Chapter 1

Introduction
1 Liver fibrosis

1.1 PATHOLOGY
Liver fibrosis is the progressive accumulation of connective tissue in the liver, which can be caused by chronic liver injury of various etiologies. The most common causes of liver fibrosis are infection with hepatitis B or C virus, metabolic disorders, alcohol abuse, schistosomiasis infection, and autoimmune diseases [1]. Liver fibrosis will eventually lead to the development of liver failure and is one of the top ten causes of death in the Western world. Patients with liver fibrosis can be asymptomatic for 15-20 years with morbidity and mortality only occurring after progression to cirrhosis, the lethal end-stage of liver fibrosis. Once clinical complications, which include liver and kidney failure, portal hypertension, variceal hemorrhage, ascites, and encephalopathy, become overt, the prognosis for patients is poor, with a 5-year survival of approximately 50%. Besides, cirrhosis is a risk factor for developing hepatocellular carcinoma [1, 2]. The poor outcome for patients with fibrosis is at least partially caused by the lack of a treatment that can prevent its progression. The most effective therapy for liver fibrosis is removal of the underlying cause. However, in many cases this cannot be achieved adequately and other means to treat liver fibrosis are required. Unfortunately, to date no effective anti-fibrotic drugs are available. Some patients benefit from anti-inflammatory drugs, from anti-oxidants, or from treatment of secondary symptoms, however, for most patients with liver fibrosis the only curative treatment is a liver transplantation.

1.2 LIVER FIBROSIS ON CELLULAR LEVEL
In general, liver fibrosis can be regarded as a chronic wound healing process, characterized by increased deposition of connective tissue. On a cellular level, the hepatic stellate cell is considered to be the key player in this process. In normal liver, stellate cells encompass approximately 5-8% of the total cell population. Their main functions are the uptake, storage, and release of retinoids, the regulation of sinusoidal blood flow, and the synthesis and degradation of extracellular matrix [3]. Stellate cells are the most important producers of extracellular matrix. During liver fibrosis stellate cells get activated and their number drastically increases due to proliferation of activated cells. Activated hepatic stellate cells are mainly responsible for the increased deposition of scar tissue. However, as discussed below, also other (myo)fibroblast populations as well as immune cells play a role in the development of liver fibrosis. Because the activation of hepatic stellate cells activates a key role in the development of liver fibrosis, the processes underlying this activation have been studied extensively. The activation of stellate cells can be divided in an initiation and a perpetuation phase [1, 4]. Triggers from neighboring cells in response to injury mediate the initiation of stellate cell activation. These triggers encompass reactive oxygen species, inflammatory cytokines, and/or growth factors and can be produced by hepatocytes, Kupffer cells, or sinusoidal endothelial cells. In addition, changes in extracellular matrix composition may play a role in this process. Although the exact role of many of these factors in the initiation of stellate cell activation is not elucidated completely yet, eventually several transcription factors within stellate cells are activated or inhibited and consequently a
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Phenotypic transformation into activated, myofibroblast-like cells takes place. During the subsequent perpetuation phase the phenotypic changes of stellate cells in combination with paracrine and autocrine stimuli result in proliferation, fibrogenesis, chemotaxis, and increased contractility of the activated stellate cells. Of the different signaling molecules involved in these processes, transforming growth factor β (TGFβ) is considered to play the key role in hepatic stellate cell activation and fibrogenesis, whereas platelet derived growth factor (PDGF) is the most potent inducer of stellate cell proliferation and plays an important role in the perpetuation of hepatic stellate cell activation. Chemotaxis of hepatic stellate cells and their subsequent migration to the area of injury is mediated by several chemoattractants among which PDGF and endothelin. The increased contractility of hepatic stellate cells is mediated via endothelin and other vasoactive compounds [5].

