

Role of liver progenitors in liver regeneration

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Abstract: During massive liver injury and hepatocyte loss, the intrinsic regenerative capacity of the liver by replication of resident hepatocytes is overwhelmed. Treatment of this condition depends on the cause of liver injury, though in many cases liver transplantation (LT) remains the only curative option. LT for end stage chronic and acute liver diseases is hampered by shortage of donor organs and requires immunosuppression. Hepatocyte transplantation is limited by yet unresolved technical difficulties. Since currently no treatment is available to facilitate liver regeneration directly, therapies involving the use of resident liver stem or progenitor cells (LPCs) or non-liver stem cells are coming to fore. LPCs are quiescent in the healthy liver, but may be activated under conditions where the regenerative capacity of mature hepatocytes is severely impaired. Non-liver stem cells include embryonic stem cells (ES cells) and mesenchymal stem cells (MSCs). In the first section, we aim to provide an overview of the role of putative cytokines, growth factors, mitogens and hormones in regulating LPC response and briefly discuss the prognostic value of the LPC response in clinical practice. In the latter section, we will highlight the role of other (non-liver) stem cells in transplantation and discuss advantages and disadvantages of ES cells, induced pluripotent stem cells (iPS), as well as MSCs.

Keywords: Liver progenitor cells (LPCs); stem cells; mesenchymal stem cells (MSCs); hepatic stellate cells (HSCs); liver regeneration; liver failure; liver cirrhosis

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Introduction

Parenchymal liver injury and the subsequent cellular response is strongly associated with the etiology and severity of injury. Mature hepatocytes represent the major cell type within the liver. Hepatocytes are unipotent cells contributing to normal cell turnover and are able to respond rapidly to injurious stimuli. In contrast, the liver progenitor cell (LPC) compartment only expands when the regenerative capacity of hepatocytes is continually and severely compromised, a feature common to most chronic liver diseases (1,2). When cell death exceeds the self regenerative capacity of the liver, liver failure occurs and orthotopic liver transplantation (OLT) becomes necessary. OLT still represents the only potentially curative treatment

option for several acute, chronic inborn or acquired liver diseases. Donor organ shortage (3) and mandatory life long immunosuppression (4) have prompted investigators to search for alternative treatment options. To date, the only therapeutic alternative to OLT which has reached clinical application is transplantation of mature hepatocytes in individuals with life threatening hepatic diseases (where organ transplantation would remain the only treatment option). Ideal candidates for this therapy are patients with acute liver failure (ALF) (5,6) and liver inborn defects of metabolism (7). Hepatocyte transplantation is less invasive as hepatocytes are delivered through the vascular system (i.e., intraportal route), which may be less harmful for critically ill patients. However, this method is limited to individuals whose liver architecture remains preserved, since it is

required for cell engraftment and expansion. Hepatocyte transplantation in patients with advanced fibrosis or cirrhosis is hampered by difficulties in engraftment and risk of portal hypertension (8). Furthermore, it remains unclear if hepatocyte transplantation can achieve a sustained improvement of liver function, since the number of hepatocytes required to improve liver function far exceeds the number of cells that can be safely administered (usually less than 1% of the liver mass). A major limitation to hepatocyte transplantation is the lack of hepatocytes of sufficient quality. For these reasons, investigators have turned to alternative stem cell based therapies, since one of the promises of stem cells is their potential to provide a renewable source of hepatocytes (9).

This review aims to provide a concise overview on: (I) the role of resident LPCs during liver reconstitution and signalling pathways involved during regeneration; (II) the implication of pluripotent embryonic stem (ES) cells and differentiated stem/progenitor cells as potential transplantable assistance for liver regeneration.

