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Pro- and anti-fibrotic agents in liver fibrosis

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Chapter 7

General discussion and perspectives

Fibrosis, which progressively deteriorates tissue function and eventually organ failure in various organs (lung, kidney, liver and skin), contributes to around 45% of deaths in the western world [1]. Although the liver has a high capacity for reversing chronic injury, persistent injury can develop to liver fibrosis and further to cirrhosis or even hepatocellular carcinoma (HCC), leading to liver failure, to which currently only liver transplantation is an effective therapy. Although it has long been thought that liver cirrhosis is not reversible, recent antiviral (hepatitis C and B) treatment provides evidence for the reversibility of liver fibrosis, even at the end-stage of fibrosis (cirrhosis) [2]. We believe that elucidating the mechanism of liver fibrosis can contribute to the development of therapies for reversing fibrosis or cirrhosis. However, liver fibrosis is a multicellular process that involves complex pathways, making it challenging to battle this disease. In this thesis, we used *ex-vivo* precision-cut liver slices to unravel the mechanism of gut-liver axis induced liver inflammation and liver fibrosis as well as evaluated two potential small molecules for their anti-fibrotic efficacy in different species.

Liver fibrosis is a multicellular process

Liver fibrosis is characterized by excessive extracellular matrix (ECM) accumulation due to the imbalance in the synthesis and degradation during chronic injury: upregulation of collagen synthesis and reduced degradation (downregulation of matrix metalloproteinase (MMPs) and increase of tissue inhibitor of matrix metalloproteinase (TIMPs)) [3]. When the liver injury is persistent, fibrosis may progress to cirrhosis, which is characterized by bridging fibrosis between portal veins, disrupted liver structure and even limiting or loss of vital liver function [4]. Activated myofibroblasts are considered to be the main player for ECM deposition [5]. Activated myofibroblast can derive from quiescent hepatic stellate cells (HSCs), bone marrow derived cells, portal fibroblast or epithelial-to-mesenchymal transition (EMT) [5]. Among these, HSCs activation is widely accepted as the key driver of liver fibrosis and a promising target cell for medicines to reverse fibrosis [6]. Toxins (*e.g.* carbon tetrachloride), viruses (hepatitis virus B, C), pathogen associate molecular patterns (PAMPs) (*e.g.* lipopolysaccharide (LPS), microbial nucleic acids and peptidoglycan) and oxidative stress or reactive oxygen species (ROS) can stimulate various pathways discussed in the next section that activate HSCs. In addition, macrophages in the liver, resident Kupffer cells and circulating monocytes developed from bone marrow cells, are instrumental in the liver fibrosis development by producing cytokines such as interleukin-6 (IL-6) and transforming growth factor-β (TGF-β) to activate HSCs [7]. Hepatocytes can sense the cytokines secreted by the macrophages, producing TGF-β and ROS to activate HSCs, which in return secrete TGF-β, IL-6 and TNF-α to stimulate hepatocytes to go into apoptosis or necrosis [8]. Taken together, liver fibrosis is a complex multicellular physiological process with intracellular communication to accomplish wound healing, however, an imbalance in this process will lead to a pathological state. The multicellular phenomena in liver fibrosis are shown in Figure 1.