Once activated, hepatic stellate cells transform into myofibroblasts and start proliferating. Myofibroblasts are responsible for the production of extracellular matrix during wound healing as well as during the development of fibrosis. Although activated hepatic stellate cells contribute significantly to the increasing amount of myofibroblasts present in the liver during the development of fibrosis, the (myo)fibroblast population involved in the fibrotic process is likely more heterogeneous. Indeed, not only hepatic stellate cells but also portal fibroblasts and possibly second-layer cells located around centrolobular veins and smooth muscle cells can transform into myofibroblasts. In addition, bone marrow derived myofibroblasts may participate in the development of fibrosis [6]. The involvement of the different subpopulations of (myo)fibroblasts may depend on the type of liver injury and the time after injury. In addition, besides activation of hepatic stellate cells and/or other fibrogenic cells, injury to the liver also results in the attraction of different types of immune cells into the liver, and in the activation of Kupffer cells, the resident liver macrophages. The immune cells infiltrating in the liver can include B and T lymphocytes, natural killer cells, and polymorphonuclear leukocytes, among which the precursors of macrophages. The specific composition of this cellular infiltrate may vary with the type and stage of liver injury. The recruitment and activation of inflammatory cells and their subsequent secretion of inflammatory cytokines can further activate hepatic stellate cells. In addition reactive oxygen species and proteases are released, which can result in increased cell damage [7]. The exact role of immune cells in the development of liver fibrosis, however, is still largely unknown [8, 9].

Thus, in response to liver injury of any etiology, hepatic stellate cells and/or other fibrogenic cells are activated and excess extracellular matrix, or scar tissue, is produced. Besides, immune cells are recruited and local Kupffer cells activated, resulting in inflammation, which can further promote the fibrotic process. The increased production of scar tissue is caused by increased production as well as decreased breakdown of matrix proteins. As mentioned, the increased deposition of extracellular matrix is mainly caused by the increased production of matrix proteins by activated stellate cells. Decreased breakdown of matrix proteins is mediated by alterations in the expression levels of metalloproteinases and their inhibitors [10]. Together, these processes result not only in accumulation of extracellular matrix and loss of remaining
hepatocyte function, but also in profound changes in the matrix protein composition, which in turn can further promote the activation and proliferation of stellate cells and thus contribute to the progression of fibrosis [11]. The extent of fibrosis and inflammation depends on the type and duration of the liver injury. When the injury is acute, the fibrogenic response is taken over by regeneration, with replacement of necrotic and apoptotic cells, removal of scar tissue, and resolution of inflammation. However, when injury is sustained, the regeneration process is insufficient and liver tissue is gradually replaced by extracellular matrix [1, 2, 4]. As this process progresses, morbidity and mortality due to disease-related complications will increase unless the causal factor is removed.

2 Treatment of liver fibrosis
Liver fibrosis and its end-stage liver cirrhosis are considered to be reversible [1]. Several studies in experimental animal models for liver fibrosis have shown that after removal of the fibrotic stimulus, spontaneous resolution of fibrosis can occur [12-14]. Similar observations were made in patients with liver fibrosis [15-17]. Thus, ideally, to treat liver fibrosis one should remove the initial stimulus, i.e. the factors that cause the chronic injury to liver cells that in turn triggers the activation of hepatic stellate cells and their production of excess extracellular matrix. However, in many cases such factors cannot be adequately removed and other means to treat liver fibrosis are required. As mentioned, to date there is no curative treatment for liver fibrosis available except liver transplantation. For this reason, extensive research is ongoing to develop new anti-fibrotic drugs. This has resulted in the discovery of several potential anti-fibrotic compounds, of which the three main categories with respect to their mechanism of action will be discussed here briefly. Pinzani et al. [18] recently provided a rather complete overview of potential anti-fibrotic agents and strategies.

