Towards treatment of liver fibrosis: Cells, targets and models
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Citation

Helm, D. van der. (2021, February 11). *Towards treatment of liver fibrosis: Cells, targets and models*. Retrieved from https://hdl.handle.net/1887/139202

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Author: Helm, D. van der
Title: Towards treatment of liver fibrosis: Cells, targets and models
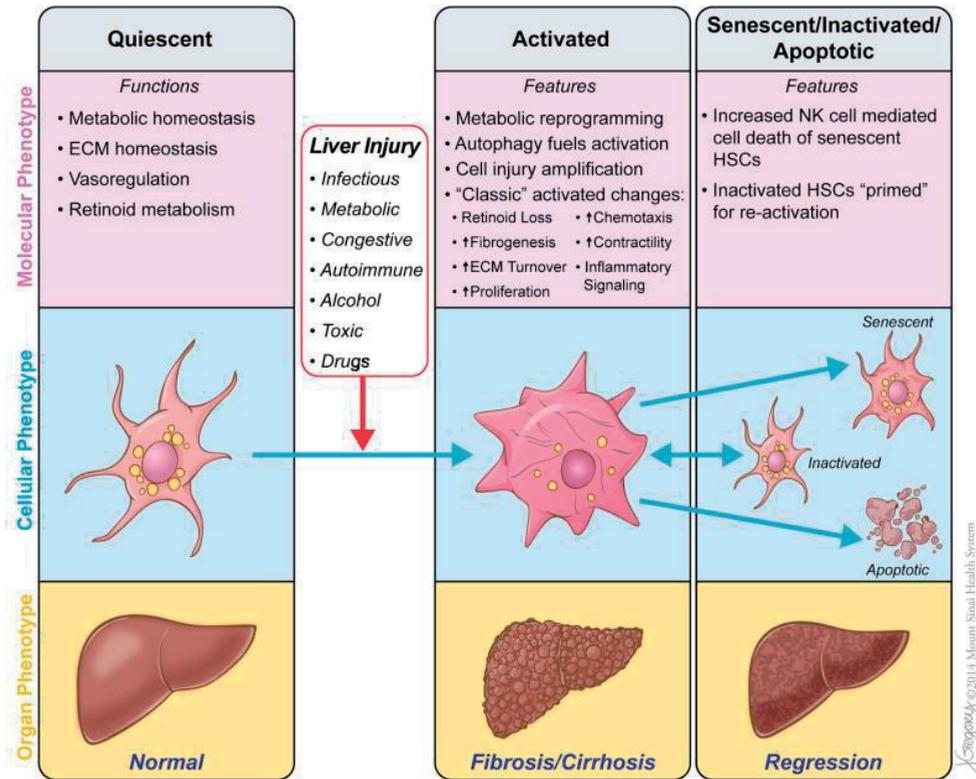**Issue date**: 2021-02-11

CHAPTER 1

1

General Introduction

Liver cirrhosis, the second phase in the fibrosis-cirrhosis-hepatocellular carcinoma (HCC) cascade, is the fourth most common cause of death in Europe (170.000 deaths per year) and the 14th worldwide (>1 million deaths per year), with an expected increasing incidence in the nearby future[1,2]. Cirrhosis is becoming a major health problem and therapeutics directly targeting the process of liver fibrogenesis, thereby preventing the progression of the disease in the fibrosis-cirrhosis-HCC cascade, are not yet available.

