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**Stem cell-based regenerative opportunities for the liver: State of the art and beyond**

Tsolaki E *et al*. Stem cells and liver regeneration

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**Abstract**

The existing mismatch between the great demand for liver transplants and the number of available donor organs, highlights the urgent need for alternative therapeutic strategies in patients with acute or chronic liver failure. The rapidly growing knowledge on stem cell biology and the intrinsic repair processes of the liverhas opened new avenues for using stem cells as a cell therapy platform in regenerative medicine for hepatic diseases. An impressive number of cell types have been investigated as sources of liver regeneration; adult and fetal liver hepatocytes, intrahepatic stem cell populations, annex stem cells, adult bone marrow-derived hematopoietic stem cells, endothelial progenitor cells, mesenchymal stromal cells, embryonic stem cells and induced pluripotent stem cells. All these highly different cell types, used either as cell suspensions or, in combination with biomaterials as implantable liver tissue constructs, have generated great promise for liver regeneration. However, fundamental questions still need to be addressed and critical hurdles to be overcome before liver cell therapy materializes.In this review we summarize the state-of-the-art in the field of liver stem cell-based therapy and the translational potential of stem cell-based therapies along with existing challenges and future perspectives, towards a successful liver cell therapy that will ultimately deliver its demanding goals.

**Key words:** Stem cells; Liver regeneration; Liver cirrhosis; Acute liver injury; Stem cell based therapy

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**Core tip:** Liver transplantation is the only effective treatment for end-stage liver diseases, but its application is limited mainly due to donor shortage. In order to fulfil the unmet medical needs in the field, alternative, cell-based therapies for the treatment of end-stage hepatic diseases are under investigation.This review aims to summarize the state of the art on SC-based approaches towards liver regeneration as well as to critically discuss and highlight new perspectives and challenges.

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**INTRODUCTION**

The liver possesses a remarkable capacity to regenerate in response to injury; however, in severe cases its regenerative capacity may prove insufficient and hepatic injury progress to end-stage disease and subsequent liver failure. Orthotopic liver transplantation is currently the only effective treatment for patients with end-stage liver diseases, including acute liver failure and hepatic cirrhosis. Nevertheless, donor shortage and waiting list mortality, postoperative morbidity and mortality, high costs and long-term side effects severely limit its application[1,2]. Hepatocyte transplantation has been suggested as an alternative approach to liver transplantation since mature hepatocytes have been traditionally recognized as the major contributors to liver repair and are functionally the most robust cell type for liver cell therapy[3,4]. Indeed, many preclinical and clinical studies have been conducted using this approach to cure metabolic and end-stage liver diseases[5]. However, the widespread application of hepatocyte transplantation is limited by organ unavailability, the negative impact of cell culture on hepatocyte viability, function and engraftment[5,6], as well as the hepatocyte susceptibility to cryopreservation damage inducing cell rupture, necrosis and apoptosis after thawing[7,8]. Therefore, alternative therapies are needed to supplement organ transplantation and bridge the gap between the need for liver transplantation and the lack of a timely available cadaveric graft.

**Adult Liver Stem/Progenitor Cells**

When hepatocyte proliferation is impaired, deficient or overwhelmed by severe liver injury, bipotent intrahepatic stem cell populations, known as resident liver progenitor cells (LPC) in humans or oval cells (OCs) in rodents, emerge and become activated, expand and actively contribute to the regenerative process by giving rise to hepatocytes and biliary epithelial cells[9-12].

The term ‘oval’ cell is used to describe small, rounded proliferating cells with a large nuclear to cytoplasmic ratio which reside in the terminal branches of the intrahepatic biliary tree, the Canals of Hering, considered along with the space of Disse as the putative hepatic stem cell niches. OV/LPC coexpress biliary and hepatocytic markers and also hematopoietic progenitor cell antigens[13,14].

Regarding the mechanism controlling OC fate in response to liver injury both in humans and in murine models, it has been proposed that during LPC/OC-mediated liver regeneration, an “inductive” niche is formed around OCs, constituting the ductular inflammatory reaction. This niche is populated by recruited macrophages and myofibroblasts and requires new synthesis/remodelling of extracellular matrix (ECM) to facilitate appropriate OC/LPC expansion and ultimately biliary and hepatocyte regeneration[15]. The role of Wnt and Notch signalling in hepatic cell fate specification has been recently recognized through the proliferation and differentiation of human LPCs into hepatocytes or cholangiocytes respectively, providing potential targets for future targeted-therapies[15,16] for the liver.

The precise identification of endogenous liver SCs and of the mechanisms that govern their proliferation and differentiation into mature hepatocytes in case of severe parenchymal extinction could facilitate their *in vitro* and *in vivo* maturation to hepatocytes and their application to clinical practice. This process was histologically identified by the description of regenerative nodules, the so called “buds” composed of small clusters of hepatocytes admixed with ductules[17]. These “buds” were suggested to be composed of new hepatocytes derived from stem cells located in the small bile ducts and the canals of Hering, thus appearing to be the structures that contain stem cell derived hepatocytes[18]. The progressive evolution of buds from stem/progenitor- cell origins to integrated mature liver parenchyma was described in a recent study using various anatomic and immunohistochemical markers including epithelial cell adhesion molecule (EpCAM), K19, CD34, glutamine synthetase (GS) and Ki-67[19].

Interestingly, hepatic stellate cells (HSTCs), considered as liver-resident mesenchymal cells[20], have been recently shown to represent a source of liver progenitor cells. Indeed, an isolated population of retinoid storing hepatic stellate cells were able to contribute to liver regeneration through differentiation. HSTCs gave rise to parenchymal and bile duct cells and ameliorated the glucuronidation defect in GUNN rats, thus providing functional hepatocytes[21].