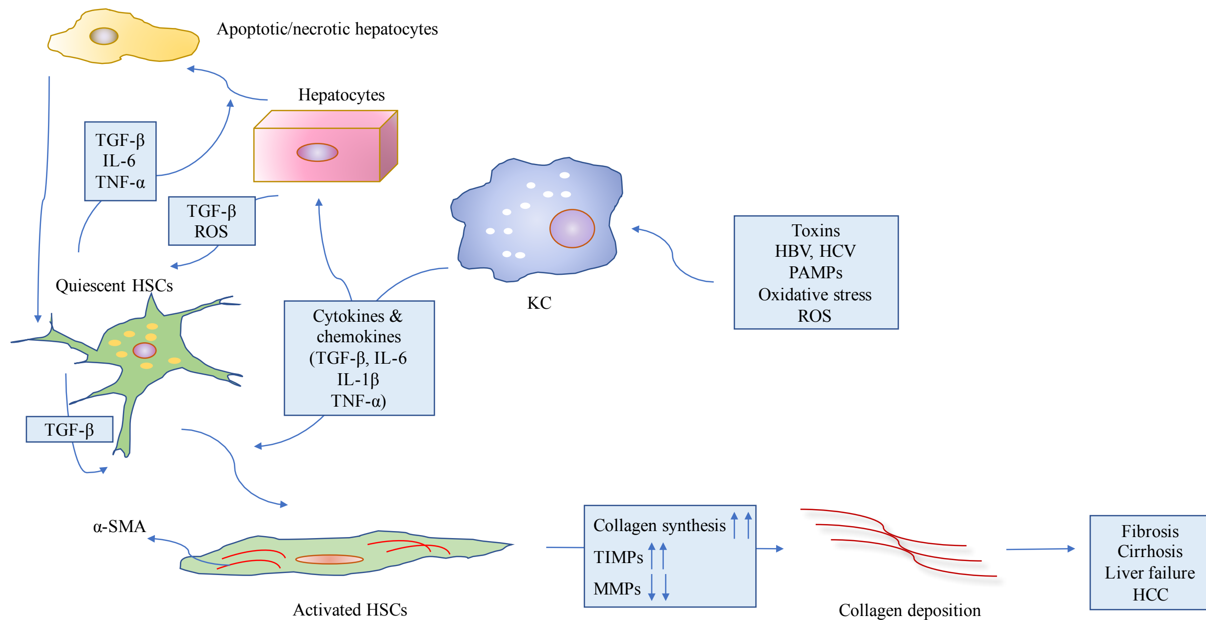

Figure 1: Simplified illustration of multicellular involvement in liver fibrosis
HSCs (hepatic stellate cells; α-SMA (α-smooth muscle actin); TGF (transforming growth factor); IL (interleukin); TNF (tumor necrosis factor); ROS (reactive oxygen species); KC (Kupffer cells); HBV (hepatitis B virus); HCV (hepatitis C virus); PAMPs (pathogen associated molecular patterns); TIMPs (tissue inhibitors of metalloproteinases); MMPs (matrix metalloproteinases); HCC (hepatocellular carcinoma).

Models used in liver fibrosis research

Mechanism of fibrosis and antifibrotic drug efficacy are mostly studied in *in vivo* animal models and *in vitro* in primary cells and cell lines. In table 1 some of the classical models as well as novel developing models in liver fibrosis are listed. *In vivo* animal models for fibrosis commonly used in research include: alcohol-induced liver disease; carbon tetrachloride (CCl_4) induced liver fibrosis; diet induced non-alcoholic fatty liver model; common bile duct ligation model and genetically modified fibrosis models [9]. Although *in vivo* animal models represent the best physiology model of a living animal, the limitations are: it takes a long time to develop (high cost, low throughput); inter-lab variability; risk of non-relevant results that do not or partially reflect the pathophysiology of human liver fibrosis and raises ethical concern for the animal welfare.

In addition, the mechanism of fibrosis and the effect of potential anti-fibrotic drugs are also studied in *in vitro* models. HSCs can be studied using primary isolated cells or immortalized cell lines (*e.g.* mouse NIH 3T3, human LX-2) obtained from animal or human tissue [9]. Primary isolated cells are superior in representing the HSCs *in vivo* compared to cell lines, yet they are much harder to culture or manipulate *in vitro* and have only a limited life span [10]. In contrast, cell lines are normally easy to keep in long-term culture and can be manipulated with genetic tools, they are however poorly correlating with HSCs in their *in vivo* situation [11].