Aetiological factors that can cause cirrhosis include hepatitis viruses (B, C and D), chronic alcohol intake (alcoholic liver disease, ALD), auto-immune hepatitis (AIH), drug-induced liver injury (DILI), genetic disorders (like α1-antitrypsin deficiency, Wilson disease and hereditary haemochromatosis), obesity and diabetes mellitus (non-alcoholic fatty liver disease, NAFLD), and cholestatic diseases (like primary biliary cholangitis, PBC, and primary sclerosing cholangitis, PSC)[2-5]. The prevalence of these aetiologies is region related[2,6-8]. In the Western world, liver cirrhosis mostly evolves in a background of alcohol intake (ALD) and lifestyle-induced NAFLD[7,9,10]. Due to the increasing prevalence of overweight and diabetes over the past decades, NAFLD has become an endemic cause of liver disease[9,10]. In Asia and sub-Saharan Africa, cirrhosis is mostly induced by viral hepatitis B or C infection[6,7]. In general, these aetiological factors lead to the onset of liver fibrogenesis and eventually to fibrosis and cirrhosis[3,4,11]. Liver fibrogenesis starts by damaged and apoptotic hepatocytes which trigger the proliferation and activation of liver-resident stellate cells[3,4,11]. These activated stellate cells differentiate into myofibroblasts and subsequently start to secrete excessive amounts of extracellular matrix (ECM) leading to fibrogenesis (Figure 1). The liver has an efficient regenerative capacity to overcome acute damage induced by injuring stimuli such as toxins, viral infection, auto-immunity, cholestasis, metabolic disorders, trauma or surgical interventions[12-16]. In response to these acute injuring stimuli, stellate cells become activated and the liver starts regenerative cascades that promote survival and proliferation of endogenous liver cells. At the end of these regenerative processes, the activated stellate cells are silenced and shift to their inactivated state[14-16]. When the liver is chronically challenged, despite some regeneration, the liver will be unable to recover in the period between the injuring insults. This continuous fibrogenesis leads to diminished liver function and eventually to fibrosis and finally cirrhosis[4,11].

In a healthy steady-state situation, stellate cells reside in the space of Disse near the portal triads[17]. The space of Disse is the area between the hepatocytes and the liver sinusoids, with the fenestrated endothelium in between. During liver fibrogenesis, the activated and proliferating stellate cells migrate and populate these spaces where they secrete ECM components and form the so-called septa that eventually bridge the entire space between portal triads (porto-portal septa), between centrilobular veins (centro-central septa), and between portal triads and centrilobular veins (porto-central septa). In the clinic, the produced ECM deposition is used to assess the severity of liver fibrosis[18]. Besides these myofibroblasts and their secreted ECM components, the septa are also filled with invading Kupffer- and T-cells[18]. After a long

Quiescent

Molecular Phenotype

Functions
- Metabolic homeostasis
- ECM homeostasis
- Vasoregulation
- Retinoid metabolism

Liver Injury
- Infectious
- Metabolic
- Congestive
- Autoimmune
- Alcohol
- Toxic
- Drugs

Cellular Phenotype

Organ Phenotype

Normal

Activated

Features
- Metabolic reprogramming
- Autophagy fuels activation
- Cell injury amplification
- "Classic" activated changes:
  - Retinoid Loss
  - ↑Fibrogenesis
  - ↑ECM Turnover
  - ↑Proliferation
  - ↑Chemotaxis
  - ↑Contractility
  - Inflammatory Signaling

Fibrosis/Cirrhosis

Senescent/Inactivated/ Apoptotic

Features
- Increased NK cell mediated cell death of senescent HSCs
- Inactivated HSCs "primed" for re-activation

Senescent

Inactivated

Apoptotic

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Regression

Figure 1: Stellate cells during the induction and regression of liver fibrogenesis. In the healthy situation, quiescent stellate cells are involved in metabolic homeostasis, vaso-regulation and retinoid metabolism. Due to liver injuring stimuli, the stellate cells become activated and differentiate into myofibroblasts. This shift is accompanied by metabolic reprogramming, retinoid loss, increased ECM secretion, increased proliferation, and increased inflammatory signalling of stellate cells. Removal of the injuring stimuli may lead to the regression of fibrogenesis, which is initiated by myofibroblasts that become apoptotic or shift to their inactivated or senescent state. Reproduced from [Pathobiology of liver fibrosis: a translational success story, Y.A. Lee, M.C. Wallace, S.L. Friedman, 64(5):830-41 2014] with permission from the illustrator and BMJ Publishing Group Ltd[11].

period of sustained fibrogenesis, the blood-flow through the liver becomes hampered which leads to fewer nutrients and oxygen supply and subsequently more cell death in the liver which eventually enhances the ongoing fibrogenesis. During the last phase, the liver will fail to perform its many functions, which indicates end-stage liver cirrhosis. During this final stage there is an increased risk of decompensated cirrhosis, characterized by variceal bleeding, ascites, hepatic encephalopathy, and multi-organ failure. Furthermore, cirrhosis also predisposes towards development of hepatocellular carcinoma (HCC)[19,20].