**Fetal Liver Stem Cells**

Fetal liver SCs appear during embryogenesis, after the establishment of the hepatic endoderm and when the liver bud is growing. Hepatoblasts, resident cells in the developing liver bud, express the signature marker α-fetoprotein (AFP) and are considered bipotential, being able to give rise to both mature hepatocytes as well as bile duct epithelial cells (cholangiocytes)[22]. Many experimental studies have focused on the regenerative capacity of fetal hepatic progenitor cells as, in contrast to adult hepatocytes, fetal liver stem cells can be readily isolated while they are highly proliferative, less immunogenic and more resistant to cryopreservation[22-25] and as such, could be of clinical benefit in treating liver diseases.

Indeed, their capacity to repopulate the liver upon transplantation has been demonstrated in animal models[26-28] and clinical trials (Table 1)[29,30]. In a clinical study, twenty-five patients with liver cirrhosis of different etiologies, were infused with human fetal liver-derived stem cells. The procedure proved safe and efficient, offering a potentially supportive modality to organ transplantation in the management of liver diseases[29]. In another study, immune-sorted, human fetal biliary tree cells were safely administered to two patients with advanced liver cirrhosis who were monitored through a 12-mo follow-up period. Immuno-suppressants were not required, and the patients did not experience any adverse event or immunological complications. Both patients showed biochemical and clinical improvement within the first 6 mo whereas the second patient maintained benefits for 12 mo[30].

The ability of fetal liver stem cells to expand clonogenically *in vitro*, their pluripotency, and the evidence that they yield mature liver cells, encourage their clinical utility for transplantation and generation of bioartificial livers. However, ethical issues and the possibility of teratoma/teratocarcinoma formation in the recipients, justify their reserved use mainly in preclinical or pilot studies.

**Extrahepatic stem/progenitor cells**

Apart from endogenous liver stem cells, several populations of exogenous stem/progenitor cells have shown potential to contribute to the liver healing process and are discussed below.

***Embryonic stem cells***

Human embryonic stem cells (ESCs) are pluripotent cells, derived from the inner cell mass of blastocyst stage embryos, having the ability to self-renew indefinitely while maintaining the potential to give rise to all cell types in the human body when provided with the appropriate differentiation signals[31]. Because of this plasticity and the unlimited capacity for self-renewal, ESC regenerative therapies have been proposed for tissue replacement after injury or disease.

ESCs are able to differentiate efficiently into hepatocyte-like cells *in vitro*, producing cells which possess some of the properties of mature hepatocytes[32-34]. ESC-derived hepatocyte-like cells contribute to the recovery of injured liver tissue in mice, not only by cell replacement but also by delivering trophic factors that support endogenous liver regeneration[32,35]. *In vitro* ESCs-derived hepatocytes, bearing the typical mature hepatocyte morphology and expressing hepatocyte-specific genes, colonized liver tissue upon transplantation and rescued liver-injured mice from death[36].

ESCs provide a valuable tool for studying the molecular basis of hepatocyte differentiation and form the basis for cell therapies. However, despite remarkable progress and the development of sophisticated differentiation protocols mimicking the normal embryonic development, ESC-derived “hepatocyte-like” cells usually fail to fully function as “true” hepatocytes. In addition, the risk for immunological rejection of the transplanted cells as well as ethical and legal concerns, hamper their use as cell replacement therapies[37,38].

***Induced pluripotent stem cells***

Induced pluripotent stem cells (iPSCs) are embryonic-like SCs produced *in vitro* via reprogramming of somatic cells through the transient, forced expression, of key transcription factors such as OCT4 (O), SOX2 (S), KLF4 (K), and c-MYC (M) (so called OSKM cocktail) or O, S, NANOG (N) and LIN28 (L) (so called OSNL), traditionally by using, permanently integrated, retroviral vectors[39,40].

Since the factor expression is not required beyond the end of the reprogramming process and the semi-random integration of retroviral vectors has been associated with insertional mutagenesis[41], several investigators explore techniques for iPSC generation using more clinically relevant methodologies of reprogramming, such as excisable vector systems[42], non-integrating DNA vectors[43], DNA-free methods[44,45] and small molecules[46].

iPSCspossess unique characteristics of pluripotency that render them extaordinatory tools for cell and gene therapies, such as (1) the unlimited self-renewal capacity *in vitro*, a feature that allows their indefinite maintenance in culture as cell lines; and (2) the potential for directed differentiation to any cell type. In addition to their potential for regeneration, iPSCs provide a novel platform for *in vitro* disease-modeling[47] and drug-screening[48].

It has been shown that iPSCs can be efficiently induced to differentiate into hepatocyte-like cells (HLCs)[49-52], whereas transplantation of iPSCs-derived HLCs reversed lethal fulminant hepatic failure, enhanced liver regeneration and improved the performance status of NOD-SCID[52], fumarylacetoacetate hydrolase-deficient[53] or CCl4-injured[54] mice. In an acute hepatic failure model, iPSCs were reprogrammed from human dental pulp-derived fibroblasts into iPSCs (DP-iPSCs) capable to differentiate into hepatocyte-like cells (iPSC-HLCs). An injectable carboxymethyl-hexanoyl chitosan hydrogel (CHC) with sustained hepatocyte growth factor (HGF) release (HGF-CHC) was developed to improve iPSC-HLCs engraftment. Intrahepatic delivery of HGF-CHC-iPSC-HLCs rescued liver function and the recipients through high anti-oxidant and anti-apoptotic activity that shrinked hepatic necrotic areas[55]. Engineered donor grafts derived from iPSCs, including re-cellularized biomatrix[56] and liver buds produced from iPSCs[57], may someday provide “autologous” organs for liver transplantation, thus highlighting their enormous potential for treating liver failure.