To mimic the multicellular aspect of fibrosis, co-cultures of the various cell types involved represent another model that is used in fibrosis research. Liver cells can be co-cultured to obtain the cell-cell interactions important during progression of fibrosis. Incubating or 3D culturing of hepatocytes with HSCs, Kupffer cells, sinusoidal endothelial cells and a mixture of non-parenchymal cells is possible [12-14]. However, due to the different cellular sources and protocols the validation and reproducibility of these co-culturing systems are a concern [14, 15]. Another possible 3D culture is represented by the hepatic organoids, which are 3D mini livers that can be obtained from actively dividing stem cells (Lgr5+) derived from damaged mouse liver [16]. In addition, the same group also developed organoids from adult bile duct-derived bipotent progenitor cells, which could differentiate into in *vitro* and *in vivo* functional hepatocytes that could engraft into damaged mouse liver [17]. This system provides a promising model for screening drugs for efficacy or toxicity or transplantation of patient derived organoids to a damaged human liver [18, 19]. However, the relevance of this system to the human organ needs to be further explored and validated, since only 33-50% of the cells in the organoid differentiate into functional hepatocytes [20]. In addition, the organoids do not contain HSCs and other liver cells, making them less suitable for fibrosis research. The Liver-on a chip is a 3D micro-physiological cell culture system on a microchip that aims to contain *in vivo* tissue components, mimic the functions, and maintain biochemical signals and the microenvironment [21-23]. Although it is a promising model to replace animal testing, and can be used to study multi-organ interactions or even personalized drug testing, careful validation of the protocols and cellular sources and functional characterization needs to be carried out before it can be widely applied [23]. In addition, inter laboratory variances exist, as there are no standard “chips” available [23].

Compared to all discussed *in vitro* systems, the multicellular character of precision-cut liver slices (PCLS) seem to be more relevant to the *in vivo* situation as they are maintaining the cellular architecture and interactions in their original context. Moreover, human PCLS overcome translation between animal species and men and this makes it an appealing model for investigating the mechanism of fibrosis, anti-fibrotic drug efficacy or toxicity in a setting closer to man.

Even though none of the above-mentioned models are perfect for representing the complex human physiology or pathology, they could provide valuable evidence to elucidate the mechanism of fibrosis.

Table 1: Representative *in vitro* and *in vivo* models of liver fibrosis (modified from [9])

	Models	Advantages	Disadvantages
In vivo			
Chemical induced	Alcoholic	Clinically relevant	Low throughput
	Carbon tetrachloride (CCl_4)	High reproducibility; Close to human liver fibrosis	Ethical concern Low throughput
Diet induced	Methionine-deficient and choline-deficient diet (MCD); High-fat diet; Choline-deficient L-amino acid defined diet	Mimics human non-alcoholic fatty liver disease or steatohepatitis	Inter lab variances due to differently standardized diet Low throughput
Surgery based	Common bile duct ligation	Close to human cholestatic injury	Low throughput
Genetically modified	Mouse multidrug resistance-associated protein 2 (Mdr2) knock-out	Close to human chronic biliary injury	Partly resembling the human pathophysiology
In vitro			
Primary cells	Primary HSCs	Relatively good representation to HSCs *in vivo*	Hard to culture & manipulate Limited life span Limited human material supply
Cell lines	LX-2	Easy to culture & manipulate with genetic tools	Poor correlation with HSCs in *in vivo*
Co-cultures	Hepatocytes with HSCs, Kupffer cells or sinusoidal endothelial cells	Presence of cell-cell interaction	Validation and reproducibility of the system is uncertain
Liver-on a chip	Liver cells with or without cells from other organs	Maintaining *in vivo* microenvironment Real time monitoring of culture system and metabolism Multi-organ interaction	Protocols and platform varies between labs
Hepatic organoids	Stem cell derived hepatocytes	Use of adult cell from patients, no genetic manipulation needed	No presence of non-parenchymal cells Relevance of human organ needs validation
Precision-cut liver slices	All resident cells the liver	Cell-cell interaction Standard protocol Physiologically relevant	Limited life span Limited human material supply