Treatment of liver fibrosis and cirrhosis is limited to removal of the injuring stimuli, such as anti-viral therapies or refraining from alcohol consumption[21,22]. For example, sustained response to antiviral therapy in patients with HBV- or HCV-induced fibrosis can lead to the reversal of

the relative stage of fibrosis[23,24]. Due to this successful treatment strategy, less patients with HCV-induced cirrhosis are now in need of orthotopic liver transplantation (OLT)[25]. Nevertheless, OLT is currently the only curative treatment for end-stage cirrhosis with deteriorating function and decompensation[9,21,24]. Although OLT is performed for decades already, it is still a major intervention with substantial risks[26,27]. Furthermore, the possibility to perform OLT depends on the general condition of the patient and on donor availability[28,29]. Recently, hepatocyte and liver organoid transplantations were tested as alternative treatment strategies for end-stage liver cirrhosis. These therapies were found to improve liver function and overall survival in mice with fulminant liver failure[30,31]. However, in mouse models for liver fibrosis, these treatments were ineffective in resolving fibrosis, and also showed low engraftment in the damaged liver. Altogether these observations indicate the need for alternative treatment strategies which preferably directly target fibrogenesis[32-34].

Mesenchymal stromal cells (MSCs)

The applicability of MSC therapy is well studied in a variety of diseases, and research in this context made huge progress in the basic and functional characterisation of this cell type[35-37]. Some of these studies showed that MSCs have functional characteristics that might be applicable to reverse liver fibrogenesis[33,37-39]. MSCs can easily be isolated from different tissues such as adipose-, umbilical cord-, and bone marrow-tissue, and are identified by their ability to adhere to plastic, their ability to differentiate into osteoblasts, adipocytes and chondrocytes, and their expression of certain membrane markers[37,38,40]. The literature, however, is less unambiguous regarding the precise subset of these membrane markers[41]. In general, mouse-derived MSCs are known to express CD29 (β1-integrin), Stem Cell Antigen-1 (SCA-1) and CD44 but not the hematopoietic cell marker CD45 and endothelial cell marker CD31[42,43]. Endoglin (CD105) and vascular cell adhesion molecule 1 (VCAM-1, CD106) membrane expression are inconsistently used as identification markers for MSCs[41,44-47].

MSCs exert multiple unique features that make them of interest for therapeutic use. One of these features is that resting MSCs are not expressing MHC class II proteins, unless activated, and are therefore not recognised and not rejected by the host immune system after transplantation[48]. Furthermore, MSCs can easily be expanded *in vitro* while maintaining their phenotype and can easily be cryopreserved, which makes it possible to treat multiple patients with the same MSC product[38].

In relation to their potential therapeutic use, MSCs are known to be able to inhibit inflammatory responses, for example suppressing T-cell responses and promoting anti-inflammatory macrophage differentiation[49,50]. Because of these immune-regulatory properties, MSCs have already been used after kidney or bone-marrow transplantation for the prevention of rejection[36,51,52]. Furthermore, MSCs promote regeneration and repair of damaged tissue as, for example, observed in the MSC treatment of perianal fistulas in Crohns disease[53]. Tissue

repair, tissue regeneration and immune-responses are also important processes during the regression of liver fibrogenesis, and therefore the use of MSCs might be of interest as potential treatment strategy for liver fibrosis[40].