In addition to acquired liver diseases, HLCs differentiation from iPSCs isolated from patient somatic tissues could provide patient-specific hepatocyte sources for treatment of inherited liver diseases, combining *ex vivo* gene correction and cell transplantation[58].

iPSCs have renewed hopes for regenerative medicine because they could deliver personalized therapies and their production from somatic, patient-specific cells, without the use of embryonic tissues or oocytes, may overcome ethical concerns and the risk of rejection. Despite these hopes with iPSCs, the issues that still need to be addressed before moving this exciting new technology from proof of concept to the clinic are: (1) the optimal reprogramming method, using clinically relevant methodologies; (2) the avoidance of teratoma formation and tumorigenicity; (3) the development of novel and rapid differentiation protocols for the generation of mature cell types from iPSCs by cost-efficient manufacturing procedures and ultimately; and (4) the long-term safety, tolerability, and efficacy of the iPSC-based treatments.

***Annex stem cells***

Annex stem cells derived from umbilical cord, umbilical cord blood, placenta and amniotic fluid, are an easily accessible source of pluripotent SCs capable to giving rise to haematopoietic, epithelial, endothelial and neural cells both *in vitro* and *in vivo*[59], thus constituting an attractive target for cell-based therapy. Human umbilical cord blood SCs, when infused into NOD-SCID mice with induced liver damage, can differentiate into hepatocyte-like cells in the absence of fusion events[60], boost regeneration and reduce mortality[61]. *In vitro* expanded and differentiated umbilical cord SCs exhibited hepatocyte-like morphology, expressed up-regulated levels of markers of hepatic lineage and were capable of *in vivo* liver repopulation and expression of hepatic markers upon transplantation in mice[62,63].

Placenta-derived multipotent cells have also been shown to differentiate into multilineage cells including hepatocyte-like cells. These cells not only expressed characteristics of human liver cells, but also demonstrated several functions of typical hepatocytes[64,65].

**Extrahepatic Adult Bone Marrow Stem Cells**

As already mentioned, liver regeneration is mainly an endogenous process, driven by mature hepatocytes[3,4] and resident intrahepatic stem cell populations[9,10]. Bone marrow (BM) is the largest reservoir of pluripotent stem cells in adults and traditionally considered as giving rise to only hematopoietic cell lineages. This concept was challenged by reports demonstrating that BM-derived stem cells (hematopoietic, mesenchymal and endothelial cells) can generate a variety of adult cell types that express non-hematopoietic cell markers and contribute to the liver healing process after tissue injury[66–72].

***endothelial progenitor cells***

endothelial progenitor cells (EPCs) may contribute to the repair and regeneration of the damaged liver mainly by promoting the secretion of factors supportive of the host’s endogenous repair mechanisms. EPCs transplantation halted established liver fibrosis in rats by suppressing activated hepatic stellate cells, increasing matrix metalloproteinase activity, and regulating hepatocyte proliferation[73]. BM-derived liver sinusoidal EPCs recruited to the injured rat liver promoted hepatocyte proliferation and contributed to organ recovery[74]. Antifibrogenic and regenerative effects of engrafted EPCs, in transplanted rats, were mediated by increased expression of endogenous and exogenous growth factors, such as hepatocyte growth factor (HGF), transforming growth factor (TGF)-alpha, epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF) which triggered the generation of a new vascular network and promoted hepatocyte proliferation, ultimately resulting in liver regeneration[75-77].

***Mesenchymal stromal cells***

Bone marrow stroma contains a subset of mesodermal progenitor cells, named mesenchymal stromal cells (MSCs) which are fibroblast-like, plastic-adherent, multipotent cells rapidly expanding *in vitro* under standard culture conditions. MSCs are most frequently isolated from bone marrow (BM-MSCs)[78] but can be also isolated from a variety of tissues including umbilical cord blood (UC-MSCs)[79], trabecular bone[80], synovial membrane[81], adipose tissue (AT-MSCs)[82], placenta (hPMSCs)[83], amniotic fluid (AF-MSCs)[84], fetal lung (FL-MSCs) and blood[85]. MSCs have the capacity to differentiate into tissues of mesodermal origin (bone, cartilage, fat) but also to give rise to cells from unrelated embryonic layers such as nerve cells and hepatocytes. In addition, they have low immunogenicity and possess immunomodulatory properties which allow them to evade the host immune surveillance[86]. Because of these features, MSCs have been proposed as a cell therapy source with increased therapeutic potential for a wide range of diseases[87-91], including acute and chronic liver diseases. Studies conducted both in rodents[92-94] and humans[95-100] have shown that MSCs derived from BM, AT, AF, dental pulp, UC and FL under specific culture conditions, are able to transdifferentiate *in vitro* into hepatocyte-like cells which express genes and fulfill some metabolic functions typical to hepatocytes.

BM-, the first and the best characterized source reported to contain MSCs, AT-MSCs, an abundant and easily accessible source of MSCs, and UC- MSCs, attainable by the least invasive method, have been comparatively tested in terms of morphology, enrichment in MSCs following isolation and expansion, colony formation, multilineage differentiation capacity, and immune phenotype. While there weren’t distinct morphological or immune phenotypic features among the three sources of MSCs, AT provided 100% success rate in MSCs isolation and the highest colony frequency while UC-derived MSCs reached the highest rates of proliferation in culture, thus suggesting UC and AT as attractive alternatives to BM in obtaining MSCs[101].

***MSCs and acute liver failure***

The therapeutic effect of MSCs in models of acute liver failure has been elucidated in various studies. Indicatively, MSCs derived from BM, placenta and AT showed differentiation potential into hepatocytes *in vitro* and *in vivo*, ameliorated liver damage, reduced mortality, exerted immunoregulation by suppressing intrahepatic natural killer T (NKT)cells and inhibiting inflammatory signalling, inanimal models of induced acute liver failure[102-106].