Liver reconstitution by resident LPCs in the niche

During the early stages of liver damage, inflammatory cytokines trigger hepatocytes to enter the cell cycle. In moderate liver damage, differentiated hepatocytes can replace neighbouring cells within few days. The LPC compartment is only activated when insufficient numbers of healthy hepatocytes remain to perform regenerative processes (see *Figure 1*), i.e., in most chronic liver diseases as well as ALF. Katoonizadeh *et al.* suggested, that at least 50% hepatocyte loss and hepatocyte replicative senescence are necessary to trigger LPC activation (10). The bipotential LPCs, in rodents historically termed “oval cells”, reside in the Canals of Herring (CoH), located in the niche of the biliary-hepatocytic interface (11). In human liver, activation of LPC compartment is referred to as “Ductular Reaction” (DR) due to the more ductular proliferation phenotype that arises during LPC expansion. LPCs are able to infiltrate along the liver plate towards the central vein and differentiate into hepatocytes as well as cholangiocytes. In the niche, LPCs are surrounded by epithelial cells, non-parenchymal cells (i.e., hepatic stellate cells, HSCs), as well as immune cells and extracellular matrix (ECM). Thus, activating signals from various sources can easily reach the LPCs. The activation and expansion of the LPC compartment occurs roughly over 7 days, while the process of LPC differentiation into intermediate hepatocytes

requires an additional 7 days (12,13). Thus, the LPC response is a much slower regenerative process compared to hepatocyte replication.

LPC activation has been described in various hepatic disease conditions such as acute liver necrosis (14,15), hemochromatosis (16), chronic cholestatic diseases (14), alcoholic liver disease (17) and chronic viral hepatitis (18). In contrast, conditions of extrahepatic biliary obstruction reveal typical ductular reactions, which show proliferation of mature cholangiocytes only, but not of LPCs (19). LPC activation is also absent or minimal during liver regeneration after partial hepatectomy (PH) to an extent of up to 2/3 of rat liver (20). In consistency with the findings mentioned above, Lowes and colleagues could furthermore describe a positive correlation between severity of hemochromatosis, alcoholic liver disease, chronic hepatitis C and increase in the number of oval cells. In addition, this observation is consistent with the hypothesis that oval cell proliferation is associated with increased risk for development of hepatocellular carcinoma during chronic liver disease (16).

Cell signalling axes regulating LPC mediated regeneration

The regenerative process upon liver injury is orchestrated by a complex cross-talk between different liver cell compartments, and is mediated by cytokines, mitogens and several growth factors (see *Figure 1*) (21). Chronic liver disease is characterized by apoptosis, necrosis and senescence of hepatocytes (22) and is associated with activation and expansion of resident LPCs (23,24). Cytokines including Interleukine 6 (IL6), tumor necrosis factor alpha (TNF alpha), Osteopontin, transforming growth factor beta (TGF beta), transcription factors nuclear factor kappa B (NF-κB) and CCAAT enhancer binding protein beta, as well as growth factors hepatocyte growth factor (HGF) and epidermal growth factor (EGF) (25) are putative factors which promote LPC-mediated regeneration. Additionally, hormones (e.g., Somatostatin, Insulin) (26), adipokines (adipose tissue derived cytokines) (27,28), and neurotransmitters (serotonin, epinephrine, norepinephrine) also regulate LPC function and stimulate the proliferation and differentiation of LPCs to mature hepatocytes and cholangiocytes. There is increasing evidence that morphogenic signals (factors regulating embryonic development) orchestrate the LPC response.

The following axes have been previously described to play a pivotal role in modulation of LPC mediated liver

