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12 *The Open Tissue Engineering and Regenerative Medicine Journal*, 2013, *6*, 12-25

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# Recent Advances in Derivation of Functional Hepatocytes from Placental Stem Cells

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**Abstract:** End-stage liver diseases are one of the leading causes of death in the world. Often orthotopic liver transplantation represents the final therapeutic choice. The limits of this approach are the scarcity of donor livers available, and the many side effects related to the administration of immune suppressants to the patients.

Cellular therapy for liver diseases is increasingly being viewed as a promising strategy to provide hepatocytes to replenish the parenchymal cells of the organ. This technique suffers of some important limitations, such as the difficulty in isolating sufficient cell numbers (e.g. when adult or foetal hepatocytes are used for transplantation), the limited viability of isolated hepatocytes and, when applicable, the limited differentiation of stem cells (when hepatocyte-like cells are derived from hepatic or extra-hepatic progenitor populations).

In recent years, perinatal stem cells have been proposed as reliable cellular populations which may be successfully used to derive hepatocyte-like cells. These cells feature key advantages over other adult stem cells: may be easily sourced from the tissues of origin, can be expanded ex vivo to obtain high cell numbers, may be differentiated towards hepatocyte-like cells. In addition, these cells feature relevant immunomodulatory and anti-inflammatory activities, and their sourcing is not limited by ethical concerns

In the present review we analyze the molecular basis of hepatocyte biology and development, and discuss the recent advances in deriving hapatocyte-like cells from perinatal stem cells. Very recent papers on mesenchymal stem cells derived from bone marrow and adipose tissues are also comparatively discussed as prototypes of the use of adult extra-hepatic stem cells. In our opinion, perinatal stem cells do represent a promising tool for liver regenerative medicine, and recent research reports further strengthened this perception and fostered further efforts by multiple research groups worldwide.

**Keywords:** Mesenchymal stem cells, Wharton's jelly, amniotic fluid, amniotic membrane, immune modulation, umbilical cord, hepatocyte differentiation, functional assays, inflammation, fibrosis, regenerative medicine, tissue repair.

## INTRODUCTION

The liver is a parenchymal organ which performs various critical functions: First, hepatocytes convert circulating glucose into glycogen, and may perform the inverse conversion when needed. Liver may act both as an exocrine gland (*via* bile production), as well as an endocrine one (for the production of blood clotting factors, plasma proteins and other molecules). Moreover, the liver converts ammonia in urea (which is then excreted by the urinary system) and performs xenobiotic detoxification [1, 2].

Hepatocytes represent about 80% of total liver mass, the remaining part of the organ is constituted by cholangiocytes, Kupffer cells, stellate (Ito) cells, hepatic specific natural killer and endothelial cells. Hepatocytes are polyhedral cells which are organized in anastomized laminae constituting the bulk of the morpho-functional units of liver: the hepatic lobules. The microscopic anatomy of these cells clearly shows the existence of a basal surface (vascular pole)

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through which the hepatocytes secrete plasma proteins into sinusoids and perform all the other exchanges with blood. Their apical surface (biliary pole) is involved in bile secretion into intralobular biliar capillaries (which are bordered by hepatocytes) that later join the perilobular bile ducts (which are bordered by cholangiocytes) [3].

Liver diseases globally constitute a major health concern worldwide, with hundreds of millions of affected people. Non-alcoholic fatty liver disease (NAFLD), autoimmune diseases, alcoholism, chronic hepatitis C and B, and drug-induced hepatic disorders are the main causes of hepatopathies. Most of these conditions, if not prevented or properly treated, may lead to progressive liver injury, which may evolve into liver fibrosis, cirrhosis, portal hypertension, and even cancer [4].

Orthotopic liver transplantation is still the final treatment for a number of end-stage liver diseases. However adverse events such as rejection and complications associated with long term immunosuppressant administration limit its therapeutic potential. Moreover, scarcity of donors and the high costs for the health system are other factors which caused the increased demand of alternative strategies.

Hepatocyte transplantation is viewed as a simpler and less invasive procedure. Despite the positive results obtained so far with this therapeutic approach, for example in the treatment of metabolic liver disorders, its usefulness for cirrhosis and acute liver failure is still limited. Moreover, scarcity of cadaveric livers, the limited replicative potential of infused hepatocytes, the damages induced from cryopreservation and the elevated cell numbers needed for transplantation, currently constitute important limits to the potential of hepatocyte transplantation [5].

Also for these reasons, many researchers searched alternative sources of suitable cells for transplantation, such as adult stem cells. Two different adult stem cells groups were shown to possess differentiative capacity towards hepatocytes: hepatic stem cells (liver-resident progenitor cell populations) and extra-hepatic stem cells, such as bone marrow-derived mesenchymal stem cells (BM-MSC), hematopoietic stem cells (HSC), adipose tissue-derived mesenchymal stem cells (AT-MSC), and extra-embryonic stem cells [6, 7].

## PHYSIOLOGICAL LIVER REGENERATION AND MECHANISMS UNDERLYING LIVER FUNCTION RESTORATION BY HEPATOCYTE TRANSPLANTATION

When liver is subjected to partial hepatectomy (HP), the hepatocytes undergo one or two replicative cycles to restore loss hepatic mass. Therefore, this organ features a strong self-regeneration ability, when compared to other organs in human body. The regenerative process is tightly influenced by several cytokines, hormones and growth factors. In particular liver-resident macrophages, known as Kupffer cells, are involved during the initial response of the regenerative process, by activation of tumour necrosis factor α (TNF-α) pathway [8, 9]. Then, after the secretion of the pro-inflammatory cytokine IL-6 by Kupffer cells, hepatocyte proliferation begins. At this point, other factors such as hepatocyte growth factor (HGF), epidermal growth factor (EGF) and key receptors as TGF-αR are involved. Then hepatocyte proliferation continues until the liver mass is restored [8-11].