When AT-, UC blood- (UCB) and human BM-derived MSCs, either as undifferentiated MSCs (U-MSCs) or as MSC-derived hepatocyte-like cells (DHLCs), were compared for their capacity to reverse acute fulminant hepatitis in an animal model, it was demonstrated that undifferentiated MSCs and DHLCs from AT and BM sources equivalently regenerated the damaged liver, thus implicating that hepatopoietic pre-differentiation of MSCS may not be necessary in liver repopulation. In addition, AT-MSCs because of their abundance and accessibility as well as their consistent hepatocyte expression profile upon differentiation, suggested AT as an excellent stem cell source for liver-regenerative procedures[107].

The conversion of MSCs into hepatocyte-like cells has been repeatedly demonstrated[108,109], and effort has been made to characterize hBMSCs-derived hepatocytes *in vitro* and *in vivo*. Towards this end, tissue inhibitor of metalloproteinases 4 (TIMP-4) and follistatin (FST) expression have been associated with transdifferentiation events and suggested as two potential novel biomarkers for the characterisation of hBMSCs-derived hepatocytes[110]. However, accumulating evidence supports the notion that the therapeutic effects of MSCs in acute liver injury are mediated to a large degree via paracrine mechanisms releasing trophic and immunomodulatory factors, rather than true transdifferentiating events. This is reinforced from experiments with MSC-conditioned medium where soluble factors contained in MSC-conditioned medium (IL-6, VEGF, HGF, and insulin-like growth factor binding proteins) seem responsible for reduced hepatocyte apoptosis[111], downregulation of proinflammatory cytokines, increased hepatocyte proliferation[112] and decreased mononuclear cell infiltration in the liver[113]. Indeed, secreted molecules in culture supernatant from both hFL-MSCs and hepatocyte progenitor-like cells derived from hFL-MSCs had a therapeutic effect in a CCl4-induced acute liver injury model[114] whereas transplantation of different origin MSCs rescued acute liver failure and repopulated mouse liver through paracrine effects that reduced the inflammatory response, inhibited apoptosis in the liver and stimulated endogenousregeneration mechanisms[115,116].

***MSCs–based therapy for liver cirrhosis***

The beneficial effect of MSCs in liver cirrhosis has been extensively demonstrated both in animal and clinical studies. Infused BM-MSCs have been shown to engraft into host liver and ameliorate fibrosis in a time-dependent manner by decreasing α-SMA expression, reducing collagen deposition and improving recovery of damaged hepatocytes in animal models of experimental liver fibrosis[117-119]. Recently, AT-MSCs have attracted much interest as liver repopulating cells in different models of cirrhosis. AT-MSCs, transplanted intraportally, rather than through tail vein, inhibited the proliferation and activation of hepatic stellate cells *in vitro* and ameliorated liver fibrosis in CCl4-treated rats by improving the microcirculation of the fibrotic liver[120,121]. In a murine steatohepatitis cirrhosis model, injected AT-MSCs resided in the liver and expressed albumin, ultimately restoring albumin expression in hepatic parenchymal cells. Gene expression profiling of AT-MSCs revealed that the amelioration of hepatic fibrosis in this model correlated with induction of anti-inflammatory and regeneration/repair pathways as well as suppression of pathogenic helper T-cell activation[122].

In contrast to the similar hepatic integration between undifferentiated AT-MSCs and AT-MSCs pre-differentiated to hepatocyte-like cells shown in acute liver injury models[107], other liver injury models suggest that pre-differentiation of AT-MSCs to hepatocyte-like cells may facilitate liver engraftment. In a xenogeneic transplantation model of liver regeneration, long-term engraftment of human AT-MSC-derived hepatocyte-like cells was demonstrated and it was significantly improved when *in vitro* pre-differentiated AT-MSCs, instead of undifferentiated MSCs were used, reaching repopulation rates of more than 10% along with functional hepatic regeneration[123].

Fibroblast growth factor (FGF)-pretreatment of AT-MSCs facilitated their transdifferentiation towards hepatic lineage *in vitro* and the infused FGF-pretreated AT-MSCs reduced hepatic fibrosis in mice[124]. In chronic liver injury models, FGF-treated AT-MSCs led to enhanced hepatocyte proliferation and induction of hepatic stellate cell apoptosis through the activation of JNK-p53 signaling in hepatic stellate cells[125], while BM-MSCs pretreated with hepatocyte growth factor **(**HGF) and FGF4 or with injured liver tissue showed increased homing and hepatic differentiation ability providing therapeutic benefit in injured mice[126,127].

It seems that MSCs exert their therapeutic effects predominantly by releasing trophic and immunomodulatory factors rather than trans-differentiating into parenchymal hepatocytes. MSCs modulate the function of activated stellate cells via paracrine secretion of IL-10, HGF and Nerve Growth Factor, providing a plausible explanation for the protective role of MSCs in liver inflammation and fibrosis[128-130]. Additionally, MSCs may alleviate hepatic cirrhosis through the expression of matrix metalloproteinases (MMP-9, MMP-13), enzymes capable of degrading the extracellular matrix, thus exerting a direct antifibrotic effect in the injured liver[131,132].