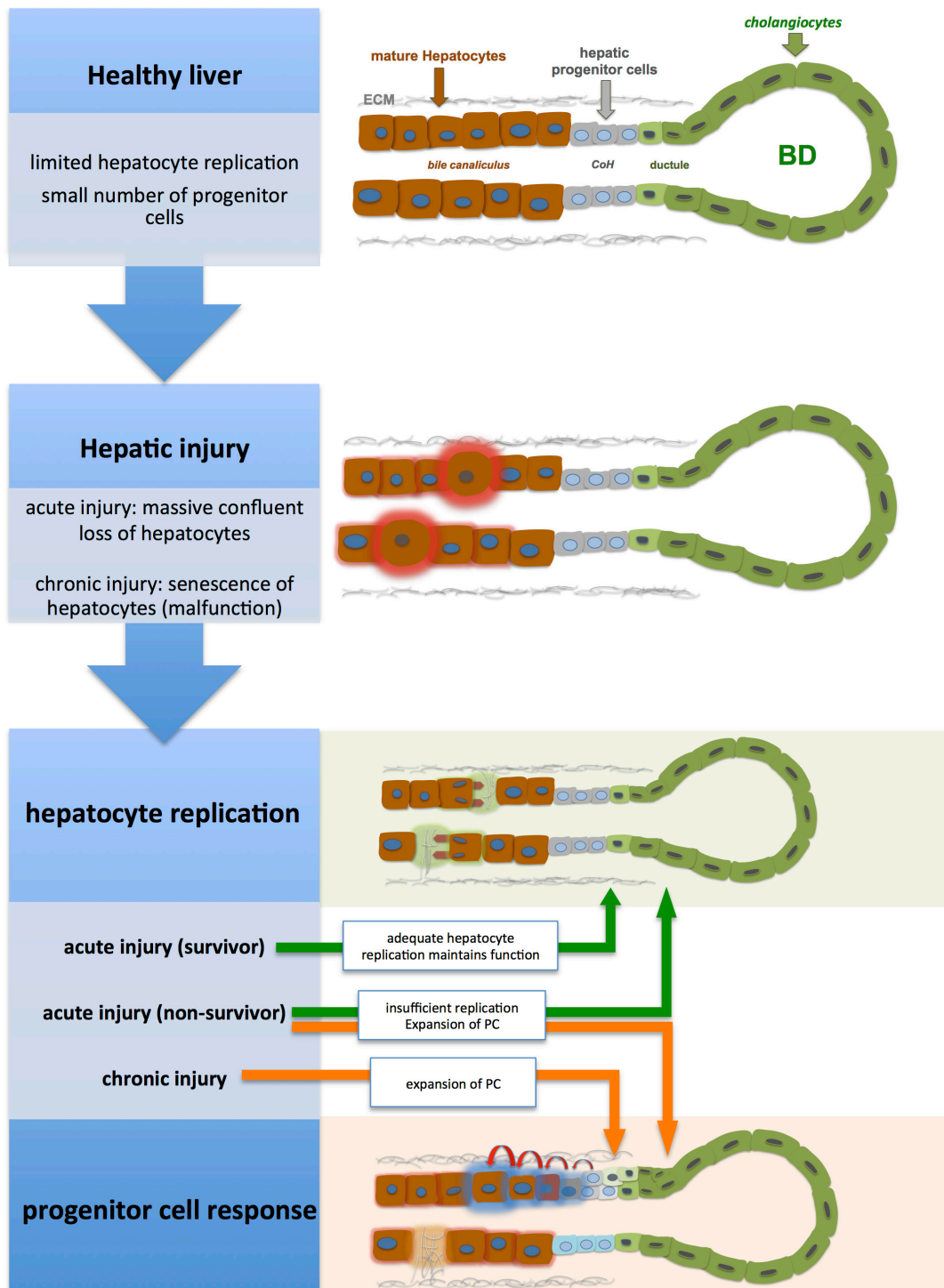


Figure 1 Depiction of liver progenitor cells (LPC) residing in the Canals of Herring (CoH) in the healthy liver tissue. Upon initial loss of hepatocytes liver regeneration is carried out by resident hepatocytes. If this mechanism is overwhelmed though by massive parenchymal injury in terms of confluent loss of hepatocytes or hepatocytic senescence, the LPC niche becomes activated in order to support and enhance the regenerative process. The progenitor cell mediated tissue reconstitution is orchestrated by several cytokines (e.g., IL-6, TNF- α , TGF- β , osteopontin), transcription factors [nuclear factor kappa B (NFKB) and CCAAT enhancer binding protein beta], morphogens (Hedgehog, Wnt, Notch, HIPPO), growth factors (HGF, EGF), hormones (thyroid, sympathetic, parasympathic) and adipokines (adipose tissue derived cytokines).

tissue reconstitution:

- (I) TWEAK—Fn14: LPC response is initialised by the TNF-like weak apoptosis inducing factor (TWEAK)/Fibroblast inducible 14 (Fn14) pathway (29). Signalling through this pathway selectively promotes LPC expansion, but does not affect viability of mature hepatocytes during chronic liver injury (30-32). Using Fn14-deficient mice, Karaca and colleagues proposed that the TWEAK/Fn14 axis could directly stimulate LPC expansion in a setting of acute liver injury (33). Bird and coworkers also demonstrated that a single bone marrow cell (BMC) infusion could directly activate a DR. Interestingly, they could show that the macrophage subset within BMC was necessary for the DR and the expansion of clonogenic LPCs. Furthermore, macrophage-derived TWEAK/Fn14 signalling was the key driver of the DR since recombinant TWEAK administration resulted in a similar expansion of LPC *in vivo*. TWEAK then signals via the TWEAK receptor Fn14 and downstream NF- κ B to induce LPC proliferation (34). Another known source for TWEAK are CXCR4+ T cells, which can be recruited by chemoattractant cytokines (i.e., SDF-1). Thus, TWEAK signalling might be a promising target for future LPC mediated therapy trials, i.e., by agonists activating Fn14 (35);
- (II) Hedgehog (Hh) signalling: recent studies showed that Hh pathway activation occurs during liver regeneration after PH (32,36-38). The Hh pathway usually orchestrates fetal tissue and organ development, and stimulates the expansion and viability of stem cells (39). In a rodent study performed by Ochoa and coworkers a 70% PH (a model of liver regeneration) was followed by expansion of liver progenitors. These progenitor cells were Hh-responsive and secreted Hh-ligands. Interestingly, inhibiting the Hh pathway with cyclopamine attenuated the LPC response, repressed expression of progenitor markers, and reduced overall survival (32). Hh ligands over-expressed during liver injury could directly stimulate LPCs to secrete chemokines that lead to the additional recruitment of cells orchestrating regeneration (40). In addition to a direct effect on LPCs, Hh ligands could also enhance LPC proliferation indirectly via activation of HSCs into matrix-producing myofibroblasts (41). Grzelak *et al.* could demonstrate in a mouse model of thioacetamide (TAA), representative of chronic liver injury, that injured hepatocytes produce Hh ligands. As described above these ligands induced expansion of the LPC population via primary cilia, cellular structures which are crucially required to transduce the Smoothed-dependent Hh signal *in vivo* (42,43). Taken together the Hh pathway seems to be crucial for involvement of LPCs for liver regeneration;
- (III) Thyroid hormone signalling: extra-hepatic signals influencing liver regeneration are of major interest to facilitate regeneration (44,45). The thyroid hormone T3 modulates cell growth, differentiation, and metabolic functions via interaction with thyroid hormone nuclear receptors (TRs). In an experimental rodent model of 70% and 90% hepatectomy, administration of exogenous T3 significantly enhanced liver regeneration (increased liver body weight ratio and Ki67 index) (46). The underlying mechanisms by which T3 mediates these regenerative effects are unclear, but may be due to upregulation of cyclin D1 and subsequent cell cycle entry (44,45). T3 may also directly induce LPC activation. In a rodent model of combined 2-acetylaminofluorene feeding and PH, László *et al.* reported that T3 administration enhanced LPC differentiation into hepatocytes (47). Future studies are needed to dissect the downstream signals by which T3 modulates LPC function;
- (IV) Fibroblast growth factor 7 (FGF7): HSCs are known to interact with LPCs physically and by paracrine signals (48). During liver injury, Thy1⁺ mesenchymal cells (Thy1⁺ MC) were found to expand in the periportal region along with, and in close proximity to, LPCs (49). Expansion of Thy1⁺ MCs in the periportal region secreting FGF7 stimulated LPC activation upon liver injury (50). On LPCs expression of the FGF7 cognate receptor FGFR2b was confirmed. In a mouse model Takase and colleagues confirmed, that FGF7-deficient mice exhibited markedly depressed LPC expansion upon toxin-induced liver injury (51). These findings provide evidence that FGF7 might play a role as LPC regulator and thus might be a potential therapeutic target for liver diseases;
- (V) The β -catenin/Wnt pathway: β -catenin is implicated in the regulation of liver development, homeostasis, metabolism, regeneration, and carcinogenesis. It plays a pivotal role in stem cell maintenance and renewal, proliferation, differentiation, cell migration, and polarity. In the canonical Wnt signalling pathway, β -catenin is the central component that mediates

the Wnt signalling from the cell membrane to the cytoplasm (52).