When HP is higher than 70%, hepatocytes do not proliferate anymore, reaching a state known as "quiescent senescence". To this regard it has been demonstrated that some liver-resident progenitor cells (oval cells in rodents or hepatic progenitor cells in humans), located in the proximity of Canals of Hering, may undergo replication and differentiate into both hepatocytes and cholangiocytes [12, 13]. It was demonstrated that active Notch signalling is needed for cholangiocytes differentiation pathway, while inhibition of this signalling cascade promotes hepatocyte differentiation [14, 15].

End-stage liver diseases all share the failure (or exhaustion) of liver self-repair mechanisms. Therefore, hepatocyte transplantation has been developed to infuse a cellular population which may undergo in-organ replication to favour hepatocyte repopulation and restoration of organ physiology. When liver architecture is not compromised by the underlying disease, the portal vein is a valid site for the infusion of hepatocytes, which then migrate to the liver via the portal system [16]. The presence of a physiologically intact extracellular matrix may help engrafted hepatocytes replication. On the contrary, when liver architecture is compromised, for instance in fibrotic diseases, it may be preferable to infuse the hepatocytes into ectopic sites such as the spleen, which can provide a site for hepatocyte proliferation [17]. To date, hepatocytes transplantation therapy was performed only for three categories of liver diseases, such as acute liver failure, inherited metabolic liver diseases and cirrhosis. Acute liver failure causes rapid deterioration of liver functions, therefore cell therapy could provide restoration of the main functions such as metabolism of toxins, secretion of proteins and stabilization of hemodynamic parameters. Hepatocytes transplantation in patients with acute liver failure were performed both via splenic artery or portal vein infusion [18-20].

Transplantation of hepatocytes into the peritoneal cavity was also considered a promising approach to create a bridge to the spontaneous liver regeneration. Whereas infused hepatocytes in peritoneal space showed a limited lifespan, the use of alginate embedding or microcarriers for cell transplantation has been investigated. It was demonstrated that transplantation of microcarrier-attached hepatocytes into rats subjected to total hepatectomy improved long-term survival rates [21]. Cell therapy provided further encouraging results for the treatment of inherited metabolic liver diseases: in a girl with Crigler-Najjar syndrome Type I with hyperbilirubinaemia, infusion of hepatocytes into the portal vein resulted in a partial correction of plasma bilirubin levels for over 11 months [22]. Other positive results were obtained in a woman with glycogen storage disease [23] and in a 4-year-old girl with infantile Refsum disease [24].

In end stage liver diseases such as cirrhosis, cell transplantation is certainly more problematic, because the loss of functional hepatocytes contributes to the decrease of liver function and disruption of organ architecture. To this regard, hepatocytes infusion in ectopic sites such as spleen may be preferable. Studies on animal models of stable liver cirrhosis demonstrated that rat or porcine hepatocytes [25],

syngeneic rat hepatocytes [17] or immortalized rat hepatocytes [26] infused into the spleen improved liver function and increased survival rate. A few clinical applications were performed in cases of decompensated chronic liver disease. As stated above, the use of microcarriers or other support media for hepatocytes transplantation in advanced cirrhosis could result in prolonged survival of engrafted hepatocytes [27].

Recent studies showed that extra-hepatic stem cells can differentiate into endodermal cellular lineages such as hepatocytes. In particular, many groups investigated the therapeutic potential of hepatocyte-like cells derived from extra-hepatic mesenchymal stem cells [28, 29].

Recent data from Yang and co-workers showed that co-culture of human placenta-derived MSCs with hepatoma-derived cells, resulted in upregulation of the metabolic functions in the latter, thus opening new paths for the therapeutic approaches based on bioartificial livers development [30]. To this regard, very recent data from Wang *et al.* showed that HGF exposure and direct MSCs contact act in a synergic fashion to inhibit liver stellate cells activation. This mechanism of fibrosis reduction may thus provide a new therapeutic option for liver diseases [31]. Another recent report on the use of MSCs in alleviating fibrosis showed that exosomes, released by human umbilical cord MSCs, reduced liver fibrosis, inflammation and collagen deposition in CCl4-induced fibrosis [32]. In addition, also matrix metalloproteinases, which have multiple roles in physiology and pathophysiology [33-35], have been suggested as key players in overcoming fibrosis effects [36].

## PHENOTYPICAL CHARACTERIZATION AND IMMUNOLOGICAL FEATURES OF MESENCHYMAL STEM CELLS

The bone marrow (BM) is certainly the most common source of MSCs. Friedenstein and colleagues were the first to demonstrate that mesenchymal stem cells from bone marrow (BM-MSCs) are able to undergo ex vivo expansion, growing on plastic surface, and differentiate in various cellular lineages such as adipocytes [37, 38] osteocytes, chondrocytes, tenocytes and nervous tissue cells [39].

