Towards treatment of liver fibrosis: Cells, targets and models

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CHAPTER 7

General Discussion

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Liver fibrogenesis is the underlying process that leads to the onset and progression of the fibrosis-cirrhosis-hepatocellular carcinoma (HCC) cascade[1,2]. This process is initiated by etiological factors that damage and destroy hepatocytes, and subsequently triggers the activation of the hepatic stellate cells. These activated stellate cells proliferate and differentiate into myofibroblasts which start to produce high levels of extracellular matrix (ECM)[3-6]. While effective treatments for some of the underlying etiological factors that trigger fibrogenesis, like viral hepatitis, become increasingly available, treatments which specifically target the process of fibrogenesis and thereby prevent progression of the disease cascade are not yet available[1,2,7-9]. Mesenchymal stromal cells (MSCs) are thought to stimulate tissue regeneration as well as to modulate inflammatory responses[10-15]. These features make MSCs an attractive tool for the resolution of liver fibrosis where these specific processes need to be restored. In that context, MSCs are thought to support survival of liver cells and directly target fibrogenesis by silencing the myofibroblasts and by inhibiting the activation and proliferation of stellate cells[16-22]. Currently, MSCs have been tested in clinical trials with promising, but also sometimes disappointing, results regarding the reversal of fibrosis and cirrhosis[14,23-25]. In the present thesis several studies are described which addressed the different aspects, which might shed light on the potential cause(s) of these sometimes even contradictory results. Furthermore, a novel treatment strategy is proposed where the beneficial features of MSCs are combined with the innate regenerative ability of the liver[26,27].

Study design might be an important factor for effective MSC therapy

MSC therapy for liver fibrogenesis is still in its infancy and an optimal and standardised treatment protocol is not available yet. The use of diverse protocols makes it difficult to compare and explain the contradictory findings observed in literature. Variables in study design which might have led to different study outcomes include the route of administration (local vs systemic), dosage of MSCs, disease stage (fibrosis vs cirrhosis), trigger for regeneration (e.g. partial hepatectomy), and possibly the existence and use of different subpopulations of MSCs[28,29]. In the *in vivo* experiments of **chapter 2** we observed that a partial hepatectomy effectively reduces the fibrotic stage of fibrosis. This phenomenon has not been described before and it would be of interest to verify these outcomes in patients.

Furthermore, our *in vivo* studies also showed that local administration of MSCs had a smaller effect than the regenerative response after partial hepatectomy. However, when these two approaches were combined, this reinforced the effectivity of both therapies. This additional effect of MSC administration was not observed after systemic infusion, indicating the importance of local administration[26]. MSCs, when injected intravenously (i.v.), can easily get trapped in the lungs, which leads to fewer, if any, cells homing to the liver, which in turn might very well be the possible explanation for the ineffectiveness of this route of administration of MSCs in

reverting liver fibrosis[30]. After local administration, the MSCs did not migrate from the injection sites and were only effective in that part of the diseased liver. Nevertheless, our study is the first describing an on-site effect of MSC therapy on fibrogenesis and gives further reasoning to the ineffectiveness of i.v. MSC treatment.

Due to the limitations in time and of the mouse model for liver fibrosis, we did not assess the potency of portal- or liver artery-infusion of MSCs. Nevertheless, since portal- and liver artery-infusions are local administration routes one might expect similar results as observed for the local treatment in the present studies. CCL4-mouse models for liver fibrosis are frequently used to study potential therapeutic interventions. However, the severity of the induced disease is rather diverse between studies and this might potentially affect study outcomes[31,32]. We showed that the described combination therapy (partial hepatectomy plus local MSC administration) effectively resolved both fibrosis and cirrhosis, illustrating that the disease stage is less relevant for the functionality of the described therapy (**chapter 2**). Furthermore, a dose-dependent response in the resolution of fibrosis between $1x10^6$ and $2x10^6$ MSCs treated mice was observed, which suggests that the effectiveness of therapy is related to the dose of MSCs. As suggested in the studies of Parekkadan et al., this observation could be explained by the myofibroblast/MSC ratio[22]. For example, in **chapters 3 and 4** we described that MSCs express HGF, which is thought to directly target and subsequently silence the myofibroblasts. When this hypothesis is true, one could imagine that higher dosages of MSCs lead to more expression of HGF and consequently induce a larger therapeutic effect. In relation to the treatment of fibrosis in patients, a therapy could be considered consisting of both a trigger for regeneration, by partial hepatectomy, and multiple local MSC injections. This approach is comparable to the successful treatment of perianal fistulas in Crohn's disease by our group, where fistulas received a trigger for regeneration by curettage of the fistula tract and subsequently local MSC administration at multiple injection sites[33].