Fibroblasts and MSCs have multiple phenotypic similarities which makes it somewhat difficult to distinguish these cell types. Literature suggests that MSCs, in contrast to fibroblasts, are positive for SCA-1 and that this marker therefore may be used to distinguish both cell types[54]. It is also suggested that fibroblasts and MSCs share some functional characteristics in immunomodulation and tissue regeneration[54-57]. However, the comparison in their ability to reverse liver fibrogenesis has not been studied before.

MSC therapy as potential therapeutic strategy to resolve liver fibrosis

Sakaida et al. published in 2004 *in vivo* studies showing that MSC treatment could inhibit and prevent the induction of liver fibrosis[58]. Since that time several *in vivo* and clinical studies assessed whether liver fibrosis and cirrhosis could be reversed by MSC therapy[39,40,59-61]. Most of these studies revealed positive and promising results showing that MSCs are able to effectively reverse liver fibrogenesis and thereby ameliorate fibrosis or cirrhosis. Furthermore, no serious side-effects or unsafety signals were observed in all these studies. In literature, different working mechanisms have been suggested. One of the suggested theories includes the ability of MSCs to stimulate the survival and proliferation of endogenous liver cells upon tissue damage (Figure 2). For example, Fouraschen et al. showed that livers that underwent a partial hepatectomy regenerate faster with MSC therapy[12]. It was suggested that MSCs express and secrete hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), and stromal derived growth factor-1 (SDF-1) and thereby stimulate survival and proliferation of hepatocytes, that might explain the pro-regenerative capacities of MSC therapy[12,62-66]. In relation to the anti-inflammatory capacities of MSCs, it is thought that MSCs reverse fibrogenesis by suppression and/or redirecting of innate- and adaptive-immune responses (Figure 3). For example, MSCs are known to directly inhibit B- and T-cell proliferation, thereby inhibiting immune-responses. In relation to the innate immune system, MSCs are thought to secrete IGF-1 and interleukin-10 (IL-10) in response to the fibrogenic environment, which stimulates macrophage M2 polarization. M2 macrophages are anti-inflammatory and are able to silence some of the immune-reactions which occur during fibrogenesis[63,67]. Furthermore, MSCs are also known to suppress dendritic and NK cell function (Figure 3).

Another suggested mechanism is a direct anti-fibrogenic effect of MSCs by the release of cytokines such as HGF which directly targets the stellate cells and myofibroblasts. HGF is known to directly inhibit the activation and proliferation of stellate cells, thereby directly targeting the initiation steps of fibrogenesis. Furthermore, HGF is also known to silence myofibroblasts (activated stellate cells), thereby directly silencing fibrogenesis (Figure 2)[37,66,68-70]. MSCs are
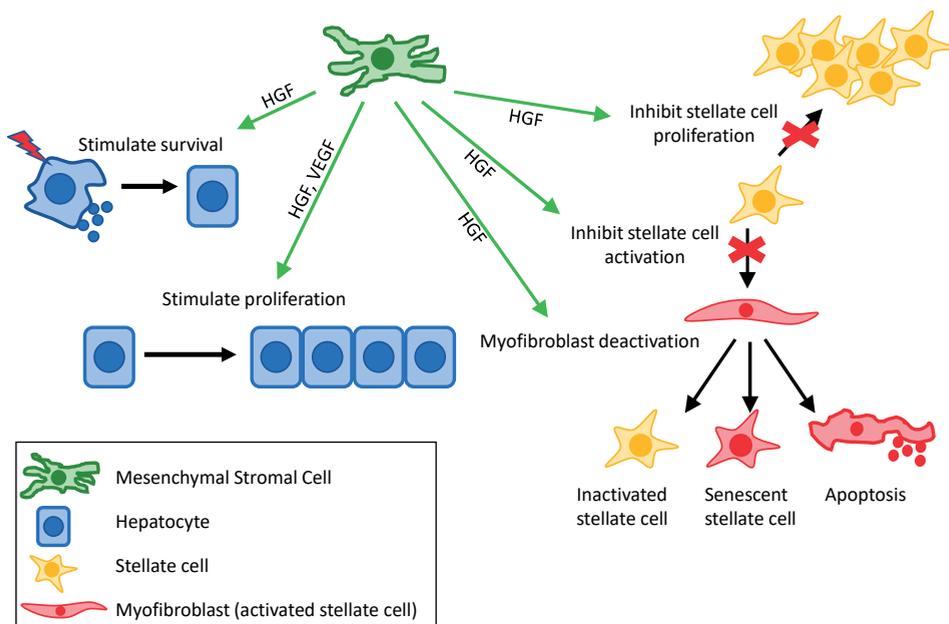
Figure 2: Potential therapeutic interactions of MSCs with endogenous liver cells for the treatment of liver fibrosis. Schematic overview of suggested working mechanisms of MSC therapy for the regression of liver fibrogenesis.

