Targeting TGFβ signaling pathway in fibrosis and cancer

Karkampouna, S.

Chapter 2

TGFβ signaling in liver regeneration

Sofia Karkampouna[1,*], Peter ten Dijke[1], Steven Dooley[2], Marianna Kruithof-de Julio[1,*]

[1]Department of Molecular and Cell Biology, Centre of Biomedical Genetics, Leiden University Medical Center, Leiden, The Netherlands
[2]Molecular Hepatology - Alcohol Associated Diseases II Medical Clinic Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany
[*] Corresponding author

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Abstract

Adult organ regeneration occurs in many systems, such as in liver, skin, intestine and heart, indicating that postnatal life is not a static or quiescent state but a dynamic and complex process. The liver is a spectacular organ, exhibiting high regenerative capacity crucial for homeostasis and tissue repair: injuries induced mechanically or chemically, can be completely restored. Regeneration involves extensive cell division, inflammation and extracellular matrix remodeling processes. At the molecular level, one of the key mediators of regeneration response is the secreted cytokine transforming growth factor-β (TGFβ). TGFβ is a profibrogenic and anti-proliferative protein with pleiotropic functions depending on the cellular context. In this review, we discuss the role of TGFβ in the development of the liver and in adult liver regeneration, with particular emphasis on its role in regulation of hepatocyte regeneration and hepatic progenitor cell-induced regeneration. Finally, we give an overview of the current direction of liver research towards cell replacement therapies.

Keywords

Liver- regeneration- TGFβ- hepatocytes- hepatic progenitor cells

Abbreviations

PH	partial hepatectomy
TGFβ	transforming growth factor β
TGFβR	transforming growth factor β receptor
HSCs	hepatic stellate cells
ECs	endothelial cells
BECs	biliary epithelial cells
MFBs	myofibroblasts
HPCs	hepatic progenitor cells
BMPs	bone morphogenetic proteins
FGFs	fibroblast growth factor
SMAD	Drosophila mothers against decapentaplegic
AFP	α-fetoprotein
Hnf	hepatocyte nuclear factor
Foxa	Forkhead box A
HGF	hepatocyte growth factor
EGF	epidermal growth factor
EMT	epithelial to mesenchymal transition
miRNA	micro ribonucleic acid molecule
DDPIV	dipetidyl dipeptidase IV
EpCam	epithelial cell adhesion molecule
CTGF	connective tissue growth factor
PAI-1	plasminogen activator inhibitor-1
OV-6	oval cell protein-6
Trop-2	tumor-associated calcium signal transducer 2
HCC	hepatocellular carcinoma
CCl_4	carbon tetrachloride
iPSCs	induced pluripotent stem cells
iNs	induced neuronal cells
αSMA	alpha-smooth muscle actin

1. Introduction

In the adult organism, the liver has a prominent role in maintaining homeostasis under both normal and pathogenic conditions. Cholesterol and glucose metabolism, lipoprotein, bile acid and serum protein synthesis and secretion are complex functions performed in the liver[1,2]. Dysregulation of these vital functions not only impairs the organ function itself but also affects all other organ systems; this may explain the phenomenal regenerative capacity of the liver throughout adult life. Following 60-70% partial hepatectomy (PH)[3,4] the remaining intact part of the liver can compensate for the tissue loss by restoring the initial size and mass, while maintaining full liver functionality. Interestingly, hepatic progenitor cells (HPCs) are not required for this regeneration process; however, if liver function is impaired HPCs take over the regeneration capability[5,6]. PH is a standardized strategy to study regeneration of the entire organ within a time frame of 5-7 days in rodents[7], in humans it is employed to enhance organ repair[8].

A unique feature of liver regeneration is that it does not depend on a single stem cell population as in other tissues such as blood, intestine, and skin[9,10]. All liver cell types are involved through rounds of mitosis and apoptosis: hepatocytes, stellate cells (HSCs), endothelial cells (ECs), biliary epithelial cells (BECs or cholangiocytes) and macrophages (Kupffer cells). Hepatic cells are organized in symmetric structures, liver lobules, which contain a central vein, in the centre of each lobule, and six surrounding portal triads (portal vein, hepatic artery and bile duct). From early response, through *de novo* synthesis of transcription factors to termination of proliferation and size adjustment, each step is tightly regulated. Cell-cell and cell-extracellular matrix interactions are crucial for the liver architecture. After injury, the hepatocytes, the main functional liver cell type, become apoptotic and release signals that stimulate cell proliferation of the remaining hepatocytes. Cell proliferation follows a consistent pattern, starting from the portal triads of the liver lobule and proceeding towards the central vein. Hepatocytes maintain high contact with sinusoidal cells during regeneration, a process named hepatocyte-sinusoid alignment[11], ECs and BECs start proliferating, stellate cells progress from a quiescent to an activated state, differentiating to myofibroblasts (MFBs). MFBs contribute to the final stages of liver regeneration as they are responsible for extracellular matrix protein synthesis such as collagen, fibronectin, laminin, integrins[12] and matrix protein remodeling. Only after chronic liver damage, when the hepatocyte proliferation is exhausted, the hepatic progenitor cells, also referred as HPCs in the mouse, become activated and can differentiate into both hepatic and cholangiocytic lineages.

