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REVIEW

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

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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 liver has 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 emerges. In this review, we summarize the state-of-the-art in the field of stem cell-based therapies for the liver 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

Core tip: Liver transplantation is the only effective treatment for end-stage liver diseases, but its appli-

cation 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 stem cell-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 prove insufficient and hepatic injury may 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 because 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 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 (SC) 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 SC niches. OC/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 or remodeling of extracellular matrix to facilitate appropriate OC/LPC expansion and ultimately biliary and hepatocyte regeneration[15]. The role of Wnt and Notch signaling in hepatic cell fate 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 the case of severe parenchymal extinction could facilitate their *in vitro* and *in vivo* maturation to hepatocytes and their application in 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 SCs located in the small bile ducts and the canals of Hering, thus appearing to be the structures that contain SC-derived hepatocytes[18]. The progressive evolution of buds from stem/progenitor cells 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, and Ki-67[19].

Interestingly, hepatic stellate cells (HSTCs), considered as liver-resident mesenchymal cells[20], have recently been 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 and are considered bipotential, being able to give rise to both mature hepatocytes and bile duct epithelial cells (cholangiocytes)[22]. Many experimental studies have focused on the regenerative capacity of fetal hepatic progenitor cells (HPCs) as, in contrast to adult hepatocytes, fetal liver SCs 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 the treatment of 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, 25 patients with liver cirrhosis of different etiologies, were infused with human fetal liver-derived SCs. 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. Immunosuppressants 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 and one maintained the benefits for 12 mo[30].

The ability of fetal liver SCs 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 SCs, 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 SCs (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* ESC-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 therapy[37,38].

Induced pluripotent stem cells

Induced pluripotent SCs (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].

As 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 have explored 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].

iPSCs possess unique characteristics of pluripotency that render them extraordinary tools for cell and gene therapies, such as (1) unlimited self-renewal capacity *in vitro*, a feature that allows their indefinite maintenance in culture as cell lines; and (2) 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 iPSC-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 of differentiating into HLCs (iPSC-HLCs). An injectable carboxymethyl-hexanoyl chitosan hydrogel (CHC) with sustained hepatocyte growth factor (HGF) release (HGF-CHC) was developed to

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	80×10^{6}	6 mo	Improved liver function and MELD score
30	Fetal liver-SCs (EpCAM+)	2: hepatic artery	Advanced cirrhosis	42×10^{6} and 60×10^{6}	12 mo	Biochemical and clinical improvement
133	BM-MSCs	4: peripheral vein	Decompensated liver cirrhosis	31.73×10^{6}	12 mo	Well tolerated and safe procedure; improved liver function
134	MSCs from iliac crest	8: peripheral or portal vein	End-stage liver disease	30×10^{6}-50×10^{6}	24 wk	No adverse effects; improved MELD and liver function
135	BM-MSCs stimulated to hepatic lineage	20: control 10: intrasplenic 10: intrahepatic	post-HCV end-stage liver disease	2×10^{7} in a total of 2×10^{8} MNCs	6 mo	Improved ascites, MELD and CP score; no difference between intrahepatic and intrasplenic groups
136	BM-MSCs	105: control 53: treated/hepatic artery	post-HBV liver failure	3.4×10^{8}-3.8×10^{8}	192 wk	No serious side effects or complications; improved ALB, TBIL, PT and MELD score
137	Differentiated BM-MSCs *vs* undifferentiated	10: control 15: treated/intravenous	post-HCV liver cirrhosis	1×10^{6}/kg body weight	6 mo	Improved MELD score, BIL, ALB and PC
138	BM-MSCs	20: intrasplenic	post-HCV liver cirrhosis	10×10^{6}	6 mo	Decreased TBIL, AST, ALT, PT; improved ALB, PC, PT, INR
139	BM-MSCs	11: hepatic artery	Alcoholic cirrhosis	5×10^{7} 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	0.5×10^{6}/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	0.5×10^{6}/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	0.5×10^{6}/kg	48 wk	No obvious side-effects; decreased serum ALP and GGT
143	Autologous MSCs	12: control 15: treated/peripheral vein	Decompensated cirrhosis	195×10^{3}	12 mo	No beneficial effect
166	BM-MNCs	9: peripheral vein	Liver cirrhosis	$5.20 +/- 0.63 \times 10^{9}$ MNCs	24 wk	No major adverse effects; improved ALB, CP scores
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: 2.31×10^{6}/kg and 4×10^{6}/kg	30 to 34 mo	Safe and well tolerated procedure; improved CP and MELD scores
177	Autologous G-CSF-mobilized CD34^{+} cells	3: portal vein 2: hepatic artery	Liver insufficiency	CD34^{+} cells: 1×10^{6} to 2×10^{8}	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	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	G-CSF: 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	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: 5×10^{6}	30 d	Improved liver function; wide areas of regeneration in liver biopsy