Gut-liver axis and liver fibrosis

Gut microbiota, considered as “the new virtual metabolic organ”, got rapid and global scientific interest because of its undeniable effect on the host physiology and during disease progression [24]. In liver diseases, also the quantity and quality of gut-microbiota changes (dysbiosis) [25]. Especially in liver fibrosis the gut permeability increases during disease progression, this will lead to increase of the pathogen-associated molecular proteins (PAMPs), the components of the gut microbiota, reaching the liver via the portal circulation [26]. This interaction between the liver and the gut is the so-called gut-liver axis. In alcoholic liver diseases, specifically in actively drinking patients with cirrhosis or severe alcoholic hepatitis patients, serum levels of LPS, a component of the Gram-negative bacteria, is dramatically increased, which may lead to liver injury through gut-liver axis [27]. Not only in alcoholic but also in the non-alcoholic steatohepatitis, the inflammation induced by the PAMPs is an important link between the initial metabolic stress, death of hepatocytes and fibrosis [25]. Toll like receptors (TLRs), which are responsible for sensing PAMPs, are one family of pattern recognition receptors in the innate immunity [28]. Most studies that studied TLRs and PAMPs and their effect on liver disease progression are described in Table 2. After activation of TLRs by PAMPs, various cytokines are produced by macrophages. Including IL-6, which promotes survival and proliferation of HSCs, TNF-α responsible for survival of HSCs and IL-1β, which activates HSCs [29-31]. Although inhibiting the inflammatory response is necessary to control the liver fibrosis progression, targeting of an individual cytokine might not be enough to inhibit fibrosis due to the complexity of the inflammatory reaction in liver fibrosis.

Up until now, this discussion has been focusing on the liver side of the gut-liver axis, however managing the dysbiosis in the gut may also be a promising way of improving liver fibrosis progression. Untargeted approaches like changing diet, using probiotics/antibiotics or faecal microbial transplantation are possible to manage the gut microbiota [27]. However, personalized, precision bio-engineered bacterial strains or drugs that target specific bacteria seem more promising to accurately modulate the gut microbiota [32].

Although the gut microbiota is considered to be a bad player in liver disease development, our finding in **Chapter 2** showed a positive role of the gut microbiota of providing a certain level of reduced sensitivity to LPS. Similar to our observation, the detrimental effect of the absence of the gut microbiota on liver pathology has been described: germ free (GF) mice are more responsive to chemical- or alcohol-induced liver injury and biliary tract injury [33-35]. Collectively, when using the GF rodents to study liver fibrosis or gut-liver axis, the hypersensitivity of them to a certain stimulus must be considered.

The gut-liver axis related liver fibrosis is mainly studied in animal models as abovementioned. In **Chapter 3**, we investigated the effect of gut-liver axis derived LPS on human PCLS. During the different stages of liver disease, the LPS derived from the gut-liver axis may play different roles as shown in **Chapter 3**. At the initial state, LPS are promoting both inflammation and fibrosis, initiating the whole spectrum of the liver’s wound healing process; at the end-stage liver disease, LPS can only promote an elevated inflammatory response but will not lead to further progression of fibrosis. The enhanced inflammation might be due to a lower level of

anti-inflammatory factor present (such as interleukin 1 receptor antagonist, IL-1Ra) compared to the situation in the healthy liver; in addition, the cirrhotic liver shows more regeneration-related processes (represented by among others vascular endothelial growth factor (VEGF) and granulocyte-colony stimulating factor (G-CSF)) instead of further progression into fibrosis, unlike what was found for the healthy livers when encountering LPS. Furthermore, LPS upregulated a series of cytokines in the cirrhotic PCLS, and some of these cytokines have a role in cancer progression [36-40]. Thus, LPS might play a role in the progression from cirrhosis to hepatocellular carcinoma (HCC). It remains to be studied whether the above-mentioned effect is also true for other PAMPs.