even thought to be able to differentiate into hepatocytes or hepatocyte-like cells[33,38]. These differentiated cells exert similar functional properties as observed in normal hepatocytes, such as glycogen storage, low density lipoprotein (LDL) uptake, and the production of albumin and urea. However, while these hepatocyte-like cells, like hepatocyte organoids, may improve liver function, they show low engraftment in the liver and are also ineffective for induction of regression of ongoing fibrogenesis[32-34]. The precise working mechanisms of MSCs are still largely unknown, but probably encompass a combination of the above mentioned mechanisms that contribute to their efficacy in the observed reversal of fibrogenesis.

The importance of study design and MSC characterisation in MSC-related therapy

Despite the promising previously performed studies and the proposed mechanisms, the use of MSC therapy for liver fibrosis is still in its infancy. Most *in vivo* and clinical studies are using different study designs, which makes it difficult to compare these studies and to evaluate the overall efficacy of MSC therapy[37,59,60,71]. For example, the disease stage (fibrosis vs cirrhosis) or aetiological factors can be different between studies and these might affect the study outcome. Furthermore, the effectiveness of MSC therapy could also be affected by technical variables in the study design such as the dosage and -administration routes (i.e., local- vs intravenous- vs portal-administration) of MSCs [39,59-61,71]. Moreover, many studies are using MSCs isolated from different sources, while it is known that adipose-, umbilical cord-
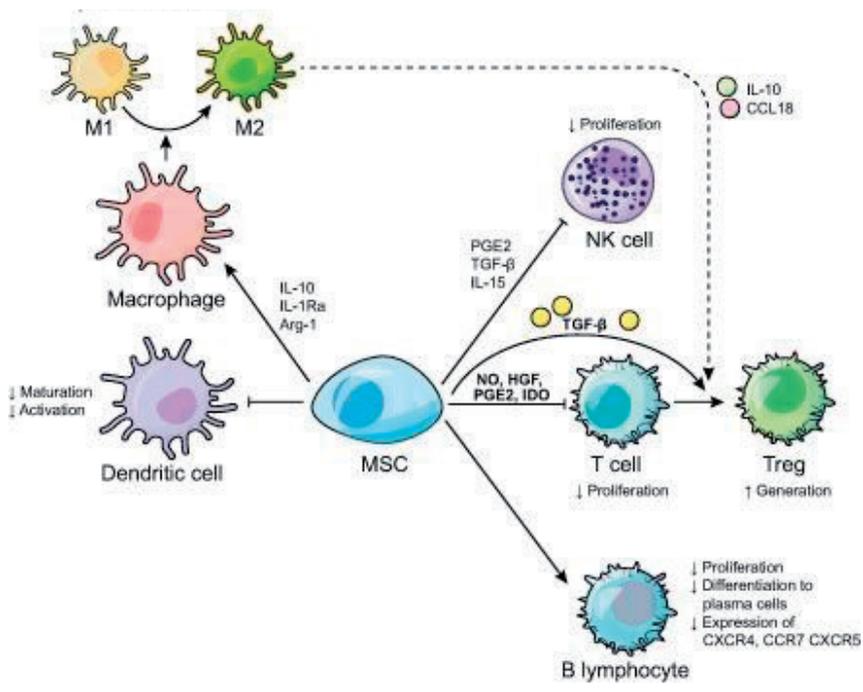
Figure 3: The putative interplay between MSCs and immune cells in the treatment of liver fibrosis. Schematic overview of suggested immunoregulatory mechanisms of MSC therapy that might lead to the regression of liver fibrogenesis. Reprinted from [Mesenchymal stromal cell therapy for liver Diseases, 68(6):1272-1285 2018, M. Alfaifi, Y.W. Eom, P.N. Newsome, S.K. Baik] with permission from Elsevier[61].