According to the general consensus, BM-MSCs express typical "core" markers such as CD44, CD73, CD90, CD105, CD166, CD49e, CD51, CD54, CD59, CD71 [5,8-11]. Specific markers of the endothelial lineage, such as CD31 and vWF [40-43] are absent in BM-MSCs, as well as hematopoietic markers (CD14, CD34, CD45, CD79, CD86, and glycophorin A-CD235a) [44, 45]. Various reports suggested that the use of MSCs in vivo should be safer with respect to formerly investigated embryonic stem cells (ESCs), since MSCs have higher chromosomal stability and do not induce neoplasm formation in the recipient host [46-48]. However, only a minor fraction of BM cells are useful for regenerative medicine applications (their frequency ranging between 0,0001% to 0,01% of nucleated cells) [49]. In addition, it has been demonstrated that BM cell numbers significantly decrease as function of donor age [50]. Therefore, many researchers investigated alternative sources of MSCs in other easily accessible tissues, such as adipose and perinatal tissues: placenta, amniotic membrane, and umbilical cord [51-54].

Moreover, in the last years the interest for MSCs in regenerative medicine did increase due also to their immunomodulatory and hypo-immunogenicity features [55]. The main proposed mechanisms of immunomodulation by MSCs involve secretion of soluble factors such as transforming growth factor-β (TGF- β), hepatocyte growth factor (HGF), prostaglandin E2 (PGE-2), indolamine 2,3 dioxygenase (IDO). This may be due to the cross-talk between MSCs and T-lymphocytes [56, 57]. Moreover, cell-cell-contacts may also have relevant roles for the immunomodulatory activities of MSCs [58]. As reported by numerous groups, main immune-related features of MSCs include the inhibition of T-cell proliferation and dendritic cell (DC) maturation and migration [59]. In addition, some studies suggested that MSCs may modulate T-cell proliferation due to their low expression of co-stimulatory molecules and the lack of class II HLA [58-60]. In addition, the immunosuppressive capacity of MSCs may also be mediated by the induction of T-cell anergy and regulatory T-cells (Tregs), with significant consequences for post-infusion therapies [60-62].

Several reports indicate that MSCs express non classical type I HLAs such as HLA-G (as well as its soluble form HLA-G5) [60,63,64], HLA-F and HLA-E [36]. To date, HLA-E expression has been observed in BM-MSC and WJ-MSCs, as well as in heart-resident MSCs [36,65-68]. It has been also demonstrated that these class Ib MHC molecules are involved in the instauration of tolerance of the mother's immune system toward the semi-allogeneic embryo and in the induction of tolerance of NK cells toward self cells [69, 70], acting coordinately with other key molecules as Early Pregnancy Factor [71, 72]. Other immune-related antigens such as CD68 and CD14 have been also reported in WJ-MSCs and cord lining (CL) MSCs [73,74]. In particular for WJ-MSCs, recent reports by us and others [75,76] demonstrated the expression of all three class Ib MHC molecules [68]. HLA-E expression has been also demonstrated in CL-MSCs and BM-MSC, after TNF-α challenge [77].

Anergy is another mechanism underlying MSC-mediated T-cell suppression. Krampera *et al.* suggested that BM-MSCs may cause T-cell arrest in G0-G1 phase through inhibition of cyclin D2 expression [78]. Further reports demonstrated that MSCs can induce immune suppression, by stimulation of the production of CD8+ regulatory T-cells, thereby inhibiting allogeneic lymphocyte proliferation [79]. MSCs may also affect dendritic cell (DC) differentiation, maturation and activation [80]. Moreover, it has been reported that MSCs may play an immunosuppressive role by influencing B-cell proliferation and activation in a dose-dependent manner, through the modulation of their chemotactic abilities and antibody production [81]. Also WJ-MSCs have been shown to possess key immunomodulatory activities [82], such as PGE2 and LIF (leukemia inhibiting factor) [83, 84]. Both molecules have been demonstrated as modulators of allogeneic lymphocytes proliferation in vitro [36]. More recently, also CD200 and its receptor have been demonstrated to be expressed in WJ-MSCs [85].

## HEPATOCYTE DIFFERENTIATION PROTOCOLS AND CHARACTERIZATION OF HEPATOCYTE-LIKE CELLS

Mechanisms acting in early liver development have been widely investigated using animal models. Liver development requires a series of inductive signals from almost three cell types: mesoderm-derived cardiogenic cells, cells of the septum trasversum and endothelial cells.

In particular, in mouse embryo, at the 7-8 somites stage, the ventral wall of the gut is adjacent to cardiac mesoderm: this releases fibroblast growth factors (FGFs), which are needed to initiate the differentiation program towards hepatic fate [86]. The septum trasversum cells, by secreting bone morphogenetic protein-4 (BMP-4) induce the outgrowth of the primordial liver. Hepatocyte growth factor (HGF) released by endothelial cells is involved in the final development of the primordial liver. In addition the liver represents a primary hematopoietic site during development: it has been demonstrated that oncostatin M, secreted by liver-resident hematopoietic stem cells, controls late hepatocyte differentiation, by increasing HNF-4α expression [87].

Based on these evidences, several *in vitro* hepatocyte differentiation protocols were developed based on the administration of hepatic inducers, either in a stepwise fashion or as a single complex mixture, to cultured cells. It has been reported that factors such as HGF and FGFs (such as FGF-2 and FGF-4), are necessary for the initial induction phase, while oncostatin M is involved in the final maturation phase of induced cells [88].

Other factors which may be added to differentiation media include: low (1% v/v) foetal bovine serum, insulin-transferrin-sodium selenite (ITS) supplement, dexamethasone at submicromolar concentrations, and epidermal growth factor (EGF). All of these factors may be added to monolayer cultures [89], to cells seeded in 3D scaffolds [90], or to co-culture systems in which foetal or adult hepatocytes are also present [91].