Results from **Chapter 2** and **Chapter 3** from specific pathogen-free mouse and healthy human PCLS indicate that interleukin-1β (*IL-1β*) and *IL-6* gene expression patterns were similar in mouse and human PCLS treated with LPS; the cytokine expression of IL-1β was similar at 24h with LPS, but not at 48h, where human IL-1β cytokine was still upregulated by LPS while this was not the case in murine PCLS. Tumor necrosis factor-α (*TNF-α*) gene expression was not upregulated at 24h in human PCLS by LPS, but it was enhanced in mouse PCLS at the same time point. However, the protein expressions of TNF-α were similar in mouse and human PCLS: upregulated at 24h and back to control level at 48h with LPS treatment. Our previous study on both rat and human PCLS showed that LPS induced *TNF-α* mRNA at 5h but not 24h, while the cytokine was upregulated at 24h by LPS [41]. These results suggest that there are time differences in the gene and protein expression of cytokines in the liver between rodent and human.

Table 2: Representative toll-like receptors (TLRs) that are involved in liver fibrosis

Receptor	Ligands
TLR-4	Lipopolysaccharide, low-molecular weight hyaluronic acid, heparin sulfate, saturated fatty acid, fibrinogen, fibronectin, heat shock proteins 60 and 70, high mobility group box-1, degraded matrix [42]
TLR-9	Unmethylated CpG DNA found in bacteria or mammalian self DNA [43]
TLR-2	Peptidoglycan [44]

Multiple pathways involved in liver fibrosis

TGF-β signaling pathway is the well-accepted master regulator of liver fibrosis [45]. All the identified three isoforms (TGF-β1-3) have been reported to be involved in fibrosis, among which TGF-β1 is the most prominent one in liver fibrosis [46-48]. The latent TGF-β complex, which is inactive, can be cleaved by proteases (e.g. MMP2&9) to release the active TGF-β; only this active TGF-β binds to TGF-β receptor 2 (TβR2) as a homodimer, recruits and activates TβR1 to activate the downstream signaling pathway [49]. This TGF-β canonical signaling pathway will lead to phosphorylation of SMAD2&3 that in turn binds to SMAD4, which is then translocated to the nucleus to initiate transcription of specific genes that are responsible for activating myofibroblasts and ECM deposition [45]. In an alternative non-canonical signaling pathway, TGF-β receptors activate, among others, mitogen-activated protein kinases (MAPK), phosphatidylinositol-3-kinase (PI3K)/ protein kinase B (AKT) and Ras and Rho-like small GTPases [45]. In this thesis, we characterized the effect of blockade of TβR1 (**Chapter 4**) and TβR1/2 (**Chapter 5**) on liver fibrosis in order to shed more light on the involvement of TGF-β signalling in the mechanism of liver fibrosis and to elucidate species differences in this. Galunisertib, studied in **Chapter 4** in human and rat PCLS, is a TβR1 inhibitor that is aimed for treating hepatocellular carcinoma, and it is still in Phase 2 study, which is estimated to complete before the 1st May 2019. *In vivo* experiments in CCl_4-treated mice showed that galunisertib exhibited an antifibrotic effect: less α-SMA positive cells, reduced fibrotic area and hydroxyproline content; but did not reduce gene expression of *TIMP1* [50]. Consistent with this observation, our results showed that galunisertib can reduce TGF-β signaling in human PCLS, reducing expression of various collagens and the enzymes involved in collagen maturation and fibril formation. On the other hand, not like in the CCl_4 mouse model, gene expression of *TIMP1* was downregulated by galunisertib in human PCLS, indicating a different effect of galunisertib on the collagen degradation enzymes in the two species. Moreover, according to our observation, the spontaneous onset of fibrosis in healthy and cirrhotic PCLS of the rat shares at least one common pathway that is due to TGF-β activation. While galunisertib as well as LY2109761 exhibited excellent antifibrotic efficacy, LY2109761 had an additional inhibitory effect on another pathway, *e.g.* bone morphogenetic protein (BMP) signaling pathway whose role in the fibrosis development is still controversial (either promoting or inhibiting) [51]. In **Chapter 5,** the overall effect of LY2109761 was anti-fibrotic. This data suggests that either BMP signalling is pro-fibrotic or that inhibiting its anti-fibrotic action does not result in increased fibrosis. Even though a broader inhibitory effect could lead to better antifibrotic properties, it might also increase the risk of additional toxicity.