and bone marrow-derived MSCs can behave differently, suggesting that the source of MSCs might also be important to induce the regression of fibrogenesis[46,72]. Furthermore, recently published studies revealed the possible existence of different subpopulations of MSCs which might explain the different findings in the literature[43,45,46,71]. With the currently used isolation protocols, a heterogeneous population of cells is isolated which are all positive for most of the known MSC characterisation markers[46,73,74]. For example, VCAM (CD106) and Endoglin (CD105) membrane expression are not used as a standard for MSC characterisation while literature already suggested that subpopulations identified by the presence or absence of these proteins might exert different functional properties[44-46]. Anderson et al. showed that Endoglin-negative MSC populations seem to have better immunoregulatory properties compared to Endoglin-positive MSC populations[45]. Other studies have shown that VCAM-positive MSC subpopulations are more pro-regenerative and immunosuppressive compared to VCAM-negative MSC subpopulations[44,46]. These findings indicate that the use of different subpopulations of MSCs probably affect therapy efficacy. Therefore, these variables might explain the different and sometimes contradictive study outcomes, warranting further research to identify an optimal MSC therapy for liver fibrosis.

Animal models to study liver fibrogenesis

Various *in vitro* and *in vivo* models are being used to study the pathogenesis of liver fibrogenesis and to test alternative treatments to reverse this pathological process[75,76]. Acute and chronic liver fibrogenesis can be induced *in vivo* by genetic modifications, mechanical alterations or administration of hepatotoxic compounds[75].

The latter is most frequently used since these models most resemble the human viral- or alcohol-induced liver diseases. Thioacetamide (TAA) and carbon tetrachloride (CCL4) are well-known and frequently used hepatotoxic compounds to induce acute- and chronic- liver injury in mice and rats[75,76]. Hepatocytes metabolise both compounds into hepatotoxic metabolites that subsequently induce apoptosis of the hepatocytes and thereby initiate the induction of fibrogenesis[75,76]. The duration of the administration-period of these compounds correlates to the severity and progression of the disease. This correlation makes it is possible to study different disease stages within the same model system[76,77]. CCL4- and TAA-induced animal models for liver fibrosis have shown to be predictable and reliable but are also expensive and sometimes acute toxicities with subsequent animal death are observed. Moreover, it takes a relative long period to induce chronic liver fibrosis (6 weeks) or cirrhosis (12 weeks). Within these periods, animals are in need of frequent check-ups and regular administration of the toxic compounds, making these models time-consuming and labour-intensive[75]. These observations indicate that CCL4 and TAA rodent models are robust but less attractive for high throughput compound screening.

Zebrafish embryos, on the other hand, are small, less expensive, easy to maintain, have a short regeneration time and also showed huge physiological similarities with man[78-80]. In relation to that latter, livers of zebrafish are constructed with the same cells as in humans and show a further resemblances of 70%[78,81]. Moreover, zebrafish embryos are suitable for high throughput screening as observed in non-hepatic related studies[80]. However, the use of these embryos in respect to liver fibrogenesis is limited, and a detailed description of a zebrafish embryo model which resembles chronic human liver fibrogenesis had not been presented yet.

