibrotic pathology in cholestatic liver injury. In Chapter 5 we therefore tested the hypothesis that food deprivation could positively influence pathology in fibrotic Abcb4−/− mouse liver. Finally, in Chapter 6 the work described in this thesis is discussed, and future perspectives are given.
References


chapter 1