2.1 INHIBITION OF HEPATIC STELLATE CELL ACTIVATION
Since activated stellate cells are considered to be the key mediator in the development of liver fibrosis, most research on the development of anti-fibrotic therapies is focused on these cells. The compounds that initially received most attention for their potential to inhibit activated stellate cells were those that interfere with the two main signaling pathways involved in proliferation and fibrogenesis: the PDGF signaling pathway and the TGFβ pathway, respectively. Of the several compounds known to inhibit PDGF signaling by directly interfering with receptor phosphorylation or downstream signaling pathways, pentoxifylline and gleevec are the only ones tested in experimental animal models for liver fibrosis, both showing anti-fibrotic effects [19-23]. In addition, a soluble PDGF receptor [24] and adenoviral expression of antisense PDGF mRNA [25] also inhibited the progression of hepatic fibrosis in vivo. Strategies to inhibit TGFβ signaling that were successful in animal models for liver fibrosis include adenoviral expression of antisense TGFβ1 mRNA, Smad7, or soluble TGFβ receptor [26-29], administration of a soluble TGFβ receptor [30, 31], and inhibition of TGFβ receptor phosphorylation [32]. Besides these two pathways, more recently also the endothelin
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signaling pathway and the angiotensin II signaling pathway were addressed as potential targets to inhibit activation or proliferation of stellate cells. Both angiotensin II and endothelin mainly regulate the contractility of hepatic stellate cells, but also play a role in the stimulation of hepatic stellate cell proliferation and migration, and in fibrogenesis [4, 5]. Inhibition of their signaling pathways using endothelin receptor antagonists [33-35], several angiotensin-converting enzyme inhibitors [36-38], and an angiotensin II receptor antagonist [39-41] was proven to be a successful anti-fibrotic therapy in experimental models for liver fibrosis. Other methods that are used to inhibit stellate cell activation or proliferation that were shown to be effective in animal models, are apoptosis induction [42, 43], stimulation of the transcription factor peroxisomal proliferator-activated receptor (PPAR), which is suggested to be important for maintaining a quiescent phenotype [4, 44, 45], and inhibition of the sodium proton exchanger, which plays a role in cell proliferation [46, 47]. Finally, to increase the drug concentration at the target site and reduce unwanted side effects, approaches are used to selectively target drugs to the hepatic stellate cells [48-50].

2.2 INHIBITION OF INFLAMMATION
Liver fibrosis is often accompanied by inflammation, which promotes the progression of the fibrotic process. Thus, inhibition of the inflammatory response is also regarded as a potential treatment for liver fibrosis. Suppression of the immune system as a treatment for liver fibrosis is especially successful in patients with autoimmune related fibrosis [51, 52]. Studies in experimental animal models for fibrosis however suggest that this treatment is ineffective in other types fibrosis [53, 54]. Local or specific inhibition rather than systemic suppression of the immune system appears to be a more promising approach to treat liver fibrosis in these cases. For instance, gadolinium chloride inhibits liver fibrosis in several experimental animal models both by a 75-80% depletion of the Kupffer cell and macrophage population [55-59], and by the induction of metalloproteinase production by Kupffer cells [57, 58]. Similarly, inhibitors of 5-lipoxygenase, which in the liver specifically induce growth arrest and apoptosis in Kupffer cells, had anti-fibrotic effects in vivo [60]. Finally, some of the strategies used to inhibit stellate cell activation are also useful in inhibiting inflammation in the liver. For instance, stimulation of PPAR results in a decreased expression of monocyte chemotactic protein 1 by hepatic stellate cells, thus reducing macrophage infiltration [61]. In addition, PPAR stimulation can directly reduce inflammation by inducing apoptosis in macrophages [62], and inhibiting cytokine expression and neutrophil infiltration [63], and was shown to reduce liver inflammation during fibrosis in rats [64]. Similarly, besides its inhibitory effects on stellate cell proliferation, pentoxifylline also inhibits pro-inflammatory cytokine expression [65], which may reduce the inflammatory response during liver fibrosis.

2.3 INHIBITION OF EXTRACELLULAR MATRIX ACCUMULATION
The removal of the excess extracellular matrix that is produced during the progression of liver fibrosis is also an important aspect in its treatment. Several strategies have been applied to reduce accumulation of extracellular matrix focusing on the synthesis and
breakdown of matrix proteins. Synthesis of collagen can be inhibited by prolyl-4-hydroxylase inhibition and by halofuginone, which both successfully inhibited experimental fibrosis in several animal models [66-70]. Remarkably, however, halofuginone treatment worsened fibrosis induced by bile-duct ligation in rats [71]. Increased breakdown of collagen was achieved by the administration of antibodies directed against tissue inhibitor of metalloproteinase-1 (TIMP-1) [72] and by adenoviral expression of metalloproteinases, resulting in reduction of fibrosis in vivo [73-75]. Although the main mechanism of action of these compounds is the inhibition of collagen synthesis, also inhibitory effects on hepatic stellate cell activation were reported [66, 69, 72]. It was suggested that these effects might be indirect [69, 72], because some collagens are known to induce hepatic stellate cell activation [76], and TIMP-1 can inhibit apoptosis of hepatic stellate cells [77]. Thus the removal of collagens or the inhibition of TIMP-1 expression may have the additional benefit of removing a stellate cell activation or survival stimulus, respectively. The other way around, the inhibition of hepatic stellate cell activation also results in decreased deposition of collagens, since these cells are the most important producers.