Despite the high regenerative capacity of adult liver, compared to other organs, liver abnormalities such as cirrhosis and liver cancer still occur with high incidence. Epidemiologic data indicate that hepatocellular carcinoma is the fifth most prevalent type of cancer and the second cause of cancer death worldwide[13]. The puzzle of the molecular mechanisms controlling liver regeneration has only begun to assemble and our current understanding is that several signaling pathways are involved and act dependently to each other. One of the main orchestrators of the injury-induced response in the liver is transforming growth factor β (TGFβ) signaling pathway and in this review, we will discuss the recent advances regarding the function of TGFβ during regeneration and how this knowledge can be used in cell replacement therapies. Bone morphogenetic proteins (BMPs), also members of the TGFβ family, while important players in liver regeneration, will not be discussed and we refer instead to other reviews[14,15]. Moreover, fibrosis and hepatocellular carcinoma will not be

addressed in this review[16]. Finally, the role of other signaling pathways, such as Fibroblast Growth Factor (FGF) and Wingless/Integrated (Wnt) will be addressed by others in this edition.

2. Liver architecture and function

2.1. Hepatic development

During embryonic development, the formation of definitive endoderm at gastrulation is the first step towards hepatogenesis. Endoderm germ layer forms a primitive gut tube consisting of three subdomains, the foregut, midgut and hindgut and gives rise to liver, pancreas, lungs and thyroid tissue[17]. The first hepatic formation, called the hepatic diverticulum, can be distinguished, around 9 dpc, adjacent to the developing cardiac tissue. Fetal hepatic progenitor cells or hepatoblasts, that will generate the main functional liver cell types, hepatocytes and BECs, delaminate from the epithelium at 9.5 dpc and migrate to the septum transversum mesenchyme (STM) to form the liver bud. During 10-15 dpc, the liver bud undergoes growth and eventually becomes the main site for fetal hematopoiesis until bone marrow takes over just before birth[18].
The embryonic liver originates from the ventral foregut[19]; however, liver tissue is in fact a mosaic of both endoderm and mesoderm-derived cell types. Epithelial cells (hepatocytes and BECs) derive from endoderm, while hepatic mesenchymal cells such as HSCs, Kupffer cells and blood vessels, have mesodermal origin[20]. Interactions among these two embryonic layers activate molecular pathways that are important for proper patterning of the liver. Initially, inhibition of mesodermal Wnt and FGF4 signaling in the foregut enables liver and pancreas induction, whereas active mesodermal Wnt signaling in the posterior gut suppresses these tissue fates[21]. Retinoic acid signaling, produced from paraxial mesoderm cells, assists in the anterior-posterior positioning of liver and pancreas[22]. In the ventral foregut, FGF signals from cardiac mesoderm and BMP signals from septum transversum mesenchymal cells induce the liver program[23,24]. Mesodermal-derived BMPs affect the levels of the endoderm specific gene Gata4, therewith blocking pancreatic and inducing liver specification, which is one of the key processes where TGFβ/BMP is required during hepatogenesis[24].

2.2. Role of TGFβ during liver development

TGFβ ligands (TGFβ1, TGFβ2 and TGFβ3) bind and activate TGFβ type I and type II Ser/Thr kinase receptors. Upon stimulation with TGFβ, the type II receptor phosphorylates the type I receptor which phosphorylates receptor-regulated SMADs (R-SMADs). The TGFβ pathway specific R-Smads, Smad2 and Smad3 subsequently form heteromeric complexes with the common co-SMAD or Smad4. These heteromeric complexes then accumulate in the nucleus, where they participate in transcriptional regulation of gene expression[25,26]. Inhibitory Smad7 antagonizes the activation of R-Smads[27].
TGFβ is part of a large superfamily of proteins that includes, TGFβs, activins, nodal and BMPs. Activins and nodal, like TGFβ, signal via R-Smad2 and Smad3, whereas BMPs signal via R-Smad1, R-Smad5 and R-Smad8[25].
TGFβ/Nodal signals are present in the embryo already at onset of gastrulation, inducing endoderm formation[17,28] and their disruption leads to abnormalities in all endoderm-derived organs. Regarding the liver, TGFβ ligands are expressed in the bud mesenchyme[29]. Embryos with double heterozygous mutations in Smad2 and Smad3 nuclear effectors of TGFβ

downstream signaling, have impaired liver organogenesis due to defective endodermal competence[30]. Proper dosage of Smad2 and Smad3 in the embryo is necessary for activation of Forkhead family member Foxa2 (Hnf-3) and Homeobox class protein (Hex) expression, two genes required for hepatic development[31]. Foxa2 is expressed early in development and regulates activation of hepatocytic gene expression program, including that of Hex and α-fetoprotein (AFP)[31,32]. In detail, Foxa2 binds to p53/Smad binding element sequences (SBE) of the AFP gene[33,34] and the related Foxa1 transcription factor also interacts with TGFβ-activated Smad2/Smad4 complexes to facilitate chromatin access and subsequent DNA binding and gene regulation[35], suggesting the possibility of Smad proteins to cooperate with other transcription factors towards activation of AFP during development. AFP protein has a crucial developmental function and is expressed in fetal and neonatal mouse hepatoblasts but is silenced in adult hepatic progenitor cells or mature hepatocytes[36].
However, reactivation can occur in HPCs of adult regenerating rat liver following liver damage[37]. Also, in human hepatocellular carcinoma, postnatal silence of AFP is activated and facilitates re-entry into the cell cycle of differentiated hepatocytes[34]. Interestingly, once these cells are stimulated with TGFβ1, endogenous AFP protein expression is diminished[34]. TGFβ directly participates in the regulation of postnatal AFP repression and disrupting its signaling pathway leads to reactivation of AFP. Therefore, TGFβ signaling via Smad proteins is important not only for activation of developmentally important genes, such as AFP, but also for repression of these genes after birth.
From another developmental aspect, studies on liver and pancreas organogenesis showed that progenitors of each lineage emerge from neighboring parts of the ventral foregut endoderm[38]. TGFβ/BMP and FGF signaling act in parallel and dynamic ways to restrict specification of hepatic and pancreatic progenitors[39]. Moreover, the TGFβ pathway plays an important role in hepatoblast specification[40,41]. Differentiation of bi-potential hepatoblasts to either hepatocytes or BECs fate follows a very distinct, spatial pattern. Hepatoblasts of liver parenchyma give rise to hepatocytes, while hepatoblasts located in the mesenchyme next to the portal vein, differentiate into BECs[30,40]. In particular, high activin/TGFβ signaling near the portal vein is needed for differentiation of BECs. On the contrary, activin/TGFβ must be suppressed in liver parenchyma by combined action of Hnf6/OC-1 and OC-2 transcription factors in order to permit hepatocyte generation[40,42]. Thus, TGFβ is involved in multiple aspects of hepatogenesis, such as hepatic competence of endoderm and lineage specification of bipotential hepatoblasts and keeps hepatic gene expression tightly regulated.