The acquisition of a hepatocyte-like phenotype may be evaluated by means of several techniques. Differentiated cells should be able to secrete proteins such as albumin and alpha-fetoprotein, which can be dosed in conditioned media. Glycogen synthesis and accumulation may be evaluated by PAS (periodic acid- Schiff) staining; the inducible activity of cytchrome P450 isoenzymes may be assessed by specific assays. In addition, enzymatic activities related to the glucose metabolism (as that of glucose-6-phosphatase) may be assessed via specific assays [92].

As a further datum to discriminate well differentiated cells, it is known that bipotential hepatoblasts can generate two cellular populations: hepatocytes and cholangiocytes. During the maturation stage of hepatocytes, there is a characteristic "cytokeratin switch": bipotential precursors express both CK-18 and CK-19, while after proper differentiation the mature hepatocytes express CK-18 alone, while CK-19 identifies the cholangiocytes population [93-96]. In a previous work, we demonstrated that undifferentiated WJ-MSCs express CK-18, CK-19, and HNF-4α [60]. Moreover, Zemel and collaborators [97] showed that naïve MSCs derived from adipose tissue expressed early hepatic genes such as AFP, CK-18, CK-19, and HNF-4α. Other authors highlighted the expression of AFP and transthyretin (TTR) also in the yolk sac cells [98]. These data collectively support the notion that expression of such markers may not be used as the exclusive evidence to demonstrate the acquisition of the desired phenotype.

Therefore, standard parameters which may be used in parallel to characterize the outcome of differentiation experiments may comprise: expression of key liver-specific transcription factors (e.g., HNF-4α, HNF-3γ, HNF-6, GATA-6) to evaluate the genetic reprogramming of cells [99-101]; evaluation of glycogen storage capacity (eg, visualized by PAS staining procedure) (Fig. **1**) [89,102]; ammonia metabolism and urea production (determined by colorimetric or fluorometric assays) [102-104]; selective uptake of vital stains (eg, indocyanine green, which is uptaken exclusively by hepatocytes) [105]; secretion of plasma proteins (eg, albumin) [106,107]. Collectively, these assays may constitute basic reliable evidences for a successful hepatic differentiation. In addition, novel assays of prospective high specificity are being developed: recent data from Cui *et al.* further highlighted the molecular mechanisms leading to hepatic differentiation of human cord-lining mesenchymal stem cells (CL-MSCs). The authors characterized the micro RNA profile of both undifferentiated and hepatocyte-differentiated cells, suggesting that 25 RNAs were overexpressed and 46 were downregulated [108].

## HEPATOCYTE-LIKE DIFFERENTIATIVE CAPACITY BY EXTRAHEPATIC MSCS STEM CELLS: *IN VITRO* AND *IN VIVO* STUDIES

MSCs are considered useful for liver regenerative medicine due to their key features: self-renewal capacity, endodermal lineage differentiation potential and immunomodulatory activity [57]. Different populations of MSCs have been used in *in vitro* experiments and preclinical studies, to derive mature hepatocyte-like cells.

*Bone marrow mesenchymal stem cells:* In 2005, Sato *et al*. demonstrated that acute liver injury, induced by allyl alcohol in Sprague Dawley rats, was attenuated after infusion of undifferentiated BM-MSCs [109]. Later then other authors showed the attenuation of $CCl_4$-induced liver fibrosis in rats after infusion of hepatocyte-like cells derived from BM-MSCs [110].

Many reports demonstrated the capacity of BM-MSCs to differentiate towards hepatocyte-like cells (HLCs). A recent study, carried out by Ayatollahi *et al*., highlighted the hepatogenic differentiation potential of BM-MSCs, using insulin like growth factor-I (IGF-I) as a component of the induction medium. In liver cirrhosis, hepatocellular insufficiency was linked to the reduction of IGF-I levels, while IGF-I gene transfer may reduce the extent of fibrosis and improve liver function. As demonstrated by the authors, after 21 days of hepatic induction, BM-MSCs switched to a polygonal morphology, producing also higher quantities of albumin and AFP than undifferentiated cells. Such cells accumulated glycogen (assessed by PAS staining) and increased urea synthesis with respect to undifferentiated cells [111].