Activation of the TGF-β signaling pathway enhances ROS production in various liver cells [52], amplifying the liver inflammation and fibrosis. On the other hand, ROS transforms latent TGF-β into the active form to initiate the downstream signaling [53]. Thus, scavenging or balancing ROS derived oxidative stress is a challenging yet a potential substantial approach to combat fibrosis, as discussed extensively in **Chapter 6**. Future experiments with ROS-inducing and inhibiting compounds in human PCLS could shed more light on the importance of ROS in fibrosis and the potential of anti-oxidants in fibrosis therapy.

PCLS in fibrosis research

PCLS have been used by us (this thesis and [41, 54-61]) and others [62-64] in fibrosis research, and the results show that the pathways involved in fibrosis are active in this model. An important motivation to use PCLS model is to reduce discomfort in animals by inducing inflammation by LPS in PCLS (**Chapter 2**) thereby avoiding the detrimental effects of LPS *in vivo*. Furthermore, by investigating the effect of LPS in human cirrhotic livers, no diseased animal models with high discomfort had to be used and human specific data were obtained (**Chapter 4**). Reduction in the number of animals and refinement were also accomplished by preparing and culturing liver slices from one animal and studying different conditions in one liver, while in an *in vivo* model only one condition can be studied per animal (**Chapter 2 and 4**). The anti-fibrotic effect of galunisertib *in vivo* [50] was successfully mimicked in human and rat liver slices (**Chapter 4**). In addition, we showed that there are time differences in the gene and protein expression of cytokines by treating PCLS with LPS in the rodent and human liver (**Chapter 2 and 3**). Utilizing human PCLS can contribute to the translation from animal results to man (**Chapter 3, 4 and 5**), however more studies are necessary, when in the end anti-fibrotic drugs are available, to confirm to what extent PCLS represent the *in vivo* situation in fibrosis research. Limitations of the PCLS model are the relative short life span of the slices and the absence of blood-derived immune cells in the PCLS. Extending the life span of human PCLS to 5 days has been achieved by choosing an enriched medium [65] and future research will show if this life span can be further extended. In addition, it might be interesting to culture the slices in the presence of blood-derived immune cells. Currently, we are trying to genetically modulate the precision-cut lung slices on gene and protein levels using siRNA [66], yet the application of this silencing technique in liver slices remains a challenge. Moreover, the scarce availability of vital human liver for preparing slices limits this technique for extensive use in laboratories.

Conclusion

Liver fibrosis is a complex process not only involving various cells, but also multiple pathways: at least innate immune signaling (as discussed above), proliferative and fibrogenic pathways (among others TGF-β) are involved [6]. In addition, reactive oxygen species (ROS) produced by TLRs stimulated macrophages and damaged hepatocytes induce paracrine signals (for instance ROS and TGF-β) to activate HSCs [67] (Figure 1). Thus, targeting one cell type or signaling pathway may not be adequate for fibrosis treatment, multi-target strategy might provide more powerful impact on combatting liver fibrosis. The studies described in this thesis add to the conviction that human and animal PCLS could be a promising model to study the mechanism of fibrosis and exploring antifibrotic agents, and species-difference therein. Moreover, they contribute to the reduction and refinement of the use of experimental animals.

Collectively, modulating the gut microbiota, inhibiting TGF-β signaling pathway and balancing the oxidative stress in the liver would provide promising strategies to treat fibrosis. However, the multi-organ, multicellular and multi-signaling pathway complexity and species differences make it challenging for the researchers to translate the knowledge of basic research into the clinic. By utilizing appropriate models to study the mechanism of fibrosis and to evaluate the drug efficacy of antifibrotic agents would assist the translational process from bench to bed-side.

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