MSC subpopulations differently affect the resolution of fibrosis

Most researchers are not familiar with the existence of multiple subpopulations of MSCs and as a result all kinds of (mixed)populations have been used, which might contribute to the different and even contradictory findings between various studies. Only a few studies assessed the possible existence of multiple subpopulations of MSCs[34-36]. This might be caused by the lack of more precise criteria for the identification and characterisation of these cells and results in a rather heterogenous population of cells being identified as MSCs[36]. Most of the studies describe mouse-derived MSCs as cells that adhere to plastic and are able to differentiate into osteoblast, chondrocytes and adipocytes. Furthermore, CD29, CD44 and SCA-1 need to be expressed on their membranes but CD45 (haematopoietic marker) and CD31 (endothelial marker) should be absent[37,38]. However, these criteria embrace different subpopulations of MSCs as identified by their VCAM (CD106) and/or Endoglin (CD105) expression[34,35]. Until recently, only a few studies have shown different functional capacities of these MSC

subpopulations[34,35,39,40]. Furthermore, there are no studies focusing on the use of different subpopulations of MSCs in relation to the treatment of liver fibrosis. Therefore, as described in **chapter 3**, we selected MSCs double-positive, double-negative, or single-positive for either Endoglin and VCAM and evaluated their antifibrotic and pro-regenerative capacity. More cell proliferation and survival of damaged HepG2 cells was observed when exposed to VCAM-positive subpopulations compared to the VCAM-negative MSC subpopulations. In addition, in line with the studies of Du et al.[39], we observed that VCAM-positive subpopulations are more migratory than the VCAM-negative MSC subpopulations. We used the CCL4 mouse-model and optimized MSC therapy from **chapter 2** to evaluate the therapeutic potential of the described subpopulations. The results showed that VCAM-positive but not the VCAM-negative MSC subpopulations successfully reduce fibrosis, regardless of their Endoglin expression (**chapter 3**). However, the Endoglin-negative subset of the VCAM-positive subpopulations revealed an intermediate collagen reduction, which was less than for the double positive population but more than in the VCAM-negative populations ($V^{neg}E^{pos}$, $V^{neg}E^{neg}$).

Previous studies, including those of our own group, showed that MSCs express pro-regenerative and antifibrotic cytokines (HGF, VEGF, IGF-1, and TGF-β1)[19,20,22,41-47]. In the study of **chapter 3** a higher expression level of HGF and IGF-1 was found in the VCAM-positive subpopulations compared to the VCAM-negative populations. Previous studies of Han and Du et al., observed the same phenomena, however, these studies were not related to liver fibrosis[36,39]. The different expression levels of HGF and IGF-1 might very well clarify the results of our studies, since these genes are known to support tissue-regeneration and directly inhibit fibrogenesis by stimulation of cell survival, cell proliferation, inhibition of stellate cell activation, and silencing of myofibroblasts[16,19,20,24,39,42,43,48]. Anderson et al. pointed out that the $V^{pos}E^{neg}$ population are more immunosuppressive compared to the $V^{pos}E^{pos}$ population[34]. In line with this statement, we observed higher IGF-1 and TGF-β1 expression levels in the $V^{pos}E^{neg}$ subpopulation compared to the other subpopulations. These genes are thought to stimulate macrophage differentiation to an anti-inflammatory and antifibrotic phenotype and thereby contribute to the resolution of fibrosis[43].

These different gene-profiles might thus also explain the intermediate results as observed for the $V^{pos}E^{neg}$ subpopulation in the *in vivo* experiments. It might very well be that the double positive subpopulation directly targets fibrogenesis by the HGF-mediated mechanisms and that the $V^{pos}E^{neg}$ subpopulation exposes an indirect and delayed anti-inflammatory pathway mediated effect. Further studies are needed to substantiate these observations since the immunosuppressive capacities of MSCs were not evaluated in our studies. In conclusion, our research showed that VCAM-positive MSC subpopulations have advantageous properties for therapeutic interaction with regenerating fibrotic livers compared to VCAM-negative subpopulations, indicating that patients with liver cirrhosis might benefit more from the treatment with VCAM-positive MSC subpopulations. Therefore, in the context of the

resolution of fibrosis it is highly recommended to include VCAM as a selection marker in the characterization panel of MSCs before use.