2.4 PATIENTS

Although the above-mentioned approaches were shown to be successful in several in vivo and in vitro models for fibrosis, studies showing the effectiveness in human patients are still limited. Conducting clinical trials to proof efficacy of anti-fibrotic drugs in patients is difficult and expensive. Long-term prospective studies with appropriate end-points are necessary as it is expected that in most patients regression of fibrosis can only be measured adequately after several years [18]. Besides, pre- and post-treatment biopsies will be required to evaluate the progression of fibrosis in patients. This is an invasive, painful method, which is highly susceptible to sampling error. Thus, novel diagnostic measurements might be needed to assess efficacy of anti-fibrotic drugs more efficiently in patients [78].

Thus far, only a small number of the anti-fibrotic drugs described above has been tested for anti-fibrotic effects in patients with liver fibrosis. With respect to the inhibition of stellate cell activation, the angiotensin II antagonist losartan, and PPAR agonists were tested. Losartan reduced fibrosis in patients with non-alcoholic steatohepatitis (NASH), which is characterized by steatosis, inflammation, and fibrosis of the liver [79]. Besides, it was suggested that treatment with losartan may have beneficial effects in patients with hepatitis C infection, both in the early stages of hepatic fibrosis [80] and in graft fibrosis during hepatitis C recurrence after liver transplantation [81]. A drawback of the use of angiotensin II inhibitors for the treatment of liver fibrosis, however, may be its blood pressure lowering effect, which is unwanted in normotensive patients. PPAR agonists have been shown to successfully inhibit liver fibrosis in patients with NASH [82]. However, it should be noted that although these agonists are known to influence hepatic stellate cell activation, it is likely that the beneficial effect on liver fibrosis that is observed in these patients is mainly mediated by influencing the cause of liver fibrosis rather than by inhibition of the fibrotic process itself. With respect to the inhibition of inflammation in order to treat liver fibrosis, immune suppression by means of corticosteroid treatment is an effective anti-fibrotic therapy in patients with
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autoimmune-related fibrosis [83], with only a small population of patients that do not benefit from this treatment [84]. However, corticosteroid treatment was ineffective in patients with alcohol-induced fibrosis [85] and in patients with primary sclerosing cholangitis or primary biliary cirrhosis [86, 87], in whom the underlying cause of fibrosis is not, or only partly due to autoimmunity. Inhibition of extracellular matrix accumulation as a strategy to treat liver fibrosis has not been tested in patients.

3 Experimental models for fibrosis

3.1 IN VIVO ANIMAL MODELS
Several in vivo animal models for liver fibrosis are used, which are designed to mimic different causes of the development of liver fibrosis in humans as close as possible. Of these models, the four most commonly used are discussed here briefly. Firstly, as a model for toxicity-induced chronic liver injury progressing to fibrosis and cirrhosis, administration of several toxic compounds is used, including carbon tetrachloride [88], dimethylnitrosamine [89, 90], galactosamine [91], thioacetamide [92], and ethanol [93]. In addition, liver fibrosis caused by the toxic effects of the accumulation of metals like copper and iron is also mimicked in experimental animal models, mostly by administration of cupric- or ferric-nitrilo-triacetate [94, 95]. In all cases, chronic administration of these hepatotoxins causes fibrosis, which in some cases is accompanied by ascites, bile-duct proliferation and/or portal hypertension. Secondly, occlusion of the bile duct in experimental animals is often applied to mimic cholestasis-induced liver fibrosis [96]. Fibrosis in this model is thought to be caused by accumulation of bile acids in the liver, and is characterized by increased proliferation of bile-duct epithelial cells [97]. Thirdly, infection with Schistosoma can be used to induce liver fibrosis. This model closely resembles Schistosoma infection in man, which occurs regularly worldwide and is a common cause of liver fibrosis. Fibrosis development in this model results from an immune response to living Schistosoma eggs in portal venules, resulting in peri-portal fibrosis [98]. Finally, a methionine or methionine/choline deficient diet can be administered to experimental animals to induce a condition that is pathological similar to metabolic steatohepatitis, or fatty liver disease in human patients [99]. This model is characterized by fibrosis, inflammation, and increased lipid content of the liver. More recently also several transgenic animal models are developed in which genes are either over-expressed [100-103] or knocked-out [104-107] to further study the mechanisms underlying the development of fibrosis, and the involvement of specific genes.