185	Autologous G-CSF-mobilized CD34$^+$ SCs	2: hepatic artery 3: portal vein	Chronic liver disease	G-CSF: 526 μg/d: 5 d, CD34$^+$ cells: 1 × 10^6-2 × 10^8	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 × 10^6	12 wk	No side effects; improved BIL, ALT, AST, CP score and ascites
187	PBMCs from G-CSF mobilized PB	20: control 20: treated	Decompensated liver cirrhosis	5-10 μg/kg per day for 4 d. PBMC: 10^7-10^8/kg	6 mo	No major adverse effects; improved liver function

G-CSF: Granulocyte-colony-stimulating factor; TBIL: Total bilirubin; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; CP: Child-Pugh; BM: Bone marrow; UC: Umbilical cord; HSC: Hematopoietic stem cell; HGF: Hepatocyte growth factor; EpCAM: Epithelial cell adhesion molecule; MSCs: Mesenchymal stromal cells; HCV: Hepatitis C virus; PT: Prothrombin time; ALB; Albumin; PC: Platelet count; INR: International normalized ratio; PA-PE: Experimental plasmapheresis with plasma-exchange; MELD: Model for End-stage Liver Diseases; ALP: Alkaline phosphatase; GGT: γ-glutamyl transferase; UC-MSC: Umbilical cord blood-mesenchymal stromal cells; BM-MSCs: Bone marrow-mesenchymal stromal cells.

improve iPSC-HLC 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, HLC 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 for 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 reprograming 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 (4) the long-term safety, tolerability, and efficacy of the iPSC-based treatments.

Annex stem cells

Annex SCs derived from umbilical cord, umbilical cord blood, placenta, and amniotic fluid (AF) are an easily accessible source of pluripotent SCs capable of giving rise to hematopoietic, 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 HLCs 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 upregulated levels of markers of hepatic lineage, and were capable of *in vivo* liver repopulation and expression of hepatic markers upon transplantation into mice[62,63].

Placenta-derived multipotent cells have also been shown to differentiate into multilineage cells including HLCs. 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 SC populations[9,10]. Bone marrow (BM) is the largest reservoir of pluripotent SCs in adults and traditionally considered as giving rise to only hematopoietic cell lineages. This concept was challenged by reports demonstrating that BM-derived SCs (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. EPC 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 HGF, transforming growth factor (TGF)-α, epidermal growth factor, and vascular endothelial growth factor 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 also be obtained from a variety of tissues including umbilical cord blood (UC-MSCs)[79], trabecular bone[80], synovial membrane[81], adipose tissue (AT-MSCs)[82], placenta[83], 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 HLCs which express genes and fulfill some metabolic functions typical of hepatocytes.

BM-MSCs, the first and the best characterized source reported to contain MSCs, AT-MSCs, an abundant and easily accessible source of MSCs, and UC-MSCs, obtainable by the least invasive method, have been tested comparatively in terms of morphology, enrichment in MSCs following isolation and expansion, colony formation, multilineage differentiation capacity, and immune phenotype. While there were no distinct morphological or immune phenotypic features among the three sources of MSCs, AT provided a 100% success rate in MSC isolation and the highest colony frequency, while UC-derived MSCs had the highest rates of proliferation in culture, suggesting UC and AT as attractive alternatives to BM for 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. MSCs derived from BM, placenta, and AT showed potential for differentiation into hepatocytes *in vitro* and *in vivo*, ameliorated liver damage, reduced mortality, and exerted immunoregulation by suppressing intrahepatic natural killer T cells and inhibiting inflammatory signaling, in animal models of induced acute liver failure[102-106].