3. Liver regeneration

Liver regeneration is a perfectly orchestrated process, able to restore liver architecture and mass within a very short period in rodents and human. Organ re-growth occurs in mammals and reptiles and usually recapitulates mechanisms of embryonic development[43]. Liver regeneration does not rely on an embryonic-like multipotential cell population, but mainly depends on the regenerative capacity of fully differentiated cells such as hepatocytes, HSCs and BECs. In the adult liver, mature hepatocytes are normally quiescent but they rapidly start proliferating after tissue damage. Their ability to reactivate telomerase activity prevents accumulation of mutations during cell divisions[44]. The combined action of all liver cell types is sufficient to reconstitute liver mass loss after liver damage induced by different causes such as viral infections, metabolic problems and 70% hepatectomy [8].
Adult liver regeneration and hepatogenesis are quite distinct processes, with differential

requirements regarding molecular signals, expression status and properties of the involved cell types. Although, regeneration is considered a postnatally-restricted process, recent studies[43,45-48] discuss the potential capacity of embryonic liver for regeneration, in terms of cell proliferation and organ size growth as a response to injury or developmental defects. Such a paradigm was addressed in chimeric embryos generated with Hex null cells. The Hex gene is required for the formation of liver bud epithelium and thus, Hex chimeric embryos have disrupted integrity of the epithelium. Hex-null cells cannot form pseudostratified epithelium and were excluded from the epithelium by wild-type cells, which respond by increasing their proliferative rate in order to allow regrowth of the liver bud[47]. Therefore, hepatic endoderm cells can undergo compensatory growth to maintain the appropriate size of the developing liver bud, which indicates liver regeneration occurrence in the embryo. Another study has provided evidence of regrowth of developing liver following partial destruction of hepatic progenitor cells right after their emergence. Such elimination of progenitors was expected to lead to a smaller organ as it was observed with pancreas[43]. In contrast, by birth the liver had expanded up to the normal size, comparable to that of wild type animals. In addition, rat embryos that have undergone in utero partial hepatectomy have the potential to restore liver organ size within two days[48]. It is remarkable that not only adult liver has regenerative capacity but also embryonic liver can compensate for progenitor cell and growth factor deficiency, even at the crucial stages of embryogenesis.

3.1. Molecular mechanisms controlling liver regeneration- implication of TGFβ signaling

The liver regenerative response is mediated by many signals. The main growth factors involved are hepatocyte growth factor (HGF), epidermal growth factor (EGF), interleukin-6 (IL-6) and TGFβ[49]. TGFβ ligands play a controversial key role in adult liver regeneration. A summary of the genetic models of TGFβ family members and the liver developmental or pathologic phenotype can be found in **Table 1**. In early stages of regeneration, the hepatocytes must proliferate, therefore the inhibitory proliferation signals exerted from TGFβ are not needed, however, a few hours after liver damage, TGFβ is expressed in both liver and serum[50], suggesting a central role for regeneration. TGFβ is also required at the end of this process for induction of EMT, inflammation, restoration of the original tissue architecture and cell-cell interactions[51,52]. The implication of TGFβ signaling in liver regeneration has been studied using different approaches, depending on the type of liver damage and cell type response (**Table 2**).

There is equilibrium among hepatic growth factors and mitoinhibition by TGFβ signaling. During regeneration initiation, cells must immediately divide in response to injury, thus, there is requirement for growth factors such as HGF and EGF. At this time point, TGFβ is upregulated, but the synchronous activation of HGF causes urokinase activation dependent removal of TGFβ. Urokinase protease degrades extracellular matrix proteins, in particular decorin, an inhibitory TGFβ binding protein[53]. TGFβ is, therefore, released from the liver matrix into the blood stream to allow cell proliferation and growth induction by HGF[8,54], firstly removed from the portal area and progressively from the central area[55], allowing hepatocyte proliferation[56].

A required signal for termination of cell proliferation is the shift of expression balance from HGF towards TGFβ-like factors. The purpose is to allow transfer of TGFβ back to the liver parenchyma to block further hepatocyte proliferation and restore cell-cell interactions and

extracellular matrix integrity[57]. The signals regulating inhibition of HGF and influx of TGFβ from circulation into the liver parenchyma are mainly unknown, but it is likely that a direct reciprocal antagonism between HGF and TGFβ components exists. In lung fibroblasts, TGFβ directly downregulates the expression of HGF[58], which could also be the case in the liver. Molecular antagonism among HGF and TGFβ signals converges at the level of common miRNAs; HGF inhibits the fibrogenic action of TGFβ-induced myofibroblast transition and epithelial to mesenchymal transition (EMT) by upregulating miRNA-29. TGFβ itself aborts HGF inhibition by directly downregulating miRNA-29[59]. Other miRNAs have also been identified as regulators of TGFβ1/Smad3 activation during termination of regeneration[60]. The pro-fibrogenic and inflammatory action of TGFβ and its importance for regeneration termination has been extensively reviewed[8]. In this review we will highlight the role of TGFβ signaling in regeneration of both hepatocytes and hepatic stem/progenitor cells.