The use of in vivo models for the study of liver fibrosis has several advantages over currently available in vitro models. Firstly, in vivo models can incorporate the effects of the extracellular milieu during hepatic stellate cell activation and fibrogenesis. In addition, these models allow for the inclusion of possible effects of the immune system, the central nervous system, and other organs in the development of liver fibrosis. Importantly, the course of fibrosis and the effects of anti-fibrotic compounds can be studied for different types of fibrosis, with different underlying causes, which closely mimic the different causes of fibrosis development in human patients. An important
disadvantage of experimental animal models however is that these models are largely restricted to rodents and may be of limited predictive value for human disease due to possible interspecies differences. In addition, *in vivo* models give high discomfort to the experimental animals and should therefore be avoided when possible.

### 3.2. IN VITRO MODELS

**Primary cells**

The study of hepatic fibrosis *in vitro* is mainly focused on the hepatic stellate cell, the key player in the fibrotic process. The first approach used to study hepatic stellate cell activation *in vitro* was the use of primary isolated cells. Liver cells can be isolated using enzymatic digestion of the liver and subsequent density gradient centrifugation and/or centrifugal elutriation, which separates the different cell types. Using this method, however, the obtained stellate cell fractions are still contaminated by other liver cell types and cell debris [108, 109]. Further purification requires culturing for 1-3 days and subsequent removal of contaminants. The purity of primary hepatic stellate cell cultures can be further increased by pronase treatment prior to separation of the cells to selectively kill hepatocytes, by using side-scattering cell sorting instead of density gradient centrifugation [109], or by selective killing of Kupffer cells *in vivo* [110] resulting in cell purities of >95%. However, none of these methods can completely prevent contamination of the cell fractions by other liver cell types.

Primary isolated cells closely resemble their *in vivo* state directly after isolation and offer an important tool to study hepatic stellate cell activation *in vitro*. However, their phenotype is depending on the culture conditions, as will be discussed later. In addition to primary isolated cells, cell lines have been developed for the research of stellate cell biology. The major reason for this development is a practical one. Isolation procedures for primary hepatic stellate cells are time-consuming, yields are relatively low, considerable variation between the different batches exists, and the possibility to subculture primary hepatic stellate cells is limited. In addition, human liver tissue suitable for the isolation of stellate cells is scarce.

**Cell lines**

Current available hepatic stellate cell cell lines and their characteristics are listed in table 1. The first method used to generate these cell lines was selection of spontaneous immortalized primary cells in culture. Using this method, several cell lines were developed from primary stellate cells or myofibroblasts isolated from normal or pathological human or rodent liver tissue. A second method used to develop cell lines is genetic manipulation to immortalize stellate cells in culture. In general these cell lines are derived from primary hepatic stellate cells isolated from normal human or rodent liver tissue, with the exception of TWNT-1, which was derived from the L90 cell line. Genetically induced immortalization can be achieved by transfection with the large T antigen of simian virus 40 or polyoma virus [111-114]. In addition, stabilization of the telomere length by transfection with human telomerase reverse transcriptase can extend the life span of primary hepatic stellate cells or cell lines [115, 116].
Table 1. Characteristics of cell lines used for the study of hepatic stellate cells and fibrosis.
HSC - hepatic stellate cells; MFB - myofibroblasts, CCl4 - carbon tetrachloride, SV40 - Simian virus 40, n.r. - not reported