Upon canonical Wnt signalling activation, free β -catenin translocates to the nucleus and binds to TCF/LEF transcription factors, inducing transcriptional activation of target genes, such as NF- κ B, resulting in release of pro-inflammatory cytokines (53). Numerous studies in animal models implicate, that β -catenin signalling plays a role in progenitor cell induction and proliferation. Hu and coworkers could demonstrate that DDC diet induced Wnt signalling parallel to significantly increased progenitor cell activation in mice (54). Since the contribution of Wnt signalling to hepatocarcinogenesis is still unclear, clinical trials of Wnt pathway modulation to ameliorate liver disease should be considered with caution;

- (VI) HIPPO—YAP pathway: the transcriptional regulators YAP and TAZ as effectors of the HIPPO signalling cascade are the focus of intense interest due to their remarkable biological properties in development, tissue homeostasis and cancer. The Hippo/YAP-signalling pathway seems to play a crucial role in regulation of the liver size (55,56). Manipulation of HIPPO pathway activity leads to profound changes in liver cell proliferation. YAP overexpression results in approximately a 4-fold increase in liver size within weeks (55,56). The precise mechanism of YAP/TAZ-dependent regulation of cell proliferation remains unclear. It is postulated, that the interaction with TEAD cofactors might be essential for promotion of cell growth in some contexts.

LPC activation and expansion parallels fibrogenesis

As described above the LPC niche consists of epithelial (hepatocytes and cholangiocytes) cells, HSCs, and immune cells (i.e., Kupffer cells) interwoven with the ECM. Cumulative evidence shows that the LPC compartment is intricately linked to the ECM (57-59). For example, laminin-LPC interactions are crucial for LPC-mediated parenchymal reconstitution, and the inhibition of ECM remodelling impairs LPC activation and expansion (60). Moreover, direct interaction of LPCs and HSCs has been demonstrated via lymphotoxin beta (LTb) (61). LPCs seem to produce LTb in response to TNF signalling (62) to recruit other LPCs, HSCs, and leukocytes to sites of injury. Blocking of the LTb receptor on HSCs abrogated the fibrotic response to dietary induced liver damage in a mouse

model (61). Another mediator of HSC activation by LPCs might be the above described TWEAK. Macrophages and NK cells comprise the main producers of TWEAK, which induces proliferation of LPCs (30). In various mouse models of liver damage knockout of Fn14 (the TWEAK receptor) or antibody treatment against TWEAK blocked the fibrogenic response associated with LPC expansion (63,64). Though, it is not clear if TWEAK itself stimulates HSC activation via Fn14 or if LPCs directly activate HSCs by LTb secretion. These findings demonstrate that the LPC response (DR) occurs parallel to a fibrogenic response (65). Thus, short term accumulation of collagen matrix might be a beneficial prerequisite for physiological repair (which precedes parenchymal cell reconstitution). In analogy to the findings in mouse models, the fibrous tissue scaffold is the LPC niche that facilitates LPC activation and differentiation. To further characterize how ECM composition modulates the LPC response during liver regeneration, future studies investigating the effects of modifying ECM components and the resulting effects on LPC activation, proliferation, differentiation and expansion are required.

Transplantation of stem cells as potential source of liver regeneration

Stem cells and their descendants (progenitor cells) are capable of sustained proliferation and differentiation into specialized cells (66). Stem cells are defined by their ability to self-renew to maintain a cell population with identical properties by symmetric and asymmetric cell division (67).

Stem cell based therapies might be applied in conditions of ALF (68), inborn errors of metabolism, autoimmune or viral hepatitis, toxic injury or (non) alcoholic liver disease (69). Common to all cell transplantation based therapies is the need of a well preserved liver architecture as prerequisite, facilitating regeneration. Stem cells can broadly be divided into pluripotent ES cells and multipotent, adult/differentiated stem/progenitor cells (see *Figure 2*), the latter can be found in various fetal and postnatal tissues (e.g., bone marrow, adipose tissue, blood). Due to combined abilities of unlimited expansion potential and pluripotency, ES appear to be an ideal source for tissue replacement after injury or in metabolic disease. However, there are currently no approved treatments utilizing ES for several reasons: the accumulation of spontaneous mutations and chromosomal rearrangements degrades their practical utility (70). Furthermore, ethical issues and technical