Several clinical trials (Table 1) have investigated the therapeutic potential of MSCs derived from BM or UCB in liver cirrhosis, providing however, conflicting results. In two pilot, phase I and I-II, studies, autologous BM-MSCs were injected into peripheral or portal vein of a small number of patients with end-stage liver disease. Liver function and clinical features were improved while the procedure was safe and well tolerated[133,134]. Safety and short-term efficacy of autologous BM-MSCs stimulated towards hepatic lineage and injected via intrasplenic or intrahepatic routewas evidenced in two groups of 20 patients with post-HCV end-stage liver cell failure. Patients significantly improved their Child and MELD score, fatigue scale and performance status over the control group who received conventional supportive treatment[135]. In 53 patients with post-HBV liver failure, autologous transplantation of BM-MSCs through the hepatic artery provided short-term efficacy in respect to several clinical and biochemical parameters, but long-term outcomes were not markedly improved[136]. Similarly, in a phase II trial with autologous transplantation of BM-derived, undifferentiated and differentiated, MSCs in 15 post-HCV cirrhotic patients, follow up at 3 and 6 mo postinfusion, revealed partial improvement of liver function tests and decline of elevated bilirubin and MELD score[137]. Another study in post-HCV cirrhotic patients, suggested the safety, feasibility, and efficacy of intrasplenically administered autologous BM-MSCs in improving liver function[138]. Eleven patients with alcoholic cirrhosis safely received autologous BM-MSCs through the hepatic artery in a phase II clinical trial; histological and clinical (by Child-Pugh score) improvement was observed in 54.5% and 90.9% of patients respectively, while the levels of TGF-b1, type 1 collagen, and a-smooth muscle actin were significantly decreased[139]. Similarly, UC-MSC infusion was well tolerated in patients with decompensated cirrhosis, acute in chronic liver failure and in patients with primary biliary cirrhosis, resulting in significant improvement of liver function and increased survival rates[140-142].

In contrast to the above mentioned studies, a randomized, placebo-controlled trial using peripheral administration of autologous MSCs to cirrhotic patients, failed to show a beneficial effect of MSCs in cirrhotic patients. Indeed, 3 of 15 patients who received MSCs died in the first 5 mo following cell administration while the absolute changes in Child and MELD scores, serum albumin, INR, serum transaminases and liver volumes did not differ significantly between the MSC and placebo group at 12 mo-follow-up, indicating that further studies with higher number of patients are warranted to clarify the true impact of systematic or liver-directed MSC infusion in cirrhosis[143].

***Considerations on the clinical application of MSCs***

The unique properties of MSCs including easy access and expansion, engraftment capacity, paracrine secretion, trans-differentiation and immunomodulation render them ideally suited for cell therapies. Importantly, compared to embryonic stem cells, MSCs do not raise ethical issues and presumably have a safer profile in terms of tumorigenesis. Up to date, a considerable amount of preclinical and clinical evidence is currently available as regards the promise of MSCs as a relatively safe and effective approach in improving liver disease. However, several issues still need to be addressed before MSCs-based liver therapy passes to the clinical practice and these are discussed below.

There is a lack of uniformity in the design of clinical trials, characterized by different MSC sources, doses and routes of administration, all of which may influence the outcome of MSC infusion on the basis also of the underlying disease; MSCs engrafted into injured or regenerating livers only after intrahepatic but not intrasplenic injection[144] whereas intravenously injected BM-MSCs migrated and engrafted into normal and injured liver parenchyma, under conditions of chronic but not acute injury[145]. On the contrary, the systematic administration of MSCs in a randomized trial with cirrhotic patients failed to provide efficacy over placebo[143].

In terms of safety, and despite the absence of severe adverse events in the clinical trials conducted thus far, a pro-fibrogenic potential of MSCs and unwanted differentiation into myofibroblasts has been described in several studies[144-146]. To avoid this unwanted differentiation, some groups have suggested that BM-SCs should be induced to differentiate into hepatocyte-like cells before their infusion[123] Alternatively, others have proposed the microencapsulation of MSCs in alginate-polyethylene glycol microspheres as a means to prevent scar formation through the artificial interruption of the cell-to-cell interactions but still the enablement of releaseof soluble molecules[147].

Although MSCs are at low risk of malignant transformation, concern exists on their potential to promote tumor growth *in vivo*[148-150]. Thus, screening of MSCs for a gene expression signature before administration, could serve as a safety measure[151]. *In vitro*, the spontaneous transformation of MSCs resulting in tumorigenesis was a rather rare event and occurred only after extended (beyond five weeks) culture. On the contrary, because of their immunomodulatory properties, MSCs may exert an antitumor effect by modulating the inflammatory environment that characterizes many tumors and by inhibiting signalling pathways associated with tumor growth and cell division[152-156].

***Hematopoietic stem cells***

Bone marrow has been considered as a source of liver-repopulating cells that contributes to the liver healing process after tissue injury, thus challenging the dogma of BM as giving rise to only hematopoietic cell lineages. It has been reported that BM-derived stem cells can differentiate into a variety of adult cell types that express non-hematopoietic cell markers[69-72], including hepatocytes[157]. The group of Grompe first suggested that functional hepatocytes may arise from hematopoietic stem cells (HSCs)[66], and in early 2000s, several groups demonstrated that SCs originating in the BM or circulating outside the liver participate in liver regeneration, not only in experimental animal models[67] but also in human livers[157,158]. Numerous studies followed, highlighting the contribution of HSCs in ameliorating liver damage.

Hepatic injury caused by surgical liver resection or cirrhosis in humans, triggered BM CD34+ or CD133+/c-kit+/bcrp-1+ cell trafficking towards the liver and putatively the differentiation of various populations of hematopoietic progenitor cells into hepatocyte-like cells[159-161]. BM cell transplantation or infusion of macrophages in a mouse model of liver fibrosis indicated that the migrated to the liver cells reduced liver fibrosis and significantly improved survival rate compared with control injured mice[162,163] while BM–derived hepatocytes were identified in lethally irradiated mice transplanted with HSCs[164]. In patients with malignant liver lesions, the combination of portal vein embolization (PVE) with administration of CD133+ BMSCs, substantially increased hepatic regeneration compared with PVE alone[165] whereas cirrhotic patients safely underwent autologous BM cell infusion and improved their Child-Pugh score and albumin levels (Table 1)[166].