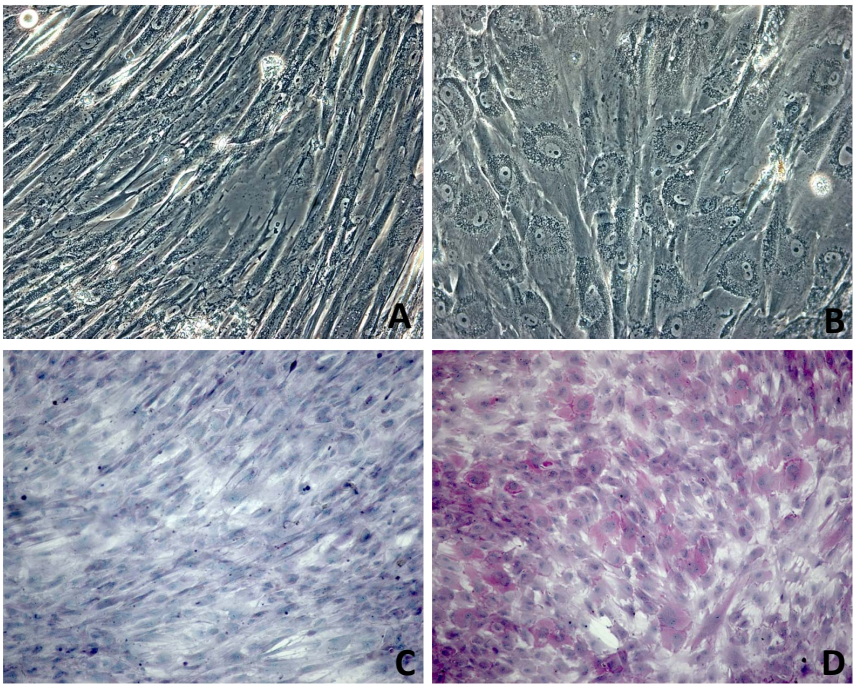


**Fig. (1).** Representative panel of morphofunctional features of human hepatocyte-like cells (HLCs) differentiated from WJ-MSCs. HLCs show a polygonal morphology (**B**) compared to control cells which maintain their fibroblastoid aspect (**A**). In addition, glycogen accumulation may be demonstrated in HLCs by PAS staining (**D**), while control cells show no stain (**C**). Magnification 20x (**A**,**B**) and 10x (**C**,**D**).

In a more recent report, authors differentiated BM-MSCs according to a previous protocol developed by Snykers group [112], highlighting the change of morphology by HLCs and expression of some hepatic markers such as CK-18, Hep-Par antigen, alpha-1anti-trypsin (AAT) and albumin. In addition, differentiated cells were able to store glycogen and were competent for LDL uptake. However, results from *in vivo* experiments were of limited extent, since administration of $4x10^8$ HLCs, to a patient with familial hypercholesterolemia, did not improve significantly serum cholesterol levels [113].

One of the main liver functions is xenobiotic biotransformation (operated by cytochrome isoenzymes, CYP450). The evaluation of the activity of these enzymes is considered a reliable proof to confirm the acquisition of HLCs phenotype by MSCs. In a recent study, the authors investigated the upregulation of CYP450 expression in immortalized HLCs derived from BM-MSCs. As reported, both immortalized MSCs and control cells were induced to differentiate towards HLCs. Human hepatocellular carcinoma cell line (HepG2), and primary human hepatocytes were used as controls. The immortalized HLCs contained about 70% more albumin than undifferentiated cells and their conditioned medium featured higher urea levels in comparison to HepG2 cells. Moreover, the expression of hepatogenic-specific genes in control cells and HLCs was evaluated by real time PCR, and it varied on the basis of the hepatocyte maturation phase. However, the expression levels of hepatic nuclear factor4α (HNF-4α), glucose-6-phosphatase (G-6Pase) and tyrosine aminotrans-ferase (TAT), were still reduced with respect to human primary hepatocytes. In addition, CYP450 isoenzymes were clearly upregulated by inducers at a level that outperformed the HepG2 cells used as controls [114].

Other very recent reports were focused on the induction of hepatocyte differentiation by co-culture. Cross-talk between cells involves both secretion of soluble factors and cell-cell contact, which are important in the induction of the differentiation processes.

Deng and co-workers suggested that Kupffer cells, activated by liver stellate cells (LSCs), may induce BM-MSCs differentiation towards HLCs, a mechanism mediated by HGF secretion [115]. More recently, Lange *et al*. demonstrated that rat BM-MSCs may be differentiated towards hepatocyte-like cells in presence of foetal liver cells (FLCs). The authors suggested that the presence of MSCs in the co-culture system was essential for both expansion and differentiation of FLCs [116].

As stated above, different groups demonstrated that BM-MSC-derived hepatocyte-like cells may reduce $CCl_4$-induced liver fibrosis in mice. It is still unclear which may be the mechanism underlying this improvement, also related to the best way of cellular infusion. To this regard, recent data provided a comparison of the therapeutic effects obtained using three different protocols of MSCs infusion (intraperitoneal, intravenous and intrahepatic transplantation), to treat $CCl_4$-induced liver injury in rats. In particular, after differentiation towards HLCs, differentiated BM-MSCs labelled with DAPI were infused into different sites in rats. This study highlighted that intravenous injection resulted the best option for two reasons: first, for the higher number of engrafted cells and upregulation of interkeukin-10 (IL-10); in addition, IL1β, IL-6, TNFα and TGF-β levels (that are associated to liver fibrosis) were lower than in the other groups [117].

As confirmed by many studies, the use of three-dimensional scaffolds can result in better features of

transplanted cells and in an improved interaction with the local microenvironment. Kazemnejad and colleagues compared the hepatic differentiation ability of BM-MSCs in 2D and 3D systems (biocompatible nanofibrous scaffold). The authors demonstrated that HLCs grown in a 3D system had an increased expression of hepatic markers with respect to cells differentiated on a 2D culture system [118].

*Adipose tissue-derived MSCs*: According to the International Fat Applied Technology Society, the term adipose-derived stem cell (ASCs) is used to describe a multipotent plastic-adherent cell population derived from adipose tissue.

In a recent report Okura and colleagues differentiated hASCs into HLCs clusters by using a floating culture technique. Differentiated cells expressed hepatic markers and exhibited functional hepatic activities. After transplantation into NOD-SCID mice with chronic liver injury, both albumin and total bilirubin serum levels were significantly improved [119]. Although several studies confirmed the hepatic differentiation ability of hASCs, the high heterogeneity of primary cells still constitutes one of the main limits towards clinical application and may lead to a lack of confidence in data interpretation. The limited characterization of cells, or the use of a mixed population in *in vivo* assays may lead to contrasting results [54]. Russo and colleagues demonstrated that MSCs transplanted in liver spontaneously gave rise to a myofibroblast population therefore participating to a fibrosis process [120]. In addition, the possibility of cancer formation by hASCs after transplantation still represents an open question.