3.2. Regulation of hepatocyte regeneration by TGFβ signaling

TGFβ-like factors, particularly TGFβ1, are produced at high levels in regenerating liver[50] by nonparenchymal cells, such as HSCs, and act in a paracrine way exerting its antiproliferative effects by inducing apoptosis of mature hepatocytes (**Fig.1A**)[61] via Smad and p38/ mitogen-activated protein kinase (MAPK) dependent mechanisms[62]. Functional roles of Smad2 and Smad3 in normal and regenerating adult liver was addressed by hepatocyte-specific inactivation of either or both Smads, which indicated that Smad3 is required for TGFβ-induced apoptosis, whereas Smad2 is dispensable[63]. Administration of TGFβ1 and TGFβ2 *in vivo* after PH causes a reduction of the fraction of hepatocytes that progress from G1 to the DNA synthesis phase during regeneration[64]. This inhibitory effect is transient and reversible, causing a delay but not complete abolishment of regeneration. Enhanced proliferation of hepatocytes, observed in presence of dominant negative TGFβ receptor mutants[65] and hepatocyte specific conditional deletion of TGFβRII[57], supports the hypothesis that TGFβ1 sustains quiescent hepatocytes in a differentiated state. Apoptosis induced by TGFβ is necessary as a growth stop signal for termination of regeneration process, but apoptosis must be circumvented at the early phase of regeneration, since hepatocytes must rapidly start proliferating at approximately 12-16 hours after injury[66]. Interestingly, during that stage, hepatocytes were described resistant to mitoinhibition of TGFβ either by downregulation of TGFβ receptors, TGFα, norepinephrine protective action[67] or upregulation of transcriptional repressors[68]. Apoptotic signals induced by TGFβ in hepatocytes require reactive oxygen species (ROS) production mediated by NAPDH oxidase NOX4[69]. Anti-apoptotic signals such as phosphatydilinositol-3-phosphate kinase or the MAPK/extracellular signal-regulated kinase (ERK) pathways inhibit TGFβ-induced NOX4 expression[70]. Such or similar mechanisms would secure normal liver regeneration even in case of TGFβ overrepresentation[71]. Disruption of TGFβ signaling by conditional knockout of TGFβRII affects hepatocyte proliferation[57], but not termination of regeneration[72]. Downstream molecules Smad2 and Smad3 may operate even when TGFβ signaling is eliminated, due to stimulation from Activin A. Only combined inhibition of TGFβ and Activin A results in high proliferation of hepatocytes and delay of termination, implicating Activin A as a TGFβ equivalent regulator of regeneration[72]. Although it seems that presence of other signals (e.g. Activin A) will compensate for TGFβ ablation at the termination stage of liver regeneration, it should not be concluded that TGFβ signaling is not required.

A remarkable aspect of liver regeneration is the transdifferentiation capacity of differentiated

hepatic cells into other cell types. Transdifferentiation may occur among hepatocytes and BECs. Failure of BEC regeneration, due to bile duct damage, induces hepatocyte transdifferentiation into bipotential progenitors which give rise to BECs facilitating their regeneration[73,74]. Gradual loss of hepatocyte-specific and acquisition of BEC-specific transcription factors[54] has been proposed as a mechanism for hepatocyte-to-BEC transdifferentiation. In the opposite direction, when hepatocytes are incapable of proliferation, BECs transdifferentiate into hepatocytes via an intermediate progenitor stage, by altering their molecular signature. Therefore, mature BECs and hepatocytes, both of which are embryonically derived from multipotent hepatoblasts, represent facultative stem cells for each other after cell damage[75-77]. This provides a paradigm of adult, fully differentiated cells, which retain and activate stem cell properties exclusively to replenish the needs of the adult organism. Indeed, the liver has evolved a unique regenerative capacity to guard its complex and vital functions and has fully capability to regenerate within. Studies on hepatocyte transdifferentiation towards BEC lineages suggest TGFβ1 signaling to participate in this process[54]. Cell tracking was performed in dipetidyl dipeptidase IV (DDPIV) chimeric rats that carry a DPPIV-positive population of hepatocytes transplanted from donor DPPIV positive rats. Hepatocytes and BECs of the recipient DPPIV negative rats do not express DPPIV. Following BEC injury by BEC-specific toxin administration and bile duct ligation, ~20% of the BEC population turned DPPIV-positive, indicating that they are derived from transdifferentiated DPPIV-positive hepatocytes[54]. An induction of TGFβ1 was observed in the hepatocytes at the area surrounding the repairing biliary ductules, resembling its expression pattern during development where high TGFβ1 signaling is also observed near the portal vein and is considered responsible for differentiation of hepatoblasts into biliary cells[54]. Thus, appearance of BEC clusters positive for the hepatocyte marker DPPIV provides strong evidence that BECs are derived from hepatocytes and that the apparent induction of TGFβ1 at the damaged portal area may be crucial for switching between hepatocyte, bipotential progenitor and BEC gene expression programs. However, the transdifferentiation concept of hepatocyte-to-BEC conversion was recently disputed by Malato et al.[78] The authors used an in situ hepatocyte fate-tracing model. They assessed that all newly formed hepatocytes are derived from preexisting hepatocytes, that bile duct proliferation is necessary for the emergence of liver progenitor cell–derived hepatocytes and could not find evidence for hepatocyte-derived biliary epithelial cells or liver progenitor cells (double labeled EYFP and CK19 where not detected in this system, indicating that EYFP positive hepatocytes are not converted into BECs).