Some studies observed that acute liver injury in zebrafish embryos leads to increased collagen and Hand-2 expression, which is indicative for the activation of stellate cells, and for the onset of fibrosis[82-85]. Furthermore, similarities to the well-known human and mouse pathogenesis of liver fibrogenesis were observed when TAA or ethanol was administered to mature zebrafish[86-88]. The zebrafish embryo might thus be an attractive high throughput model system to study chronic liver fibrogenesis. The abilities of TAA and CCL4 to induce fibrogenesis in zebrafish embryos and the possible involved pathways have not yet been described. If these compounds induce fibrogenesis in zebrafish embryos with similar pathological mechanisms as observed in humans it would be a perfect high throughput screening model for the identification of alternative therapeutics to reduce fibrogenesis.

Cripto-1: a new player in the fibrosis-cirrhosis–HCC cascade

As mentioned earlier, therapies directly targeting fibrogenesis are needed. In order to discover new targets for intervention, it is important to increase our basic understanding and knowledge of the fibrosis-cirrhosis-HCC cascade and the underlying pathological mechanisms.

In 2018, Zhang et al. described elevated Cripto-1 (Teratocarcinoma-Derived Growth Factor 1; TGDF1) protein levels in blood of patients with HBV- and HCV-induced cirrhosis[89]. Cripto-1 belongs to the epidermal growth factor-Cripto/frl/cryptic (EGF-CFC) family and is a GPI-anchored signaling protein that is important during embryogenesis and believed to be silenced after birth[90-92]. Surprisingly, recent discoveries indicate that Cripto-1 is re-expressed postnatally in different neoplastic processes but a link to fibrogenesis was never observed[90]. Oncogenesis, embryogenesis, fibrogenesis, tissue repair, and tissue regeneration are different processes but also share multiple similarities including cell proliferation, cell survival, and cell differentiation[14-16]. Cripto-1 is known to be an important protein for these cellular features during embryogenesis and oncogenesis[92,93]. One might therefore speculate that Cripto-1 expression during fibrogenesis could be involved in the survival, proliferation and plasticity of liver cells as protective mechanism to overcome the injuring stimuli. When this would be true it could imply a functional role for Cripto-1 in the fibrosis–cirrhosis-HCC cascade. Altogether, these observations warrant further research to disentangle the contribution of Cripto-1 in liver fibrogenesis, which in the future may contribute to the identification of new leads for antifibrotic therapy.

Cripto-1 in Hepatocellular carcinoma

Hepatocellular carcinoma is the second leading cause of cancer-related death worldwide[94]. HCC mostly arises in a background of cirrhosis in the last phase of the fibrosis-cirrhosis-HCC pathological disease course[95,96]. HCCs are known to be invasive and to have a high metastatic potential leading to poor prognosis of patients. The treatments for early and intermediate tumor stages include resection, OLT and/or minimally invasive image-guided therapies such as local ablation by trans-arterial chemoembolization (TACE) or radiofrequency ablation (RFA)[97,98]. For advanced tumor stages, systemic treatments such as Sorafenib and Regorafenib are being used[98,99]. However, these palliative systemic therapies can have substantial side-effects, are effective in only a minority of the patients and lead to an average survival benefit of only 6 months[98,100]. Despite these different treatment strategies the overall patient prognosis for HCC remains poor due to tumor recurrence and non-response to therapy[98].

Biomarkers for HCCs that correlate with tumor stage and which are able to predict the progression of the tumor could be of help in the early detection and treatment of HCC[101]. In the clinic, alpha-fetoprotein (AFP) is used as a biomarker but as sole marker is insufficient for diagnosis since it does not predict disease stage and serum levels are not elevated in 30% of the HCCs[98,102]. However, in the cases where AFP is elevated those serum levels do