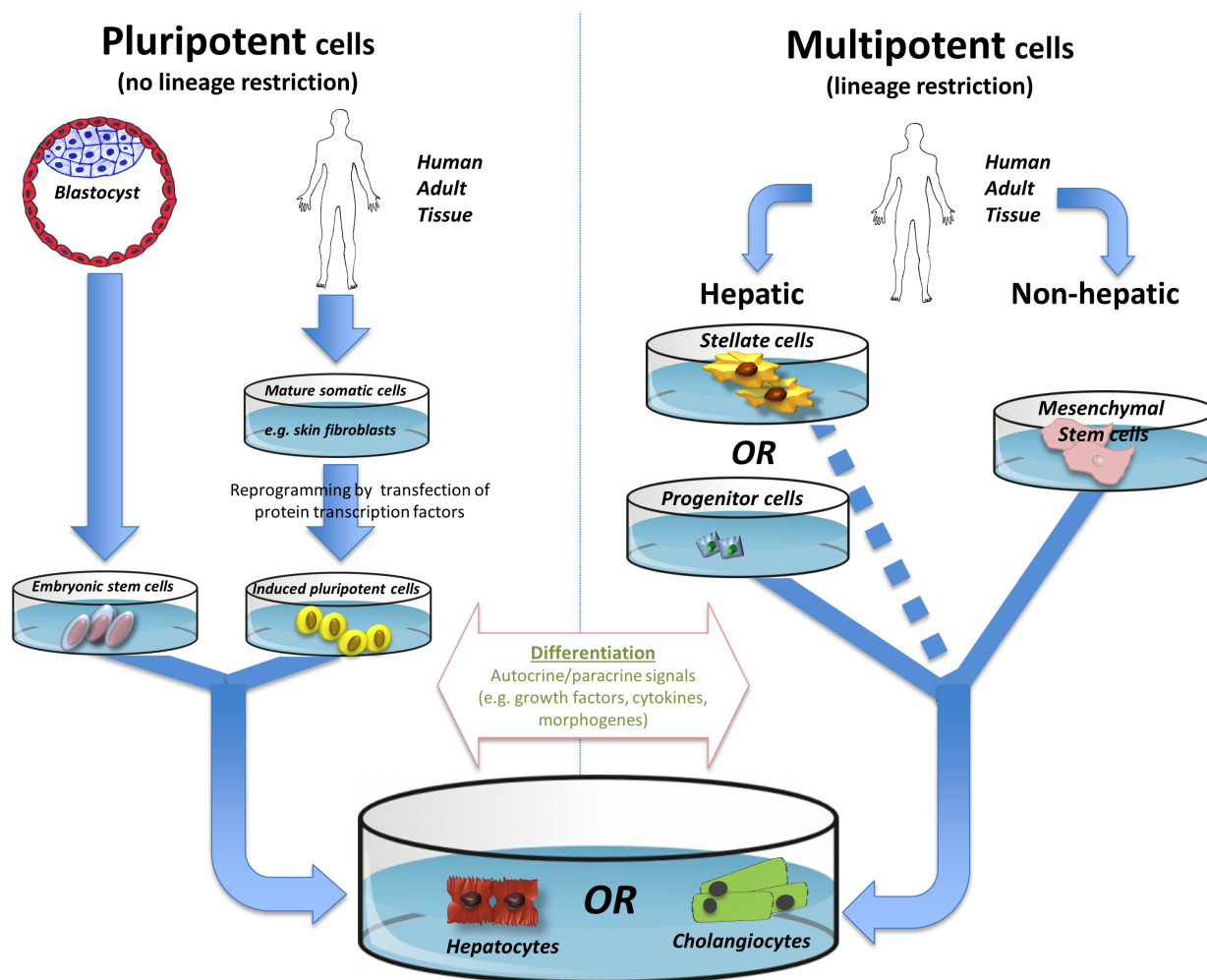


Figure 2 Overview of different sources of stem cells that can give rise to hepatocytes/cholangiocytes by differentiation, mediated by autocrine/paracrine signalling. Stem cells can be divided into pluripotent and multipotent cells, the latter are restricted to one lineage. Pluripotent cells can be divided into embryonic stem cells (ES) which can be extracted from blastocysts, or induced pluripotent stem cells that can be obtained from various adult human tissues (e.g., derived from skin tissue) by reprogramming with retroviral transfection of protein transcription factors. Multipotent cells are obtained from either human liver (HSCs or progenitor cells) or other tissues (mesenchymal stem cells) and differentiate towards either cholangiocytic or hepatocytic lineages.

hurdles, such as differentiating ES cells into usable cells while avoiding transplant rejection are current problems preventing a broad medical implication at present.

Another subgroup of pluripotent stem cells, so called induced pluripotent stem cells (iPS), can be generated by reprogramming mature somatic cells (71,72) (e.g., derived from human adult skin tissue) by retroviral transfection of various transcription factors. In theory, autologous administration of iPS should not even require immunosuppression (73). At present, broad clinical

application is hampered by the same hurdles as described for ES.