In addition to the existing subpopulations, previous studies claimed that MSCs are fibroblast-like cells with similar functions in immunosuppression and tissue repair[49]. However, these studies were not related to liver diseases and focussed on basic mechanistic *in vitro* studies[49-51]. In our studies fibroblasts, in contrast to MSCs, were found to be ineffective in resolving fibrogenesis *in vivo* **(chapter 2** and **4)**[26,27]. These observations could very well be correlated to the observed lower expression levels of antifibrotic genes (HGF, VEGF, IGF-1 and TGF-β) in fibroblasts compared to MSCs[13,19]. Overall, our observations illustrate the unique phenotypical and functional features of MSCs compared to fibroblasts.

MSCs also reverse fibrosis in a novel TAA-induced zebrafish embryo model for liver fibrosis

Liver fibrosis and cirrhosis in rodent models are most frequently induced by administration of hepatoxic compounds, such as CCL4 and TAA[31,32]. These models, however, are relative expensive, have a long induction period (6-12 weeks) and have a relatively high work load; they are therefore less attractive for high throughput compound screening[52,53]. To generate a model for liver fibrosis suitable for this purpose, we attempted to translate the widely used CCL4 and TAA models to zebrafish embryos. The experiments illustrated that TAA, in contrast to CCL4, induces fibrogenesis with similar mechanisms as observed in man and rodents (**chapter 4**)[27]. After 6 days of TAA treatment, increased collagen-1α1, Hand-2 and Acta-2 (the fish homologue of α-smooth muscle actin) expression levels were observed, which is indicative for the proliferation and activation of stellate cells and their subsequent differentiation into myofibroblasts, all illustrative for fibrogenesis[52,54-56]. Furthermore, this model also showed smaller liver sizes and increased collagen deposition.

However, the characteristic collagen-filled septa structures as observed in the livers of humans and rodents with liver fibrosis were not observed in our zebrafish embryo model[57]. This difference is very likely due to the different liver architecture between these species. Although, the livers of zebrafish embryos are constructed with the same cells as in humans, these livers are less well organised and miss the typical hexagonal cell organisation[52]. These fundamental differences might very well be the reason for the diffuse collagen deposition as observed in the livers of the zebrafish embryos in our model[52]. Furthermore, similar findings in RNA expression profiles and collagen deposition were observed in the livers of adult zebrafish upon ethanol treatment[56].

The applicability of this model system to analyse novel therapeutic interventions was shown by the administration of MSCs and fibroblasts as potential novel cell therapies for fibrosis. In concordance with our mouse studies (**chapter 2** and **3**) we observed that MSCs, in contrast to

fibroblasts, were able to considerably prevent the progression of TAA-induced liver fibrosis in the zebrafish embryos[26,27]. One of the limitations of our model, however, is that the immune-system of zebrafish embryos is not fully developed. Therefore, compounds that intervene in the immunological pathways during fibrogenesis cannot be tested in this model. Although we have shown the pathological similarities between species and the robustness of our model, newly discovered compounds for the reversal of fibrogenesis identified by this model still need further testing in rodent models. This second step is crucial since the rodent models have a higher resemblance to man and contain a functioning immune-system. Furthermore, the pharmacokinetic and pharmacodynamic differences between rodents and man are better understood. Zebrafish embryos are known to resist higher dosages of certain compounds than rodents and man, which illustrates the difficulty to translate the dosages between these species. Altogether, our observations indicate that TAA induces liver fibrogenesis in zebrafish embryos through mechanisms that are highly comparable to the pathogenesis of liver fibrosis in humans. The proven induction of fibrogenesis together with the low labour intensiveness, cushiness and low costs of this model provide researchers with a rapid model for future mechanistic and therapeutic studies on liver fibrosis suitable for high throughput screening purposes.

Cripto-1: a new player in the pathological pathway of fibrogenesis

As previously alluded to, Cripto-1 (Cripto) is an oncofetal protein and known to stimulate multiple processes including cell differentiation, cell survival and cell proliferation[58-61]. These features are also involved in liver regeneration and fibrogenesis and Cripto was speculated to be also important during liver fibrogenesis[62-64]. This idea was encouraged by the study of Zhang et al. which showed elevated Cripto levels in blood of patients with viral hepatitis induced cirrhosis[65]. In concordance with that study, we also observed elevated Cripto levels in plasma of patients with ALD- or HCV-induced cirrhosis. However, in addition, these elevated levels were found to normalise one year after removing the fibrosed source by liver transplantation (**chapter 5**). Furthermore, for the first time, human-, mouse-, and zebrafish embryo-livers were all found to express Cripto during fibrogenesis, which is indicative for a well preserved role for Cripto in the pathology of hepatic fibrogenesis[66]. In humans, Cripto protein expression in liver tissue positively correlated with the clinical laboratory MELD score for liver disease. Surprisingly, this correlation was not observed between Cripto levels in the blood and the MELD score. Further studies with paired blood- and tissue-samples from patients are needed to verify whether Cripto tissue-expression is reflected by Cripto levels in the blood. These studies might also help to clarify the undetectable Cripto levels as observed in a minority of the tested plasma samples (**chapter 5**). The specific role of Cripto in liver fibrogenesis is still elusive. Based on literature it is known that NANOG is expressed in hepatocytes during fibrogenesis. NANOG is a regulator of Cripto expression, which could thus contribute to the Cripto expression during liver fibrogenesis[66-68].