***G-CSF mobilization as a source of large numbers of putatively liver-repopulating cells***

HSCs can be easily forced to egress BM and circulate into the peripheral blood from where they can be apheresed and subsequently enriched by their surface expression of CD34 or/and CD133. Mobilization of BM-resident HSCs occurs at a low magnitude under specific stimuli such as tissue injury[159,167] or at high amounts after pharmacological priming with cytostatic drugs, chemokines or hematopoietic cytokines[168,169]. G-CSF is a hematopoietic growth factor and the most widely used mobilizing agent[170]. G-CSF, as a means of forced circulation of large numbers of HSCs, has been extensively investigated for its hepatic regenerative effect, both in animal models of liver injury[171-174] as well as in clinical trials[175-177]. In general, two approaches have been explored as means of liver population with mobilized HSCs, both in animal models and clinical trials; G-CSF-mobilization alone or G-CSF-mobilization followed by infusion of autologous mobilized HSCs.

As seen with BM transplantation in liver injury models and despite the higher numbers of HSCs potentially accessing the liver by G-CSF mobilization, the true contribution of mobilized HSCs to liver repopulation is low. We and others[171,173] have shown that G-CSF–mobilization of BM chimeras in induced acute and chronic liver injury models results in liver regeneration and improves survival, however, the vast majority of cells repopulating the liver originate in situ. In a comparative study of all currently available mobilizing agents (G-CSF, Plerixafor, Plerifaxor + G-CSF) as regards their liver repopulating potential, we have shown that all mobilizing modalities ameliorate liver fibrosis, by acting differentially during the healing process. In all cases, liver recovery was not ultimately mediated by the HSCs but either from a paracrine or “by stander” signaling effect of the mobilized HSCs that triggered endogenous repair mechanisms and stimulated tissue progenitor cells or/and a direct, “trophic” effect of the mobilizing agents to the liver. These effects, however, are difficult to be experimentally dissected to definitively address this question[174].

Clinical studies that evaluated G-CSF mobilization in patients with advanced liver disease provided conflicting results (Table 1). In trials with end-stage liver cirrhosis or alcoholic steatohepatitis patients, G-CSF was well tolerated[175,178], the mobilized HSCs were shown to coexpress epithelial and stem cell markers[175] and to induce hepatic progenitor cells to proliferate within 7 d of administration[178]. In acute-on-chronic liver failure (ACLF) patients, mobilization of HSCs with G-CSF promoted hepatic regeneration and more than doubled the percentage of patients with ACLF who survived for two months; it also significantly reduced CTP, MELD, and SOFA scores and prevented the development of sepsis, hepatorenal syndrome, and hepatic encephalopathy[179]. Similarly, a recent randomized open study showed that the administration of G-CSF was safe and improved liver function as well as survival in patients with severe alcoholic hepatitis[180]. In an interesting case report, experimental plasmapheresis with plasma-exchange (PA-PE), as a process to eliminate circulating toxic factors, was combined with G-CSF in a patient with acute-on-chronic liver failure (ACLF)[181]. This regimen induced mobilization of HSCs and a rapid and long lasting clinical improvement associated with a ductular reaction, in which hepatic progenitor cells (HPCs) expressing GCSF receptor (GCSFR) were observed. PA-PE might have modulated the liver microenvironment thus providing a conducive milieu to G-CSF-mediated amplification of endogenous HPCs that promoted liver regeneration. Given that G-CSFR was expressed by HPCs (G-CSFR), G-CSF might also be directly involved in modifying the HPC niche exerting a “hepatotrophic effect”[181]. On the contrary, other clinical studies reported on the safety and tolerability of G-CSF mobilization but could not demonstrate significant clinical improvement, despite effective mobilization[182,183].

The relatively easy access to large quantities of HSCs by mobilization followed by cytapheresis, renders them ideally suited as liver repopulating cells. Thus, several groups have investigated G-CSF-mobilization followed by infusion of autologous mobilized HSCs, an approach that forces a maximum SC dose to circulate at a given time, thus increasing the number of SCs that potentially home to the liver and initiate the recovery process.

We previously assessed the safety and efficacy of boost *iv* infusions of mobilized peripheral blood stem cells (mPBSCs) in two patients with end-stage alcoholic liver cirrhosis. The patients tolerated well three mobilization rounds and infusions of mPBSCs that resulted in lasting amelioration in the clinical course of a previously decompensated disease, during a 30 mo follow-up[176]. In another study, a significant biochemical and histopathological improvement was achieved in a patient with drug-induced acute liver failure after intraportal administration of mobilized CD34+ BMSCs[184].

A phase I study was performed to determine the safety and tolerability of G-CSF administration, followed by collection and intraportal or intrahepatic reinfusion of circulating CD34+ cells into patients with liver failure. An improvement of the hepatic function without significant side effects in short and long term follow-up was observed in more than 50% of the subjects[177,185]. In another trial, following G-CSF mobilization and leukapheresis, the autologous CD34+ cells were expanded *in vitro* and injected into the hepatic artery of nine patients with alcoholic liver cirrhosis (ALC). The clinical and biochemical improvement in the study group was encouraging while it proved safe to mobilize, expand, and reinfuse autologous CD34+ cells in ALC patients[186]. In one of the largest up to date trials, forty patients with decompensated, hepatitis B virus-related, liver cirrhosis were randomized to receive G-CSF alone or in combination with leukapheresis and reinfusion of peripheral blood monocytes (PBMC). A significant biochemical and clinical improvement was observed in both groups, however, the subjects receiving G-CSF plus PBMC infusion experienced the greater and longer-lasting clinical benefits during the follow-up period[187].

***Considerations on the use of HSCs as liver-repopulating cells***

The concept of BM-derived liver regeneration has been strongly questioned. Despite the improvement that was seen in several parameters of liver function, both in preclinical and clinical studies, it has become clear that, in the absence of selective pressure, the true contribution of BM to liver regeneration is extremely low to effectively support liver recovery *per se*[188-190]. The current belief is that the clinical benefit observed in the injured liver after hematopoietic stem cell (HSC) therapy is produced by the activation of endogenous progenitor cells through paracrine signaling interaction between donor and host cells providing cytokines and growth factors[190-192], rather than by transdifferentiation of BMSCs into parenchymal liver cells[158] or cell fusion with resident target cells in the host tissue[193,194].