correlate to tumor size and tumor progression, and therefore in these cases it can be used to evaluate response to therapy and follow-up of the disease[103]. The mechanisms behind the development, progression, invasion, and metastasis of HCCs are largely unknown. Elucidation of these processes might lead to the identification of new biomarkers and new (personalized)-therapies. For example, biomarkers which could distinguish Sorafenib responders from non-responders would lead to a better and more personalized treatment. As mentioned earlier, Cripto-1 is re-expressed during oncogenesis where it is involved in cancer progression and metastasis[91,104-110]. Moreover, Wang et al. recently showed that Cripto-1 expression in HCC correlates to poor patient survival and faster tumor recurrence in HCC patients but the precise contribution of Cripto-1 is unclear[92,111]. Suggested mechanisms include Cripto-1 involvement in pathways leading to faster proliferation and onset of epithelial to mesenchymal transition of tumour cells[92,112-117]. The exact function of Cripto-1 in HCC and its possible usage as a biomarker, however, need to be further studied. As described, Cripto-1 is also observed in blood of patients with cirrhosis without the presence of HCC or any other neoplasms[89]. One might speculate that hepatocytes expressing Cripto-1 during fibrogenesis may be the cells with the highest potential to become oncogenic and thereby may be identified as the "cancer stem cells". In the future, unravelling the role of Cripto-1 in the fibrosis-cirrhosis-HCC pathological disease course might lead to the identification of new targets for HCC and antifibrotic therapies.

Outline and aims of the studies described in this thesis

Currently, MSCs have been tested in clinical trials, often with promising results but also sometimes with a lack of effectivity regarding the reversal of fibrosis, cirrhosis and end-stage liver disease[39,61,118]. Results from the literature are difficult to compare since there are multiple differences in study design such as underlying disease aetiology, disease stage, administration route- and dosage- and source- of MSCs, which could affect the study outcomes[60,61,71]. Therefore, in the study of **chapter 2,** the therapeutic potential of MSCs and fibroblasts were assessed and compared, in combination with partial hepatectomy as regenerating stimulus, in CCL4-induced fibrosis and cirrhosis in mice. Furthermore, the impact of route of administration and dosage of MSCs on the therapeutic efficacy of MSCs was evaluated. Specifically the local administration of the MSCs in regenerating fibrotic and cirrhotic livers was thought to be able to ameliorate fibrogenesis.

The use of different MSC subpopulations might also contribute to the contrasting findings in literature[44-46,119]. In the study of **chapter 3,** the pro-regenerative and anti-fibrotic abilities of four different subpopulations of MSCs, selected on their Endoglin and/or vascular cell adhesion molecule (VCAM) expression, was compared. This approach was used to evaluate whether different subpopulations of MSCs could lead to different outcomes, which might explain the contradictory results observed in literature.

Rodent models for liver fibrosis have been widely used, but are not suitable for high throughput screening purposes[75]. Therefore we aimed to translate the widely used CCL4 and TAA mouse models for liver fibrosis to zebrafish embryos as a new model suitable for fast screening (**chapter 4**). The applicability to study new therapeutic interventions was evaluated by the administration of MSCs and fibroblasts as potential novel cell therapies for liver fibrogenesis.

Therapies directly targeting fibrogenesis are needed. More knowledge of the pathological mechanisms underlying the fibrosis-cirrhosis-HCC cascade could lead to identification of new leads for the development of alternative treatment strategies. Interestingly, a recent study reported elevated Cripto-1 protein levels in plasma of patients with cirrhosis[89]. This was the first study that suggested a connection between Cripto-1 expression and fibrogenesis. In order to compare Cripto-1 expression of normal and fibrogenic liver tissue of humans, mice, and zebrafish embryos a study was performed to evaluate whether Cripto-1 is expressed by liver cells (**chapter 5**). Furthermore, the Cripto-1 level in blood and its expression in liver tissue were assessed to evaluate whether it relates with the disease stage. If this would be the case, it could imply a contribution of Cripto-1 in the fibrosis–cirrhosis-HCC cascade which warrant further studies.

Cripto-1 is known for its role in cancer progression and metastasis[90]. In HCC, Cripto-1 expression correlates with poor prognosis and overall survival, however, the functional role of Cripto-1 in HCC is largely unknown[89,111]. Therefore, as described in **chapter 6** the role of Cripto-1 in HCCs *in vitro* and *in vivo* was studied. In addition it was assessed whether Cripto-1 expression might affect the use of conventional systemic therapies.

Finally, in the overall discussion of **chapter 7** the implications of the findings of the different studies is discussed and directions for future research are indicated.

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