TGFβ1 signaling may promote another transdifferentiation event, the conversion of fetal hepatocytes into a putative liver progenitor population, at least *in vitro*[79]. Fetal hepatocytes can circumvent growth inhibition and apoptosis by TGFβ1 if cultured in serum containing media. TGFβ-treated cells display a mesenchymal phenotype with loss of liver specific transcription factors (Hnf1α, Hnf4α, and Hnf6) and downregulation of gene expression signatures characteristic of differentiated hepatocytes, such as albumin and AFP. These cells are considered dedifferentiated cells, with induction of stem cell marker expression[79], according to the authors representing a population of liver progenitors derived from hepatocytes under stimulation with TGFβ1. They further propose that such progenitors, *in vitro* retain differentiation potential back to hepatocytic cell fate, and could be used as a tool for isolation of such cells, although the molecular mechanisms behind this process remain unclear. Similar spontaneous hepatocyte mesenchymal transdifferentiation events that were enhanced with TGFβ have been reported for mouse hepatocytes that were cultured as monolayer. In an EMT-like process, hepatocyte features like albumin and transferrin

expression were lost with culture duration, whereas vimentin and collagen expression were established[80-84]. In this setting, TGFβ drives a so called late signature, including direct Smad target genes, like connective tissue growth factor (CTGF), plasminogen activator inhibitor -1 (PAI-1), Snail[80,85], which has been associated with poor prognosis for HCC patients[86]. If, however, such scenario as described for cultured hepatocytes similarly occurs *in vivo* remains to be proven.

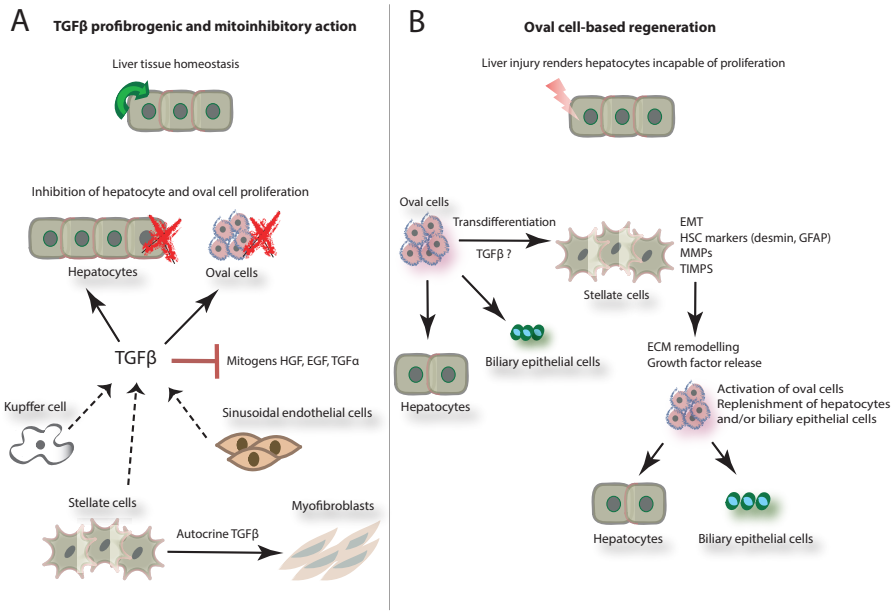

Fig.1. TGFβ profibrogenic and mitoinhibitory action in (A) quiescent state and (B) after liver injury induction (A). Stellate cells (HSCs) produce TGFβ ligands, which in an autocrine manner promote transdifferentiation of HSCs into MFBs and in paracrine way acts upon hepatocytes and biliary cells (not indicated here). Other cell types also produce and secrete TGFβ, such as Kupffer cells and sinusoidal ECs. The anti-proliferative role of TGFβ on hepatocytes and HPCs is mediated by antagonism with HGF, EGF, TGFα mitogens. (B). HPC/oval cell-based regeneration for replenishment of non-replicative hepatocytes after extensive liver injury. TGFβ signals keep HPCs in quiescent state; however, there is evidence that TGFβ assists regeneration by promoting transdifferentiation of a subset of HPCs into HSCs. The production and remodeling of ECM by HSCs causes release of growth factors, which activate remnant HPCs to proliferate and differentiate into hepatocytic and BEC lineages.

3.3. Regulation of oval cell response by TGFβ signaling

Hepatocyte proliferation is the first key regenerative response; however, after extended and chronic liver damage replicative senescence leads to problematic cell regeneration[87]. Hepatocytes incapable of proliferating, such as in prolonged hepatitis infections, cirrhosis, non-alcoholic and alcoholic fatty liver diseases[16], are replenished by hepatic progenitor cells (HPCs), which are not present in normal adult liver nor required for normal cell turnover[78]. HPCs or oval cells are believed to be bipotent epithelial cells that give rise to hepatocytes and BECs similarly to hepatoblasts. Such cells have been identified in the adult mouse and rat liver and there is debate concerning their identity, whether they comprise hepatic stem cells or transit amplifying cells[78,88].

Following liver injury, HPCs emerge around the portal vein and the canals of Hering, they express both hepatic (albumin, transferrin) and BEC specific markers (CK19 and EpCam) and also unique markers such as OV-6 and Trop2[89]. A population of Sox9-positive liver progenitors has been identified around the bile duct area during the regenerative process and is able to differentiate into hepatocytes in response to different types of hepatic injuries[77], however it is not clear whether these Sox9-expressing progenitors comprise HPCs or precursors of HPCs. Further, cells expressing Oct3/4 stem cell markers have also been identified exclusively in the periportal tract during liver regeneration [90].