Overall, from the variety of the published studies on the use of HSCs as a cell therapy source for liver diseases, it seems that mobilization of HSCs, apheresis and re-infusion is safe while it improves the quality of life and the disease parameters. As such, this approach may help to “bridge” patients to liver transplantation or reverse a decompensated cirrhosis to a compensated stage. In addition, the use of autologous mobilized HSCs as a cell source for liver regeneration, is not associated with ethical concerns while it can provide easy access to and high yields of SCs without the risks of rejection or need for immunosuppression. However, efficacy still needs to be confirmed and the route of delivery, the amount of infused cells and the timing of infusions to be clarified, stardardized and validated in well-designed large clinical trials.

***Liver tissue engineering***

Liver tissue engineering endeavours to provide novel tools for end-stage liver diseases which will, ideally, replace organ transplantation. Therapeutic approaches towards this goal include implantable hepatic tissue engineered constructs and bioartificial liver (BAL) devices.

Implantable engineered cellular tissues provide an alternative method of cell delivery and are gaining ground in the field of regenerative medicine. They are generated mainly by immobilizing or encapsulating cells using biomaterial scaffolds. Biomaterial scaffolds provide three dimensional (3D) structures resembling the extracellular matrix environment *in vivo* and have been used in association with appropriate induction medium to promote BM-derived MSCs differentiation into hepatocyte-like cells[195]. Except from alginate scaffolds[195], derived from natural polysaccharide based biomaterials, nanofibrous scaffold, synthetic polymer-based biomaterials allowing easy control of the quality and reproducibility of the product, have been used to investigate the hepatic differentiation potential of human BM-MSCs seeded on 3D nanofibrous scaffold. Nanofibrous scaffold enhanced SCs differentiation into functional hepatocyte-like cells expressing liver specific markers, as compared to 2D culture systems[196].

Similarly, the topographic properties of ultrawebnanofibers enhanced the differentiation of MSCs to hepatocyte-like cells (HLCs) which maintained functionality in long-term cultures. Differentiated HLCs homed and engrafted to the injured liver of fibrotic mice, enhanced serum ALB and rescued recipients from liver failure[197]. In another study, collagen-coated poly (lactic-co-glycolic acid) (C-PLGA) 3D scaffolds, supplemented with hepatocyte differentiation medium, provided a suitable environment for differentiation of BM-MSCs to mature hepatocytes over the control, monolayer culture system[198]. Recently, Poly 3-hydroxybutyrate-co-3-hydroxyvalerate-co-3-hydroxyhexanoate (PHBVHHx) scaffolds, made up by biodegradable polyester produced by bacteria, provided higher viability and attachment of human umbilical cord Wharton’s jelly-MSCs than other polymers tested, ultimately promoting the recovery of injured liver after transplantation in mice[199].

Bioartificial liver (BAL) devices contain functional hepatocytes that supply the important molecules to support hepatic function and remove circulating toxins. This technology, however, is limited by the complexity of liver function and the shortage of human livers to provide adequate numbers of hepatocytes. Thus, *ex vivo* differentiated hepatocytes from alternative sources have been investigated. A BAL device seeded with ES cell-derived hepatocytes or primary hepatocytes which was subcutaneously implanted in 90% hepatectomized mice improved liver function and prolonged survival over control mice whereas ES cell-derived hepatocytes in BAL developed characteristics nearly identical to those of primary hepatocytes[200].

Very recently, the 3D printing technologies, by fabricating complex 3D tissue engineering scaffolds and providing patient-specific tissue models showed promise to revolutionize liver regenerative medicine towards customized transplantation approaches[201].

For all the above technologies however, challenges still remain and dictate an in depth understanding of the specific molecular mechanisms and signaling pathways in the hepatic microenvironment that affect hepatic cell lineages and regulate efficient differentiation of stem cells[202].

**CONCLUSION**

SC-based liver regeneration is an exciting and dynamic area of research flaunting remarkable advancements in liver medicine, both in basic science and the translational field. The clinical translation for liver cell therapies however, from only a promise for cure to a treatment reality for end stage liver diseases, requires deeper understanding of SC and liver biology and the remaining unsolved aspects to be addressed.

In the liver cell therapy field there has been a lack of uniformity in preclinical and clinical studies as regards the type and the extent of injury into the liver parenchyma, the SCs sources and dose of stem cells, the therapeutic timing and route of administration of stem cells, and the primary end points. In addition, positive results in animal models have not always been translated to successful clinical trials as clear evidence of therapeutic benefit has usually been lacking from clinical trials. As such, carefully designed clinical trials will help to elucidate the most appropriate SC therapy for the different liver diseases, should the background and severity of the target disease as well as the putative functional roles of different stem cells and their biological action intended from their infusion, be considered.