In contrast, determined/adult stem cells are transiently amplifying cells during development or regeneration and restricted to one lineage. In the liver adult stem cells are represented by LPCs, which can be easily isolated and cultured (74-76). Effective use was demonstrated by transplantation via intravascular injection into the liver (76,77). In the past, the major limiting factor in the study of LPCs has been the inability to identify, isolate or purify

these cells in a reliable fashion. Recently, Cardinale and colleagues successfully isolated multipotent stem/progenitor cells from the human biliary tree by extended cell culture techniques (78) and demonstrated that these progenitor cells are capable of giving rise to hepatocytes, cholangiocytes, and pancreatic islets. Similarly a LPC isolation protocol has been developed for mouse and human liver tissue, but using fluorescence-activated cell sorting (FACS), based on the observation that progenitor cells express high levels of aldehyde dehydrogenase (ALDH) activity. FACS-ALDH1 positive LPCs in culture could give rise to functional hepatocyte-like cells as illustrated by albumin and urea secretion and cytochrome P450 activity (74). These and other novel methods of LPC isolation could well pave the way for the development of future therapies.

Therapeutic effects of mesenchymal stem cells (MSCs) in liver failure are based on their ability to differentiate into hepatocytes and to alter function of immune cells responsible for acute liver injury. They release trophic and immunomodulatory factors (74,79,80) and attenuate proliferation of NKT cells and promote apoptosis of stellate cells. MSCs are assigned to the adult stem cell population and can be found in the perivascular compartment of the liver and most other organs (81,82). They can be isolated from a variety of tissues, such as bone marrow (83), adipose tissue (84), umbilical cord tissue (85) and amniotic fluid (86,87) and can give rise to hepatocyte like cells. MSCs can be expanded *ex vivo* for multiple passages, but not indefinitely (88). As a promising source for transplantation to cure several hepatic disorders, due to their low MHC I and absence of MHC II expression, MSCs seem to have a low rejection risk upon their administration (89). Apparently MSCs play an increasing role as a source of transplantable cells in several types of liver disease. There are multiple clinical trials either completed, actively recruiting or enrolling patients suffering from cirrhosis (mainly alcoholic) but also cases of ALF, which are treated with MSC transplantation (see *Figure S1*). The majority of clinical trials conducted on MSCs for the treatment of liver diseases are based on three different sources of cells for transplantation: (I) unfractionated bone marrow, peripheral blood or cytokines (e.g., G-CSF) to mobilize cells; (II) immunoselected cell populations (CD34⁺ cells, CD133⁺ cells) from bone marrow or peripheral blood; (III) cultured MSCs or cultures treated with growth factors (e.g., HGF, EGF, fibroblast growth factor) (90).

Besides cytokines and growth factors there are also several chemical compounds (dexamethasone, etc.),

transcriptional factors (hepatocyte nuclear factor 3 β), or cell types (the human hepatoma cell line Huh7, fetal liver cells, HSCs, etc.) promoting differentiation and maturation of MSCs towards hepatocytes (91). Although multiple approaches to this method are available now, all procedures are still lacking further validation. Thus, MSC based therapy remains a challenging issue requiring close cooperation between researchers and clinicians.

Conclusions

OLT as treatment for acute and terminal chronic liver diseases depends on sufficient donor organs and requires long-term immunosuppression. Additional alternative therapeutic options like hepatocyte transplantation have emerged and are warranted. However, the potential of this latter technique has been constrained by numerous technical hurdles. Recent approaches aim to harness stem cell based alternatives, such as induction of resident liver progenitor cells, which could possibly be facilitated by ECM associated factors in the future. Another option is transplantation of non-liver stem cells. Pluripotent cells (ES, iPS) carry the risk of spontaneous mutations and chromosomal rearrangements. Transiently amplifying LPCs have shown great potential *in vitro* and *in vivo*, thus first clinical trials might be launched in the coming few years. At present, MSCs represent the most promising source of stem cells with the highest number of ongoing clinical trials. They can potentially alleviate disease conditions by immunomodulatory as well as paracrine signalling mechanisms for months to years.

Before stem cells can be utilized for liver repopulation in humans, still several problems have to be solved, such as the lack of consensus about the immune phenotype of LPCs, obtaining a sufficient quantity of cells for the desired clinical application, ethical aspects, and long term efficacy and safety (92).