Spatial specification of adult stem or progenitor cells in the periportal area of the liver lobules and the canals of Hering suggest the existence of a stem cell niche or morphogenic signals that allow stem cell activation[91,92]. In regenerating liver, upon hepatic damage due to acetaminophen (APAP) administration, label retaining assays show the *in vivo* existence of four epithelial stem cell niches, that is the proximal biliary tree, intralobular bile ducts, periductal mononuclear cells and peribiliary hepatocytes[91]. It is conceivable to hypothesize that the liver regenerative capability is mediated by host stem/progenitor, located in intra-hepatic niches that respond to microenvironment signals and contribute to the efficiency and flexibility of liver regeneration. Regarding TGFβ1 and its potential regulation of HPCs activation, it is essential to have insight on whether the spatial expression pattern of TGFβ and its downstream effectors coincide around the stem cell niches.

Indeed, TGFβ1 is expressed in rat HSCs surrounding HPCs, which migrate from the site of emergence, the periportal area, towards the pericentral part while differentiating into ductile or hepatic cells[93]. To study the effect of TGFβ1 on HPCs, Park et al., used a model of PH combined with hepatocyte ablation by administration of acetylaminofluorene (2-AAF-PH). A scenario was suggested as following; hepatocyte damage promotes HPC generation and HSC activation. HSCs secrete TGFβ1 which acts in a paracrine way to induce apoptosis of HPCs thus inhibiting their activation[93].

The stem cell compartment of human liver, as identified by expression of stem cell markers Oct4 and Stat3, also expresses the TGFβ members TGFβRII and ELF[94]. Heterozygous Smad3-adaptor protein β2-spectrin (β2SP)/ELF deficient mice have an expanded population of Oct3/4 positive progenitor cells residing in the periportal region, which highlights the inhibitory role of TGFβ on proliferation of progenitor cells[90]. Surprisingly, given the negative effect of TGFβ on HPC activation, disruption of TGFβ signaling, particularly of β2SP, does not enhance regeneration but instead causes a significant delay in regenerating human liver and in mice after PH[95]. Another aspect that highlights the role of TGFβ in liver regeneration is the increasing spatial expression of TGFβRI[55] and β2SP from periportal to central areas, which is in line with the wave of proliferating cells. Undoubtedly, TGFβ is important for tuning liver regeneration but there is a dichotomy of the TGFβ effects on differentiation, emergence of stem/progenitor cells, apoptosis, cell proliferation, which is depending on the cell type and stage of damage or disease. For instance, TGFβ ligands affect the regeneration process by stimulating apoptosis of both hepatocytes and HPCs[76] although both cell types may exhibit a differential response and sensitivity to TGFβ1, which is dependent on the cell physiological stage. Thus, in hepatocellular carcinoma, HPCs emerge and are highly proliferative in presence of the "growth inhibitor" TGFβ1. Interestingly, HPCs respond to TGFβ1, as seen by nuclear localization of phosphorylated Smad2, however they are insensitive to its mitoinhibitory actions (Ki67 staining), as compared to hepatocytes[96]. The difference in sensitivity between hepatocytes and hepatic progenitor cells in such settings has been attributed to the absence of inhibitory Smad6 in hepatocytes[96]. Thus, inhibition of hepatocyte

proliferation and the regenerative response mediated by TGFβ is circumvented by hepatic progenitor cell activation, thus providing a rescue mechanism to safeguard liver repair.
This further indicates, that apoptosis and growth control exerted by TGFβ1 signaling on hepatic progenitor cells is only one aspect, since TGFβ1 may promote transdifferentiation or differentiation of hepatic progenitor cells into other lineages, e.g. towards the HSC lineage[97]. In presence of TGFβ1, hepatic progenitor cells not only upregulate ECM genes, such as collagen, matrix metalloproteases (MMPs) or tissue inhibitor of metalloprotease (TIMPs), but also induce expression of HSC markers such as desmin and glial fibrillary acidin protein (GFAP). Such conversion of epithelial hepatic progenitor cells to mesenchymal HSCs results from an EMT process that is at least partially orchestrated by TGFβ signaling[97].
Activation of HSCs is highly correlated with the hepatic progenitor cell response during injury, e.g. upon induction in the 2-AAF/PH model, when hepatocyte function is compromised. HSC activation requires TGFβ and the generated myofibroblasts can be detected around the portal vein area and in the proximity of hepatic progenitor cells. Stellate cells excrete MMPs and TIMPs, which by ECM remodeling cause release of bound growth factors that act on HPCs and, e.g., enhance their proliferation[98]. Selective inhibition of HSC activation causes diminished HPC activation (OV-6 and AFP positive) in the periportal area and thus, severely interferes with the regenerative response[99]. Hepatic rat stem cell lines when exposed to TGFβ increase the expression of differentiation markers such as albumin and tyrosine aminotransferase[100], suggesting TGFβ as a signal required for differentiation of hepatocytes from HPCs.
In summary, TGFβ has a direct negative effect on proliferation of hepatocytes and HPCs (**Fig.1B**). During regeneration, these cells lose sensitivity to the TGFβ cytostatic program and continue to divide in presence of TGFβ. The action of TGFβ in liver regeneration should not be reduced to its proapoptotic and growth inhibitory signal, as it is usually done. The contribution of TGFβ signaling in regeneration could be exerted by activating HSCs, which in turn promote HPC activation, directing them towards hepatocyte differentiation. Thus, TGFβ has pleiotropic functions depending on the cell type and the physiological situation of the organ. Therefore, the molecular mechanism of TGFβ action should be studied in detail in such context. Further knowledge on the supporting role of TGFβ during regeneration and dissecting its interplay with other signaling pathways such as HGF and EGF can provide insight on improved possibilities for manipulation of cell regeneration processes for therapeutic applications.