Another possible explanation might be related to the well-known regenerative capacity of the liver upon tissue injury[62-64]. Recently Zhang et al., observed upregulated Cripto levels in damaged HepG2 cells stimulating the survival and proliferation of the injured cells[61]. One might speculate that Cripto is re-expressed during fibrogenesis in order to survive the injuring stimuli and support tissue regeneration. The studies on Cripto of the present thesis are indicative for an active involvement of Cripto, but further research is required to disentangle whether Cripto has a functionally relevant role in liver fibrogenesis. Such studies might contribute to the identification of new leads for antifibrotic therapy.

Surprisingly, the studies of Kim and Yun et al. both showed increased expression of HGF and VEGF when MSCs were stimulated with Cripto[69,70]. These cytokines are known to have a direct antifibrotic and pro-regenerative effect (e.g., inhibition of the activation and proliferation of stellate cells, inactivation of myofibroblasts and stimulation of hepatocyte survival) and therefore Cripto expression in fibrogenic livers might be the missing link to unravel the working mechanism for MSC treatment of liver fibrosis. In this mechanism, Cripto expressed by the fibrogenic livers may stimulate the MSCs to perform their antifibrotic function by -for example- increasing their HGF and VEGF production (Figure 1)[13,19].

Cripto expression promotes resistance to treatment in HCC

Cripto expression in HCC is corelated to faster tumour recurrence and poor patient survival, but the precise working mechanism(s) are still unknown[60,71]. Suggested mechanisms include Cripto involvement in pathways leading to faster proliferation and onset of epithelial to mesenchymal transition (EMT) of tumour cells[60,66]. The function of Cripto in fibrogenesis might be different than in HCC or one could speculate that the cells expressing Cripto in fibrogenesis are more likely to become oncogenic. Further research is needed to verify this hypothesis.

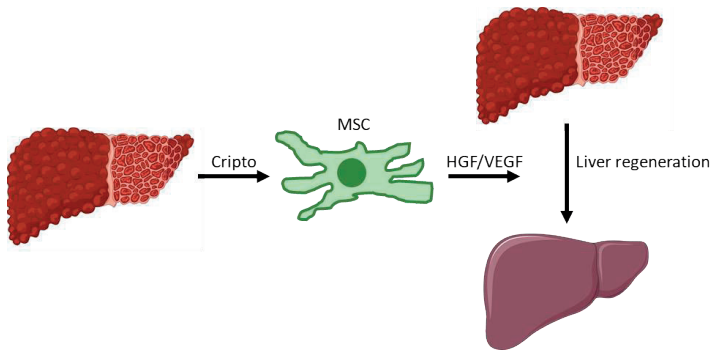

Figure 1. Cripto as one of the driving factors in effective MSC therapy. Cripto expressed by the fibrogenic livers may stimulate the administered MSCs to perform their antifibrotic function by increasing their HGF and VEGF production. These cytokines are known to silence fibrogenesis and to stimulate normal liver regeneration.

Our study revealed that Cripto expression induced an EMT gene profile, with increased proliferation and faster migration of HepG2 tumour cells (**chapter 6**). Furthermore, the PDX mouse model showed that HCCs with high Cripto expression respond less to conventional end-stage systemic therapies such as Sorafenib and that administration of Cripto inhibitors was found to sensitize Cripto-expressing HCCs for Sorafenib treatment[61,66]. However, these observations were based on one PDX tumour and one HepG2 *in vitro* study, thus additional studies to verify these outcomes in a larger cohort are needed.

Not all HCCs express high levels of Cripto and expression sometimes is lower, as observed in non-tumour cirrhotic liver tissues (**Chapter 5 and 6**). This finding illustrates that Cripto expression levels in tissues are less suitable to use as a biomarker for the diagnosis of HCC. Nevertheless, our observations are suggestive for the existence of a more aggressive subgroup of HCCs or HCC cells recognised by their high Cripto expression. Further research is needed, but these findings at least indicate that patients with a high Cripto-expressing HCC may not benefit from Sorafenib treatment.