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**Table 1 Clinical trials using stem cells for the treatment of liver diseases**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Ref.** | **Cell Source** | **No. of patients/administration route** | **Disease cause** | **No. of cells infused** | **Follow-up period** | **Outcomes** |
| 29 | Fetal liver-SCs (EpCAM+) | 25: hepatic artery | End-stage liver cirrhosis | 80 × 106 | 6 mo | Improved liver function and MELD score |
| 30 | Fetal liver-SCs (EpCAM+) | 2: hepatic artery | Advanced cirrhosis | 42 × 106 and 60 × 106 | 12 mo | Biochemical and clinical improvement  |
| 133 | BM-MSCs | 4: peripheral vein | Decompensated liver cirrhosis | 31.73 × 106 | 12 mo | Well tolerated and safe procedure; improved liver function  |
| 134 | MSCs from iliac crest | 8:peripheral or portal vein | End-stage liver disease | 30 × 106-50 ×106 | 24 wk | No adverse effects; improved MELD and liver function |
| 135 | BM-MSCs stimulated to hepatic lineage | 20:control10:intrasplenic10:intrahepatic | post-HCV end-stage liver disease  | 2 × 107 in a total of 2 × 108 MNCs  | 6 mo | Improved ascites, MELD and CP score; no difference between intrahepatic and intrasplenic groups |
| 136 | BM-MSCs | 105:control 53:treated /proper hepatic artery | post-HBV liver failure  | 3.4 × 108-3.8 × 108 | 192 wk | No serious side effects or complications; improved ALB, TBIL, PT and MELD score  |
| 137 | differentiatedBM-MSCs *vs* undifferentiated  | 10:control 15:treated/intravenous  | post-HCV liver cirrhosis  | 1 × 106/kg body weight | 6 mo | Improved MELD score, BIL, ALB and PC |
| 138 | BM-MSCs | 20: intrasplenic | post-HCV liver cirrhosis | 10 × 106 | 6 mo | Decreased TBIL, AST, ALT, PT; improved ALB, PC, PT, INR |
| 139 | BM-MSCs | 11: hepatic artery | Alcoholic cirrhosis | 5 × 107 injected twice | 12 mo | No significant side effects; histological improvement; improved CP score |
| 140 | UC-MSC | 15: control 30: treated/ intravenous | post-HBV decompensated liver cirrhosis | 0.5 × 106/kg body weight  | 1 yr | No significant side effects; improved liver function and MELD score; reduced ascites |
| 141 | UC-MSC | 19: control 24: treated/ intravenous | post-HBV acute-on-chronic liver failure (ACLF)  | 0.5 × 106/kg body weight  | 72 wk | No significant side effects; improved liver function and MELD score; increased survival  |
| 142 | UC-MSC | 7: peripheral vein | Primary biliary cirrhosis | 0.5 × 106/kg  | 48 wk | No obvious side-effects; decreased serum ALP and GGT |
| 143 | Autologous MSCs | 12: control 15: treated/ peripheral vein | Decompensated cirrhosis | 195 × 103 | 12 mo | No beneficial effect |
| 166 | BM-MNCs  | 9: peripheral vein | Liver cirrhosis | 5.20 +/- 0.63 × 109 MNCs | 24 wk | No major adverse effects; improved ALB, CPscores |
| 175 | G-CSF mobilization | 40: controls 8: treated/subcutaneous | Severe liver cirrhosis | G-CSF: 5 μg/kg every 12 h for 3 d | 8 mo | No adverse events; improved MELD score |
| 176 | Autologous G-CSF mobilized CD34+cells | 2: peripheral vein | End-stage liver disease | G-CSF :10 μg/kg per day: 4-5 d /CD34+cells: 2.31 × 106/kg and 4 × 106/kg | 30 to 34 mo | Safe and well tolerated procedure; improved CP and MELDscores |
| 177 | Autologous G-CSF-mobilized CD34+cells  | 3: portal vein2: hepatic artery | Liver insufficiency | CD34+ cells: 1 ×106 to 2 ×108 | 60 d | No complications or specific side effects; improved ALB  |
| 178 | G-CSF mobilization | 11: control 13: treated/ subcutaneous | Alcoholic cirrhosis | G-CSF: 10 μg/kg per day 2 times daily for 5 d | 12 wk | Effective CD34+ cells mobilization; increased HGF; induced HPC proliferation |
| 179 | G-CSF mobilization | 24: control 23: treated/ subcutaneous | Acute-on-chronic liver failure (ACLF) | G-CSF: 5 μg/kg for 12 doses  | 60 d | Increased survival; reduced CTP, MELD and SOFA scores |
| 180 | G-CSF mobilization | 23: control 23: treated/subcutaneous | Severe alcoholic hepatitis | GCSF: 5 μg/kg every 12 h for 5 d  | 3 mo | Safe and effective HSCs mobilization; improved liver function and survival |
| 181 | Experimental PA-PE, combined with G-CSF | 1: subcutaneous | Acute-on-chronic liver failure (ACLF) | 10 μg/kg per day for 5 d  | 2 mo | Rapid and long lasting clinical improvement; HSCs mobilization and a ductular reaction |
| 182 | G-CSF mobilization | 24: subcutaneous | Acute on chronic liver failure | G-CSF: 5 and 15 μg/kg per day for 6 d |  | Safety and feasibility of G-CSF mobilization; no clinical/biochemical improvement  |
| 183 | G-CSF mobilization | 18: subcutaneous | Liver cirrhosis | increasing doses of G-CSF daily for 7 d | 3 wk | No severe adverse events; no liver function significant modification  |
| 184 | Autologous G-CSF mobilized CD34+cells | 1: portal vein | Drug-induced hepatitis | G-CSF: 15 μg/kg/ for 5 d CD34+ cells: 5 × 106 | 30 d | Improved liver function; wide areas of regeneration in liver biopsy |
| 185 | Autologous G-CSF-mobilized CD34+ SCs | 2: hepatic artery3: portal vein  | Chronic liver disease | G-CSF: 526 μg/d: 5 d, CD34+ cells: 1 × 106-2 × 108 | 6-18 mo | No side effects; improved BIL and ALB |
| 186 | Autologous G-CSF-mobilized cultured CD34+ SCs | 9: hepatic artery | Alcoholic liver cirrhosis | 520 μg/d: 5 d/ mean TNCC:229.7 × 106  | 12 wk | No side effects; improved BIL, ALT, AST, CP score and ascites |
| 187 | PBMCs from G-CSFmobilized PB | 20: control 20: treated | Decompensated liver cirrhosis | 5-10μg /kg per day for 4 d. PBMC: 107 to108/kg | 6 mo | No major adverse effects; improved liver function |

EpCAM: epithelial cell adhesion molecule; MSCs: mesenchymal stromal cells; HCV: hepatitis C virus.