When AT-, UC blood-, and human BM-derived MSCs, either as undifferentiated MSCs or as MSC-derived HLCs (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, suggesting that hematopoietic pre-differentiation of MSCs may not be necessary for liver repopulation. In addition, because of the abundance and accessibility of AT-MSCs as well as their consistent hepatocyte expression profile upon differentiation, AT may be an excellent SC source for liver-regenerative procedures[107].

The conversion of MSCs into HLCs has been repeatedly demonstrated[108,109], and effort has been made to characterize hBMSC-derived hepatocytes *in vitro* and *in vivo*. Towards this end, tissue inhibitor of metalloproteinases 4 and follistatin expression have been associated with transdifferentiation events and suggested as two potential novel biomarkers for the characterization of hBMSC-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 (interleukin-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]. In addittion, 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 endogenous regeneration mechanisms[115,116].

MSC-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-smooth muscle actin 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 the 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 HLCs shown in acute liver injury models[107], other liver injury models suggest that pre-differentiation of AT-MSCs to HLCs may facilitate liver engraftment. In a xenogenic transplantation model of liver regeneration, long-term engraftment of human AT-MSC-derived HLCs was demonstrated and 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 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 UC blood 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 route was 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 SCs, 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-MSCs should be induced to differentiate into HLCs 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 release of 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 signaling 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 SCs 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 SCs (HSCs)[66], and in the early 2000s, several groups demonstrated that SCs originating in the BM or circulating outside the liver participated in liver regeneration, not only in experimental animal models[67] but also in human liver[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 HLCs[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, a combination of portal vein embolization (PVE) and administration of CD133+ BMSCs substantially increased hepatic regeneration compared with PVE alone[165], while 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 easily be forced to leave the 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 in high amounts after pharmacological priming with cytostatic drugs, chemokines, or hematopoietic cytokines[168,169]. Granulocyte-colony stimulating factor (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 for 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, but 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) with regard to 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 “bystander” signaling effect of the mobilized HSCs that triggered endogenous repair mechanisms and stimulated tissue progenitor cells and/or a direct "trophic" effect of the mobilizing agents in 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 in end-stage liver cirrhosis or alcoholic steatohepatitis patients, G-CSF was well tolerated[175,178], and the mobilized HSCs were shown to coexpress epithelial and SC markers[175] and to induce HPCs 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 ACLF patients who survived for 2 mo; 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 ACLF[181]. This regimen induced mobilization of HSCs and a rapid and long lasting clinical improvement associated with a ductular reaction, in which HPCs expressing G-CSF receptor (G-CSFR) 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-CSF might also be directly involved in modifying the HPC niche exerting a "hepatotrophic effect"[181]. In contrast, 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 SCs (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 trials, 40 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, but the subjects receiving G-CSF plus PBMC infusion experienced 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 an improvement 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 in effectively supporting *per se* liver recovery[188-190]. The current belief is that the clinical benefit observed in the injured liver after 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 various 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 improving quality of life and 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 and can provide easy access to, and high yields of, SCs without the risk 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 need to be clarified, standardized, 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 3-dimensional (3D) structures resembling the extracellular matrix environment *in vivo*, and have been used in association with an appropriate induction medium to promote BM-derived MSC differentiation into HLCs[195]. Apart from alginate scaffolds[195], derived from natural polysaccharide-based biomaterials, 3D nanofibrous scaffolds of 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. The nanofibrous scaffolds enhanced SC differentiation into functional HLCs expressing liver specific markers compared with 2D culture systems[196].

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

BAL devices contain functional hepatocytes that supply important molecules to support hepatic function and to 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 ESC-derived hepatocytes or primary hepatocytes which was subcutaneously implanted in 90% hepatectomized mice, improved liver function and prolonged survival over control mice, while ESC-derived hepatocytes in BAL developed characteristics nearly identical to those of primary hepatocytes[200].

Very recently, 3D printing technologies, by fabricating complex 3D tissue engineering scaffolds and providing patient-specific tissue models showed promise in revolutionizing 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 SCs[202].

CONCLUSION

SC-based liver regeneration is an exciting and dynamic area of research showing remarkable advancement in liver medicine, both in basic science and in 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.

Up to date there has been a lack of uniformity in preclinical and clinical studies, as regards the type and the extent of injury of the liver parenchyma, the source and dose of SCs, the therapeutic timing and route of administration of SCs, and the primary endpoints. 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 different liver diseases by considering the background and severity of the target disease as well as the putative functional roles of different SCs and the intended biological action by their infusion.

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