*Umbilical cord blood-derived MSCs:* Umbilical cord blood (UCB) has been described as a reliable source of hematopoietic stem cells (HSCs) compared to bone marrow and peripheral blood. In fact, the yield of proliferating HSCs derived from umbilical cord blood (UCB cells), is high and the isolation process is free of side effects or health risks for the donor, as well as ethical concerns [121, 122].

Very recent data showed the hepatic differentiative capacity of hUCB cells. HLCs differentiation was induced by the administration of hepatic inducers at different steps, for a total of 14 days (see Table **1**). Hepatocyte-like cells featured a polygonal shape, expressed AFP, albumin, G6P and CK-18. One of the novel aspects of this study was the shorter induction timeframe to obtain HLCs: only 14 days, as compared to 21 days timeframes reported in other works [123]. Clearly, more stringent assays will be needed in order to assess the adherence of these cells to the mature hepatocyte phenotype.

In another recent study, CD34+ UCB cells were differentiated in HLCs. After encapsulation and transplantation in rats with acute hepatic failure, the mortality rate was lower than that of non-encapsulated cells after 48 hours. Moreover, hematologic parameters such as ALT, AST and total bilirubin were significantly different between encapsulated and non-encapsulated cells. In addition, after 1-2 weeks post-transplantation, the authors observed the presence of fibrous tissue surrounding microcapsules in the greater omentum [124].

*Amniotic epithelial cells (AECs):* Amniotic membrane constitutes another promising placental source for tissue engineering. In particular, very recent data highlighted the ability of cells derived from the epithelial layer of amniotic membrane to be differentiated towards HLCs [125, 126]. Seminal in vivo data came from the work of Manuelpillai and co-authors [127], who demonstrated that hAECs, transplanted into immunocompetent mice with $CCl_4$-induced liver damage, successfully engrafted without evidence of rejection. Moreover, human proteins such as albumin and HLA-G were detected respectively in mouse serum and liver. In addition, hAECs transplantation did reduce hepatocyte apoptosis and serum ALT levels with respect to control animals. The number of activated stellate cells and the extent of fibrosis areas were also reduced.

Other recent data showed that GFP-expressing hAECs, when transplanted in the spleen of SCID mice, retain the ability to produce albumin and formed clusters of cells in both spleen and liver preferentially [128].

Marongiu and co-workers [129] showed that hAECs could be differentiated towards HLCs in vitro, by using a combination of extracellular matrix proteins (porcine liver-derived extracellular matrix) and growth factors (such as b-FGF, HGF, Oncostatin M). In vitro data demonstrated that differentiated hAECs expressed hepatocyte markers at levels comparable to those of fetal hepatocytes. Also at the functional level, ammonia and testosterone metabolism, and expression of fetal inducible cytochromes suggested the acquisition of a HLC phenotype. The authors also performed transplantation of naïve hAECs into retrorsine-treated animals: infused cells did express liver-specific markers (such as HNF-4-α), enzymes (such as CYP3A4 and CYP2B6) and transporters (such as BSEP), at levels comparable to adult liver tissue [129]. More recently, the same research group published relevant data on the use of hAECs to correct in vivo a liver-related metabolic defect (maple syrup urine disease) [130, 131].

As detailed earlier in this review, one of the most intriguing areas of interest for the use of perinatal stem cells is their role in immunomodulatory processes. Tee *et al* [132] reported new data on the immunogenicity and immunomodulatory properties of HLCs derived from hAECs. The authors demonstrated that differentiated HLCs, while expressing mature liver functions, also inhibited mitogen-induced PBMCs proliferation. The authors speculated that this could be due to the active secretion of immunomodulatory molecules by these cells, as demonstrated for IL-6, TGF-β1, PGE2 and HLA-G. The levels of these molecules were determined by ELISA in both undifferentiated hAECs and HLCs. The authors concluded that the retention of immunomodulatory properties may enable HLCs grafts to survive for longer periods despite their immunogenicity [132]. These promising data reinforce the concept that the maintenance of immunomodulatory properties in the differentiated progeny of hAECs may favor longer survival of grafts despite the immunogenicity of HLCs.

*Amniotic fluid stem cells*: Amniotic fluid stem cells (AFSCs) can be found in the human amniotic fluid throughout gestation and are considered promising for cell-based therapies and tissues engineering [133]. Liver diseases have also been proposed as targets for AFSCs therapy.