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Species</th>
<th>Source</th>
<th>Method</th>
<th>Retinoid metabolism</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRX</td>
<td>Mouse</td>
<td>MFB from fibrotic lesions induced by schistosomiasis</td>
<td>Spontaneous immortalization</td>
<td>Inducible</td>
<td>[117, 125-128]</td>
</tr>
<tr>
<td>CFSC</td>
<td>Rat</td>
<td>Primary HSC from CCl4-treated cirrhotic liver</td>
<td>Spontaneous immortalization</td>
<td>n.r.</td>
<td>[129, 130]</td>
</tr>
<tr>
<td>NFSC</td>
<td>Rat</td>
<td>Primary HSC from normal liver</td>
<td>Spontaneous immortalization</td>
<td>n.r.</td>
<td>[129]</td>
</tr>
<tr>
<td>L90</td>
<td>Human</td>
<td>Hepatic mesenchymal tumor</td>
<td>Spontaneous immortalization</td>
<td>Inducible</td>
<td>[118, 124]</td>
</tr>
<tr>
<td>GREF-X</td>
<td>Human</td>
<td>Primary MFB from normal liver</td>
<td>Transformation with polyoma virus large T antigen</td>
<td>n.r.</td>
<td>[111]</td>
</tr>
<tr>
<td>A640-IS</td>
<td>Mouse</td>
<td>Primary HSC from normal liver</td>
<td>Transformation with temperature-sensitive SV40 large T antigen</td>
<td>n.r.</td>
<td>[113]</td>
</tr>
<tr>
<td>HSC-T6</td>
<td>Rat</td>
<td>Primary HSC from normal liver</td>
<td>Transformation with SV40 large T antigen</td>
<td>Inducible</td>
<td>[114, 120]</td>
</tr>
<tr>
<td>hTERT-HSC</td>
<td>Human</td>
<td>Primary HSC from normal liver</td>
<td>Retrovirual transfer of human telomerase reverse transcriptase</td>
<td>n.r.</td>
<td>[115]</td>
</tr>
<tr>
<td>PAV-1</td>
<td>Rat</td>
<td>Primary HSC from normal liver</td>
<td>Spontaneous immortalization</td>
<td>n.r.</td>
<td>[119]</td>
</tr>
<tr>
<td>MG-2</td>
<td>Rat</td>
<td>Primary HSC from normal liver</td>
<td>Spontaneous immortalization</td>
<td>n.r.</td>
<td>[131]</td>
</tr>
<tr>
<td>TWNT-1</td>
<td>Human</td>
<td>L90-cells</td>
<td>Retrovirual transfer of human telomerase reverse transcriptase</td>
<td>n.r.</td>
<td>[116]</td>
</tr>
<tr>
<td>LX1</td>
<td>Human</td>
<td>Primary HSC from normal liver</td>
<td>Transformation with SV40 large T antigen</td>
<td>Inducible</td>
<td>[112]</td>
</tr>
<tr>
<td>LX2</td>
<td>Human</td>
<td>Primary HSC from normal liver</td>
<td>Low serum selection pressure of LX-1 cells</td>
<td>Inducible</td>
<td>[112]</td>
</tr>
</tbody>
</table>
All hepatic stellate cell cell lines have an activated phenotype with a fibroblast-like appearance, but many also possess retinoid related parameters and/or inducible vitamin A storage, which are characteristics of the fat-storing phenotype of stellate cells. The fat-storing phenotype of hepatic stellate cells in vivo is considered as a quiescent phenotype, however, in the case of cell lines this is less well established. It was suggested that the fat-storing phenotype of these cell lines is either an alternative state of stellate cell activation [123] or uncoupled to the activation state of the cells [120]. Therefore, when using hepatic stellate cell cell lines it is important to accurately establish their cellular and molecular features. Several cell lines have been characterized for their applicability for the study of retinoid metabolism [119, 120, 124, 125], but only LX cells have been extensively characterized with respect to stellate cell activation and fibrogenesis related parameters [112]. The absence of thorough characterization of a cell line concerning its cellular and molecular features makes interpretation of experimental results difficult. It should be taken into consideration that the process of immortalization and culturing of cell lines most likely affects multiple cellular and molecular functions. The use of cell lines for the study of stellate cell activation and fibrogenesis can therefore at best give an indication of the responses of hepatic stellate cells in vivo.