4. Hepatocyte reprogramming and liver regeneration- New prospective

With liver transplantation being the only therapy and the reduced availability of liver donors, research has shifted focus on cell replacement therapies[101]. Regarding the liver, this is particularly promising because of the powerful regenerative characteristics of differentiated liver cells such as hepatocytes. In this respect, we do not need to rely on a limited number of stem or progenitor cells but can use a source of hepatocytes, the main functional cell types, as a therapy. A barrier to this approach, however, is the incapability of hepatocytes to be maintained or expanded *in vitro*.

Several studies have managed to generate hepatic-like cells by different approaches, from embryonic stem cells, induced pluripotent stem cells or even from pluripotent germ cells[102-106]. Homogeneous populations of functional hepatic-like cells have been derived from human ES cells via a process mimicking endoderm development and hepatic specification[107]. Fetal liver stem/progenitor cells when transplanted into adult rat liver, display efficient differentiation into mature hepatocytes and high repopulating capabilities[108]. Cell lineage reprogramming of somatic cells into induced pluripotent stem cells (iPS cells) by modulation of transcription factor dosage is a promising therapeutic approach for liver diseases. Recently, direct reprogramming of mouse fibroblasts to hepatocytes (iHeps) by overexpression of two transcription factors in different combinations, Hnf4a together with Foxa1, Foxa2 or Foxa3[109] or by transduction of Hnf1a, Gata4, Foxa3 and inactivation of p19Arf has been achieved[110]. Personalized medicine with a patient-specific hepatocyte source would limit the risk of organ rejection and eliminate the need of an external source of hepatocytes. However, *in vivo* functionality and stability of the properties of these cells have to be further validated before any clinical translation. Another recent study to address the importance of generating "self" hepatocytes for transplantation purpose has been carried out by Liu et al.[111]. The authors derived iPS cells from different cell types, which retained similar epigenetic signatures. As proof of principal for retention of hepatic functionality of these differentiated cells, the authors intravenously infused mice after liver damage. Remarkably, the cells integrate in the regenerating liver with an efficiency of 8 to 15%, which is comparable to that of hepatocytes (11%)[31]. These encouraging observations point in the direction of "self"-derived hepatic like cells as clinically applicable in liver regeneration.

Finally, hepatic generation by non-hepatic cell types has also been accomplished from human mesenchymal cells[112] or by cell fusion with bone marrow derived cells[113].

An exceptional paradigm of the plasticity of hepatocytes is their potential to transdifferentiate into neurons (iN) after ectopic expression of neuronal transcription factors (Ascl1, Brn2, Myt1l)[114]. Interestingly, genes inducing iPS phenotype such as REST, Oct3/ 4, cMyc and Nanog are expressed in cultured primary hepatocytes and control cell proliferation and apoptosis *in vitro*, but may also control liver regeneration *in vivo*[115]. Another therapeutic option—relative to cell transplantation—would be to stimulate hepatic progenitor cells as a resident source, thus, avoiding the drawbacks of limited graft survival, restricted homing to the site of injury and host immune rejection. So, the hepatic progenitor cell population supported from the environment could possibly be induced to proliferate and transdifferentiate into major hepatocyte/ BDC by exogenous stimulation of stemness factor expression, to provide a resident cell based therapy, as previously successfully described for cardiomyocytes in adult heart injury[116].

The remarkable cell plasticity and regenerative ability of hepatocytes shows that they are an excellent tool for cell based therapies for liver diseases (**Fig.2**), however, efforts should also focus on the therapeutic use of hepatic progenitor cells as they have even higher proliferative potential than hepatocytes and are less susceptible to inhibition by TGFβ signals. Further knowledge of the intrinsic signals controlling liver regeneration is needed, to provide a way to manipulate these signals and stimulate *in vivo* regeneration.

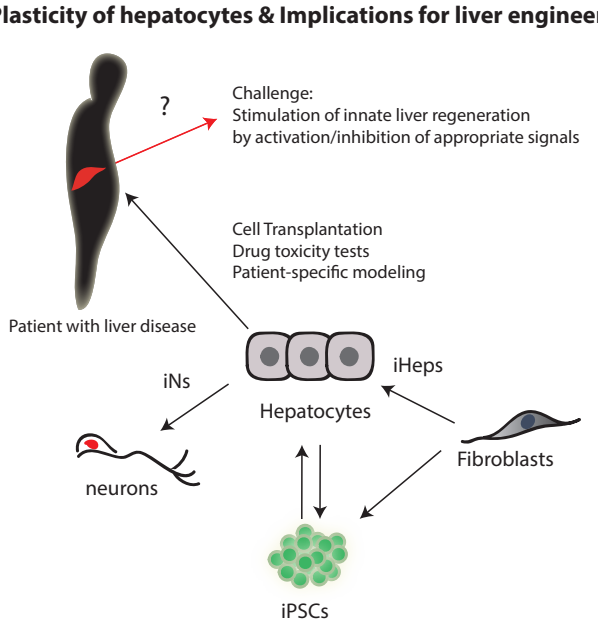

Fig.2. Model of therapeutic approaches for liver diseases
Scheme of recent advances and possibilities in the field of induced hepatocytes (iHeps) and pluripotent stem cells (iPSCs). Hepatocytes are highly regenerative and plastic cells, which can be derived from iPSCs and ES cells, and in turn can give rise to iPSCs and directly transdifferentiate into neuronal-like cells (iNs). Human induced hepatocytes could be utilized for patient-specific disease modelling, drug toxicity trials and cell transplantation. Such studies would contribute to our understanding of the molecular mechanisms that control normal and aberrant liver regeneration. Activation or inhibition of the key signals can be manipulated in a therapeutic way in order to stimulate regeneration in vivo. Such approach could be applied to the treatment of several liver diseases, such as fibrosis, cirrhosis, hepatocellular carcinoma, as well as for regeneration of other tissues.