**Table 1. List of Studies Pertinent to the Hepatocyte-Like Differentiation of Adult and Placental Stem Cells**

| Extrahepatic Stem Cells | Hepatic Differentiation Protocol Scheme | Analysis of Hepatic Markers | Functional Assays | References |
|---|---|---|---|---|
| Human bone marrow-derived mesenchymal stem cells (BM-MSC) | Two steps-21days protocol: DMEM plus 10%FBS, IGF-I, HGF, dexamethasone for 7days 14 days with addiction of OSM to differentiation medium | I Albumin, AFP by ICC II. | *In vitro* I.PAS staining for storage glycogen II. Colorimetric assay to evaluate urea production III.ELISA for albumin production | [108] |
| Human bone marrow-derived mesenchymal stem cells (BM-MSC) | Three step-40 days protocol: DMEM-LG plus FGF-4 0-3 days DMEM-LG plus HGF 3-6 days DMEM-LG with HGF, ITS and dexamethasone from 6 days until 40 days | I.Albumin,CK18, AAT, HepPar1 by IF II. Albumin, CK19, CK-19, AAT, TAT, HNF3β, AFP, LDLR, G6P, by RT-PCR III. Albumin by WB | *In vitro*: I. PAS staining for storage glycogen II.LDL uptake III.Albumin and urea production by ELISA In vivo: Differentiated and undifferentiated BM-MSC together in patient with familial hypercholesterolemia. Evaluation of cholesterol levels | [110] |
| Human bone marrow-derived mesenchymal stem cells (BM-MSC) | Three step-23 days protocol: Serum free IMDM plus EGF, bFGF for 2 days IMDM plus HGF, bFGF, nicotinamide for 7 days IMDM plus OSM, dexamethasone and ITD for 14 days | I.AFP,CYP3A4,HNF-4α by IF II. ALB AFP, CK18, G6PD, HNF-4a, TAT , CYP1A1, CYP1A2, CYP2C8, CYP2B6, CYP2D6, CYP2C9, CYP2C19, CYP3A4 and CYP2E1 by qRT-PCR | *In vitro*: I. spectrofluorometric assay to evaluate urea production II.PAS staining for storage glycogen II. Cytochrome P450 assay in BM-MSC, HepG2, and Bmi-1/hTERT immortalized MSC | [111] |
| Rat bone marrow-derived mesenchymal stem cells (rat BM-MSC) | One step-21days protocol: α-MEM plus FBS, HGF,FGF-4, EGF | I. Albumin, AFP, CK-18, by RT-PCR II.AFP, albumin by IF | In vivo: intravenous, intraperitoneal, intrahepatic injection of MSC in CCL4-induced rat injury model. I. Evaluation surviving MSC, collagen deposition II. α-SMA by IHC III.MMP2, MMP9, TIMP1, HGF, PDGF, IL1β, IL2,IL6,IL10,IL13,INFγ, TNFα, TGFβ1By RT-PCR IV.IL-10 expression by ELISA V. evaluation of albumin, ALT, TBIL by biochemical analyzer | [114] |
| Human umbilical cord blood-derived hematopoietic stem cells (UCB-HSC) | Three steps-17days protocol: Serum-free DMEM for 24 hours Serum-free DMEM plus EGF,bFGF for 48 hours H-DMEM plus 10%FBS, HGF,FGF-4, dexamethasone for 14 days | I. TO, Albumin, AFP, G6P,CK-18 by RT-PCR II. albumin, AFP by ELISA | | [120] |
| Human umbilical cord blood-derived hematopoietic stem cells (UCB-HSC) | One step-16days protocol: Pre-conditioning: DMEM-LG with TPO,SCF, Flt-3 DMEM-LG plus FBS, linoleic acid, ITS, L-ascorbic acid 2-P, FGF-4,HGF for 16 days | I. Albumin by ICC II. albumin, AFP, Gata-4, by Rt-PCR | In vitro: I albumin expression by ELISA In vivo: hepatic stem cells encapsulated and transplanted in abdominal cavity in rat with acute liver failure at 48 h after D-Galactosamine induction. I. evaluation of ALT, AST, TBIL | [121] |

**Table 1. contd…**

| Extrahepatic Stem Cells | Hepatic Differentiation Protocol Scheme | Analysis of Hepatic Markers | Functional Assays | References |
|---|---|---|---|---|
| Human Amniotic epithelial cells | Two step, 32 days protocol. Pre-conditioning: Serum-free DMEM with 100ng/mL Activin-A for 2 days, with 0.2% FBS for two more days<br>Differentiation medium: Iscove's modified Dulbecco's medium (IMDM), 5% FBS, 10ng/mL EGF, 10ng/mL FGF-2, 10ng/mL HGF, 1μM Dex for 28 days.<br>One step experiment with culture on Liver ECM substrate:<br>IMDM, 10% FBS, 10 ng/mL EGF, 10<br>ng/mL FGF2 for 48 hours and then supplemented with 20 ng/mL HGF, 1μM Dex, 1X ITS for 5 days. One additional week with 20 ng/mL OSM and without FGF2) | I: Albumin, A1AT, CYP3A4, CYP3A7, CYP2A2, CYP2B6, ASGPR by Real-time PCR; | *In vitro*:<br>I. 17-hydroxyprogesterone caproate metabolization assay;<br>II. Ammonia metabolization assay<br>In vivo:<br>Undifferentiated hAECs were transplanted in SCID/beige mice. Albumin, AFP, BSEP, CYPs and over 30 markers were analyzed by real time PCR | [124] |
| Human Amniotic epithelial cells | One step, 21 days protocol:<br>DMEM/F12 with 10% FCS, 10ng/ml EGF, 0,1μM, insulin, 0,1 μM DEX for 3 weeks. | I. GATA-4, HNF-3β, Albumin, OTC, AFP, HNF-4α by IF<br>II. TDO by RT-PCR<br>III. HLA class I and class II, CD 40, CD80, Cd86 by FC<br>IV. HLA-G, PGE2, TGF-β1, Il-6 by ELISA | *In vitro:*<br>I. PAS staining for glycogen<br>II. CYP3A4 activity assay<br>III. Urea secretion<br>IV. MLR assay | [127] |
| Human Amniotic fluid stem cells | ILR-1a transfected AFSCs and normal AFSCs cultured on collagen type I coated flasks.<br>3 steps, 18 days protocol<br>Basal medium: 60% DMEM and 40% MCDB-201 with 2% FBS, PEN/STREP, 1 mg/ml linoleic-acid, 0.1mM L-ascorbic acid, 0.03mM nicotinamide, 0.25mM sodium pyruvate, and 1.623mM glutamine.<br>Hepatogenic cytokines and growth factors were added sequentially according to the following schedule. Days 0–2: basal medium +10ng/ml FGF-4; days 3–5: basal medium +20ng/ml HGF; days 6–18: basal medium +20ng/ml HGF +1× ITS and 20 mg/l DEX +1mM trichostatin A | I. Albumin and AFP by IF<br>II. β-actin, Oct-4, AFP, and ALB by RT-PCR | *In vivo*:<br>I. AST and ALT serum levels<br>II. IL-1Ra, IL-6, IL-1β and TNF-α plasma levels | [129] |
| Human Umbilical cord matrix-derived mesenchymal stem cells (UC-MSC) | One step-until 28days in different support<br>protocol:<br>IMDM plus 15% FBS, dexamethasone, OSM,ITS,HGF, FGF-4 | I. Albumin, AFP, by ICC<br>II. AFP, albumin, MTP, TNFα, IL-5, IL-10, TGFβ by RT-PCR<br>III.CD68, α-SMA, albumin, desmin, nestin by IHC | *In vitro*:<br>I. Albumin by ELISA<br>II. Colorimetric assay for urea production<br>III.PAS staining for glycogen storage<br>*In vivo*:<br>undifferentiated UB-MSC transplanted in CCL4-induced liver injury mice<br>I. Catalase activity by BCATm protein assay Kit | [130] |