Culture matrices

It is well known that freshly isolated hepatic stellate cells cultured on uncoated plastic spontaneously activate. In addition, as discussed above, hepatic stellate cell cell lines in general have an activated rather than a quiescent phenotype. Although further stimulation of these cells can still be achieved, this spontaneous activation makes it difficult to analyze stellate cell activation induced by fibrogenic cytokines, drugs, or toxic compounds. Therefore, to prevent the activation of hepatic stellate cells during culture, several culture matrices have been developed. Morphological, quiescent and activated stellate cells can be easily distinguished. Quiescent stellate cells have cytoplasmic lipid droplets and long cytoplasmic processes, whereas activated hepatic stellate cells lack both the droplets and the processes, have a fibroblast-like appearance, and express α smooth muscle actin (αSMA) [3, 132]. Depending on the culture matrix, hepatic stellate cells in culture show different phenotypes. When cultured on uncoated polystyrene or on collagen type I, III, IV or V, laminin, or fibronectin-coated culture dishes, hepatic stellate cells have a flattened fibroblast-like shape without cellular processes, indicating an activated phenotype. In contrast, when cultured on Matrigel, a commercially available gel containing basement membrane components that closely resembles the extracellular matrix of hepatic stellate cells in vivo, stellate cells are round-shaped with no cellular processes, low proliferation rate, and a similar appearance to freshly isolated, quiescent stellate cells [121, 133-135]. Importantly, culturing activated rat or human primary hepatic stellate cells on, or in this gel can deactivate these cells, as shown by a reduction of αSMA and collagen expression and low proliferation rates [136, 137]. Hepatic stellate cells cultured on collagen gels have long cytoplasmic processes and lipid droplets, a distinct phenotype from the flattened fibroblast-like phenotype seen when culturing hepatic
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stellate cells on plastic or collagen-coated culture dishes [121, 135, 138, 139]. These cells produce less collagen and are less proliferative than stellate cells cultured on uncoated plastic [138] and it was suggested that stellate cells cultured on, or in collagen gels adapt their quiescent, normal in vivo phenotype.

The use of matrices for the culturing of primary stellate cells and stellate cell lines is promising for the development of an in vitro model in which activation of hepatic stellate cells can be more closely regulated. In addition, the possibility to induce deactivation of stellate cells [136, 137] might be helpful in elucidating mechanisms underlying the regression of liver fibrosis. During the development of liver fibrosis, the composition of the extracellular matrix alters [1, 4] and the use of this system to study the effects of different matrix components on hepatic stellate cells might be valuable to give further insight in the process of stellate cell activation and fibrogenesis. However, it should be taken into account that the culture matrix does not merely determine the phenotypic appearance of hepatic stellate cells, but also several aspects of the behavior of (activated) stellate cells, like proliferation and the production of extracellular matrix (degrading) proteins [136, 138, 140, 141]. Since these processes are closely related to the activation state of hepatic stellate cells, interpretation of responses of cells cultured on matrix to pro- or anti-fibrogenic compounds might be difficult.

Co-cultures

The lack of a physiologic cellular context when studying stellate cell activation in cell culture can be addressed by co-culturing of different liver cell types. Several methods have been developed to obtain co-cultures consisting of two or more liver cell types [142]. The conventional method of making co-cultures is to isolate different liver cell types and plate them together in monolayer culture. By varying the seeding-densities of the cell types, the degree of cellular interactions can be modulated. In addition, by plating one cell type on culture inserts or coverslips, direct cell-cell interactions in the co-culture can be prevented in order to study soluble factors influencing cell behavior. Using these methods, co-culture of primary hepatic stellate cells or cell lines with Kupffer cells, endothelial cells, or hepatocytes is possible. In addition, in several studies this method was used to co-culture all these liver cell types, however, the exact cellular composition of these cell mixtures during incubation was not described [143-146]. Another approach used to obtain a co-culture containing all liver cell types, was to culture hepatocytes in a collagen gel, and plate a mixture of non-parenchymal cells on top of this gel, thus obtaining a 3D co-culture but loosing direct cellular interactions of the non-parenchymal cells with hepatocytes [147]. Finally, co-culture models are described that use a confluent layer of pre-cultured non-parenchymal cells, plating hepatocytes on top of this layer to achieve a co-culture of the four liver cell types, thus allowing interactions between parenchymal and non-parenchymal cells [148, 149].