TGFβ component	Transgenic model	Phenotype	Organism/ Stage	Reference
TGFβ1	*Alb;Tgfβ1* (hepatocyte-specific overexpression	Liver fibrosis, decreased hepatocyte mitogenic response after PHx	Mouse, adult	[71,117]
TGFβ2, β3	*Tgfβ2*$^{-/-}$;*Tgfβ3*$^{-/-}$ *Tgfβ2*$^{-/-}$;*Tgfβ3*$^{+/-}$	Early embryonic lethality, liver malformations	Mouse, embryonic	[118]
TGFβR-II	*Alb-Cre;TgfβrII*$^{f/f}$ (TgfβrII-KO)	Increased hepatocyte proliferation in regenerating liver and increased liver mass: body weight ratio	Mouse, adult	[57]
TGFβ-R-III	*TGFβR-III*$^{-/-}$	Early embryonic lethality, liver defects	Mouse, embryonic	[119]
Activin βC, βE	*Actβc*$^{-/-}$; *ActβE*$^{-/-}$	No obvious defect in liver development, normal regeneration	Mouse, embryonic and adult	[120]
Elf/ β2-spectrin	*elf*$^{-/-}$	Midgestational death due to hypoplastic liver	Mouse, embryonic	[121]
	elf$^{+/-}$	Spontaneous development of HCC	Mouse, adult	[122]
	elf$^{+/-}$	Delayed hepatocyte proliferation followed by activation of progenitors cells	Mouse, adult	[95]
Smad2	*Smad2*$^{-/-}$	Lethal, gastrulation defects	Mouse, embryonic	[123]
	Alb-Cre;Smad$^{f/f}$ (Smad2-KO)	Increased hepatocyte proliferation after CCl_4 injection and EMT transition	Mouse, adult	[63]
Smad3	*Smad3*$^{-/-}$	Viable, postnatal immunity defects at first few months	Mouse, embryonic and adult	[124 125]
	Smad$^{dex8/dex8}$ (Smad3 KO)	Blockage of TGFβ-induced EMT and apoptosis	Mouse, adult	[63]
Smad2, Smad3	*Smad2*$^{-/-}$;*Smad3*$^{-/-}$	Lethal, lack of mesoderm, gastrulation defects	Mouse, embryo	[126]
	Alb-Cre;Smad2$^{f/f}$ *Smad*$^{dex8/dex8}$ (Smad2, 3 double KO)	High susceptibility to toxic liver injury	Mouse, adult	[63]
	Smad2$^{+/-}$;*Smad3*$^{+/-}$	Defects in definitive endoderm and liver development	Mouse, embryonic	[30,31]
Smad4	*Alb-Cr;/Smad4*$^{f/f}$	Iron overload in liver and other organs and premature death	Mouse, adult	[127,128]
Smad7	*Smad7*$^{-/-}$	Promotes TGFβ fibrogenic action via CTGF upregulation	Mouse, adult	[85]

Table 1. Representative list of available animal models of TGFβ signaling components selected with regards to defective liver developmental, functional or regenerative phenotype

Alb; albumin, cre: cre recombinase, KO; knock out, EMT; epithelial-to-mesenchymal transition, CTGF; connective tissue growth factor, f/f; homozygous floxed (loxP containing) gene locus, CCl_4; carbon tetrachloride, +/-; heterozygous mutant, -/-; homozygous mutant.

Regeneration model	Species	TGFβ component	Functional implication	Reference
Transplantation	Human	β2-spectrin	HPC/Oval cell response	[90]
Partial hepatectomy (PH)	Mouse	TGFβR-II	Hepatocyte proliferation	[57]
	Mouse	TGFβ1, LAP	Neutralisation of TGFβ1 mitoinhibitory activity upon hepatocytes	[117]
	Mouse	SnoN, Ski inhibitors	Activation status of Smad complexes, hepatocyte resistance to TGFβ mitoinhibition	[68]
	Rat	TGFβ1	Inhibition of TGFβ1 by neutralizing antibody, hepatocyte mitosis, liver regeneration	[129]
	Rat	TGFβ1, TGFβ2	Inhibition of early proliferation response	[64]
Carbon tetrachloride (CCl_4)	Mouse	Endoglin	Activation of HSCs, fibrogenesis	[130]
	Mouse	SnoN, Ski inhibitors	Activation status of Smad complexes, hepatocyte resistance to TGFβ mitoinhibition	[68]
	Mouse	TGFβ1	Activation and transdifferentiation of HSCs, collagen and αSMA expression in HSCs	[131,132]
	Mouse	Smad3	Smad3 induces collagen expression but is dispensable for HSC activation	[132]
Bile ligation	Mouse	Endoglin	Activation of HSCs, fibrogenesis	[130]
Choline-deficient, ethionine-supplemented diet (CDE)	Mouse	Smad2	Proliferation and sensitivity of HPCs to TGFβ1	[96]
Partial liver irradiation	Rat	TGFβ1	Stimulation of intrinsic regeneration by irradiation and TGFβ1 expression pattern	[133]
Dimethylnitrosamine (DMN)	Rat	TGFβR	Anti-TGFβR molecular intervention and regulation of transcription factors important regeneration	[65]

Table 2. Liver regeneration models reported to have elucidated functions on TGFβ family members
Different liver injury/ regeneration models are indicated, which have provided insight into the functional implementation of TGFβ ligands, receptors, R-Smads in the liver or in combination with therapeutic interventions such as neutralizing antibodies or small molecule inhibitors against TGFβ molecules.

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