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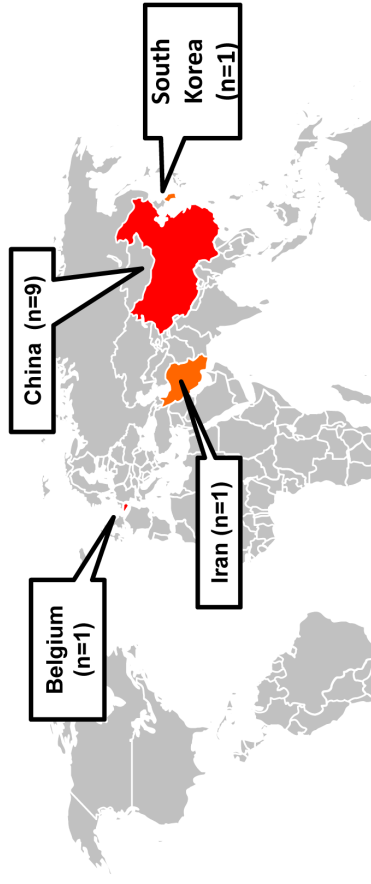
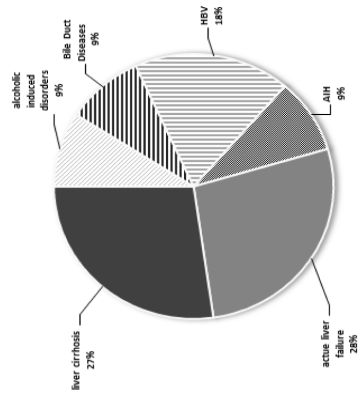
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Clinical trials applying MSCs for the treatment of liver diseases				Investigators
Status	Phase	Number of patients	Study	
recruiting	Phase 2	12	The Effectiveness and Safety for Mesenchymal Stem Cell for Alcoholic Liver Cirrhosis	Soon Koo Baik, M.D
recruiting	Phase 1 Phase 2	100	Umbilical Cord Mesenchymal Stem Cells for Patients With Primary Biliary Cirrhosis	Fu-Sheng Wang, professor
completed	Observational Model: Case Control Time Perspective: Retrospective	158	Therapeutic Effects of Liver Failure Patients Caused by Chronic Hepatitis B After Autologous MSCs Transplantation	Zhi-liang Gao, Dr
recruiting	Phase 1 Phase 2	240	Safety and Efficacy of Human Umbilical Cord Derived Mesenchymal Stem Cells for Treatment of HBV-related Liver Cirrhosis	Daiming Fan
recruiting	Phase 1 Phase 2	100	Umbilical Cord Mesenchymal Stem Cells for Patients With Autoimmune Hepatitis	Fu-Sheng Wang, professor
recruiting	Phase 1 Phase 2	70	Safety and Efficacy of Human Mesenchymal Stem Cells for Treatment of Liver Failure	Fu-Sheng Wang, Professor
recruiting	Phase 1 Phase 2	120	Umbilical Cord Mesenchymal Stem Cells Transplantation Combined With Plasma Exchange for Patients With Liver Failure	Zhi-Liang Gao, Doctor
recruiting	Phase 1 Phase 2	40	Mesenchymal Stem Cells After Renal or Liver Transplantation	Yves Beguin, MD, PhD
completed	Phase 1 Phase 2	30	Improvement of Liver Function in Liver Cirrhosis Patients After Autologous Mesenchymal Stem Cell Injection: a Phase I-II Clinical Trial	Pedram Kharaziha, MD
not yet recruiting	Phase 1 Phase 2	320	Safety and Efficacy Study of Umbilical Mesenchymal Stem Cells for Liver Cirrhosis	Hanwei Li, MD, PhD et al.
competed	Phase 1 Phase 2	20	Human Umbilical Cord Mesenchymal Stem Cells Transplantation for Patients With Decompensated Liver Cirrhosis	Chengwei Chen

Figure S1 Global distribution and different etiologies of clinical trials applying MSC transplantation for treatment of liver diseases and liver cirrhosis are depicted. [A search on www.clinicaltrials.gov (November 2014) with the term “mesenchymal stem cells” AND “liver failure”. The studies with status “completed”, “recruiting”, “not yet recruiting” were included, trials with the status “unknown” were excluded].