Despite the possibility to co-culture hepatic stellate cells with other liver cell types, only few studies are described investigating the effects of intercellular interactions on stellate cell activation and fibrogenesis using co-culture systems [150-152]. Besides, ethanol-induced collagen synthesis by stellate cells, which requires metabolic conversion of ethanol into acetaldehyde by hepatocytes, was successfully studied in a
co-culture model of hepatocytes and hepatic stellate cells [157, 158], indicating that these models are promising for the development of an in vitro model that more closely mimics the in vivo situation. However, current co-culture models cannot accurately mimic the in vivo cellular composition of the liver and its acinar structure and although cell culture models contribute significantly to the understanding of stellate cell biology, they still fall far short of recapitulating intercellular and cell-extracellular matrix interactions in the process of hepatic stellate cell activation and fibrogenesis in vivo.

**Precision-cut liver slices**

Recently, precision-cut liver slices came into notice as a potential model for the study of stellate cell activation and liver fibrosis because, unlike current in vitro models, they could enable to study these processes in vitro in a multicellular system in which cell-cell and cell-extracellular matrix interactions are maintained. Precision-cut liver slices are extensively used for the study of drug metabolism and (multicellular) toxicity [153-156] however studies describing liver slices as a tool to study hepatic stellate cell activation and fibrogenesis are still limited.

### 4 Aim of the thesis

As no effective treatment for liver fibrosis is available yet, extensive research is ongoing to develop new anti-fibrotic drugs. Activation of hepatic stellate cells and the development of liver fibrosis is a multicellular process also involving hepatocytes, Kupffer cells, and sinusoidal endothelial cells. Therefore, when studying these processes and the effects of potential anti-fibrotic compounds thereon, intercellular interactions should be taken into account. In addition, maintaining cell-extracellular matrix interactions is vital for recapitulating stellate cell activation and fibrogenesis in a relevant manner.

To date, studies on the development of effective anti-fibrotic drugs rely mostly on in vivo animal experiments. These models do incorporate the effects of the extracellular milieu during hepatic stellate cell activation and fibrogenesis accurately, but are largely restricted to rodents and therefore may be of limited predictive value for human disease. In addition, in vivo models give high discomfort to the experimental animals and should therefore be avoided when possible. By using in vitro models, the use of experimental animals can be greatly reduced because various experimental conditions can be tested using cells or tissue from only one animal, which is often not possible in in vivo experiments. In addition, in vitro research allows experimental conditions that cannot be performed in vivo. Importantly, by using human cells or tissue, species differences can be addressed and the results obtained with rodent cells or tissue can be validated for their relevance to the human situation.

The choice of in vitro models for liver fibrosis is still limited. Current in vitro models, which rely on cell cultures of primary or immortalized cells, have contributed significantly but, as discussed above, lack a physiologic milieu that can accurately incorporate effects of cell-cell and cell-extracellular matrix interactions. Hence there persists a need for an in vitro system to study the processes underlying the development
Introduction of liver fibrosis and to test the efficacy of potential anti-fibrotic compounds in a more physiologic milieu in vitro. Precision-cut liver slices could provide such a system since in the liver slice all liver cell types are present in their natural environment, thereby preserving the cell-cell and cell-extracellular matrix interactions. In addition, when using slices from fibrotic liver tissue, this enables to study progressed stages of fibrosis in a multicellular, pathophysiologic milieu, that cannot be achieved in vitro using cell culture models. Finally, the use of human liver slices avoids interpretation problems due to species differences. Liver slices are already successfully used in studies of metabolism and (multicellular) toxicity [153-156]. The aim of the research described in this thesis was to evaluate whether precision-cut liver slices are also applicable for the study of liver fibrosis and as a test-system for anti-fibrotic compounds.

5 References

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