Perspectives for the future

MSCs possess pro-regenerative, antifibrotic and anti-inflammatory properties. The studies in this thesis particularly focussed on the regenerative and antifibrotic capacities of MSCs in relation to the resolution of fibrogenesis. To further assess these aspects a follow-up study in which mice are sacrificed at multiple timepoints during the regeneration process is needed to unravel the underlying working mechanism of the proposed novel MSC therapy. Possible MSC-initiated effects on proliferation of endogenous liver cells need to be examined at an earlier time point, since in the described studies all the livers were already fully regenerated at the time of examination. Furthermore, the exact cross-talk between the administered MSCs and the fibrotic liver environment needs to be elucidated further. In **chapter 2** we showed that locally administered MSCs form specific regions and did not migrate. In the future it might be possible to select these MSC regions for RNA isolation and subsequent RNA profiling[26]. Another, more indirect approach would be to isolate and profile RNA from MSCs that are incubated with homogenates derived from fibrotic or cirrhotic livers. These experiments might lead to more knowledge of the cross-talk between MSCs and their environment, but might also identify mediators secreted by MSCs important for the resolution of fibrosis. This approach may lead to a cocktail of specific mediators, which possibly may be used for the treatment of fibrosis and cirrhosis instead of using the living MSCs themselves. For example, most of the suggested working mechanisms of MSC therapy are based on HGF and IGF-1 expression. For other diseases, such as amyotrophic lateral sclerosis (ALS) and vocal fold scar, HGF infusion has been demonstrated to be safe[72-74]. With use of the high throughput zebrafish embryo model for liver fibrosis, as described in **chapter 4**, it would be of interest to administer HGF, IGF-1 or newly discovered mediators to assess their therapeutic effect[27].

The characterisation panel of membrane-markers of MSCs differs between studies, which can lead to the use of different subpopulations of MSCs[36,40]. We showed that different subpopulations of MSCs have a different impact with regard to the reversal of liver fibrogenesis **(chapter 3)**. Therefore, it is highly recommendable to study the different subpopulations of MSCs and design different characterisation panels of membrane-markers which are tuned for purpose. For example, based on the present thesis we would suggest to add VCAM as a marker for MSCs for the resolution of liver fibrosis. However, for other purposes it might very well be better to use a different subpopulation of MSCs.

In the coming years more clinical trials, testing the efficacy of different MSC therapies for liver diseases, will be finalised (see clinicaltrials.gov). The results of these trials will lead to more knowledge regarding the effectiveness of MSC therapy. The University of Utah, for example, started a study to evaluate the potential of hepatic artery injection of autologous bone marrow-derived MSCs in patients with alcoholic liver cirrhosis. Another trial performed by the Xijing Hospital of Digestive Diseases is evaluating the effect of systemic (i.v.) administration of MSCs in patients with decompensated liver cirrhosis. These trials are using different administration strategies (portal-/local- and systemic intravenous-administration), and it would be interesting to compare those studies regarding MSC subpopulations and to assess whether the local administration is more effective compared to systemic treatment, as suggested by our studies. Unfortunately, these studies are using different doses of MSCs which might also affect their outcomes.

In **chapter 5 and 6** of the present thesis we observed that hepatocytes express Cripto during fibrogenesis and that Cripto is also involved in the progression and metastasis of HCC. Further research is needed to unravel the pathophysiological role of Cripto in fibrogenesis. Elucidation of the function of Cripto could possibly lead to new insights into fibrogenesis and might lead to alternative therapies for the resolution of fibrogenesis. HCC with high Cripto expression was found to be resistant to Sorafenib therapy, therefore a combination therapy of Sorafenib and Cripto inhibitors is advocated (**chapter 6**)[66]. However, in relation to this proposed treatment the safety of Cripto inhibitors needs to be assessed first. Meanwhile one could reconsider to prescribe Sorafenib to patients with high Cripto-expressing HCCs. Furthermore, it would be of interest to study whether Cripto plasma levels correlate with tissue expression and are able to predict the aggressiveness of HCCs. This would provide clinicians with a relatively easy tool to distinguish Cripto high- and Cripto-low tumours as more or less aggressive, which can be of help to decide on the most optimal treatment.

Finally, it is anticipated that the rapid evolvement of our understanding of fibrogenesis, MSC functionality, regeneration and oncogenesis will lead to novel therapies for liver disease in the near future.

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