**Table 1. contd…**

| Extrahepatic Stem Cells | Hepatic Differentiation Protocol Scheme | Analysis of Hepatic Markers | Functional Assays | References |
|---|---|---|---|---|
| Human placental mesenchymal stem cells (hPMSC) | Four step-35days protocol:<br>Pre-conditioning: LG-DMEM plus FBS10% for 2 days<br>IMDM plus EGF, FGF for 2 days<br>IMDM with HGF, FGF, niacinamide, ITS, for 10 days<br>IMDM plus OSM, dexamethasone, ITS for 10 days<br>HEPATPZYME-SFM for another days | I. Albumin, CK-18,CK19, AFP by ICC<br>II. Albumin, CK-18,CK19, AFP, by RT-PCR<br>III. albumin, CK-18 AFP by IHC | In vitro:<br>I.PAS staining for glycogen storage<br>II.LDL-uptake<br>III. spectrophotometer for urea production<br>IV. cytochrome P450 activity assay<br>In vivo:<br>HPMSC transplanted in Chinese miniature pigs with acute liver failure induced by D-Galactosamine<br>I. Evaluation of ALT,AST,ALP,TBIL,TBA<br>II LIF and INF-γ by ELISA | [135] |

Abbreviations: FBS, fetal bovine serum; IGF-I,insulin growth factor-I; OSM, oncostatin M; AFP, α-fetoprotein; FGF, fibroblast growth factor; ITS, insulin–transferrin–sodium selenite; CK-18, cytokeratin-18; HepPar-1, hepatocyte-paraffin-1; CK-19, cytokeratin-19; AAT, α-1-antitrypsin; TAT, tyrosine aminotransferase; HNF-3β, hepatic nuclear factor-3 β; LDLR, receptor low density lipoprotein; G6P, glucose-6 phosphatase, EGF, endothelial growth factor, bFGF, basic fibroblast growth factor; CYP3A4, cytochrome P450 3A4 subunit; HNF-4α, hepatic nuclear factor-4α;

G6PD, glucose-6-phosphate dehydrogenase; ; CYP1A1, cytochrome P450 1A1 subunit; CYP2B6, cytochrome P450 2B6, CYP1A2, cytochrome P450 1A2 subunit; CYP2C8, cytochrome P450 2C8; subunit; CYP2D6, cytochrome P450 2D6 subunit; CYP2C9, cytochrome P450 2C9; subunit CYP2C19, cytochrome P450 2C19 subunit; CYP2E1 cytochrome P450 2E1 subunit; AHR, Aromatic hydrocarbon receptor, PXR, pregnane × receptor; CAR, constitutive aldosterone receptor; MMP2, metalloproteineasi-2; MMP9, metalloproteineasi-9;TIMP-1,tissue inhibitor metalloproteinases-1;

PDGF, platelet-derived growth factor; Il1 β, interleukin-1; Il-2, interleukine-2; IL-6, interleukin-6; Il-10,interleukine-10;Il-13, interleukine-13; INFγ ,interferone- γ ; TNFα, tumor necrosis factor-α; TGFβ1, transforming growth factor β1;ALT, alanine aminotransferase; TBIl, total bilirubin levels in serum; TO, tryptophan 2,3 dioxygenase; TPO, thrombopoietin; SCF, stem cell factor; Flt-3, fetal liver tyrosine kinase-3;AST, aspartate aminotransferase, MTP, microsomal triacylglycerol transfer protein;ALP, alkaline phosphatase; TBA, total bile acids; LIF, leukemia inhibitory factor; MSC, mesenchymal stem cells;

BM-MSC, bone marrow mesenchymal stem cells, ICC, immunocytochemical; IHC, immunohistochemical, RT-PCR, reverse-transcriptase-PCR, IF, immunofluorescence, qRT-PCR, quantitative Real time-PCR.

Zheng and co-workers [134] showed that AFSCs expressing IL-1Ra (interleukin-1 receptor antagonist) may improve fulminant hepatic failure (FHF) in an *in vivo* model. The authors showed that administration of cells prevented liver failure and increased animals survival. GFP-mediated tracking showed that the cells engrafted in host liver and produced albumin. Therefore the authors suggest that this approach may be promising for the therapy of FHF.

*Wharton's Jelly MSCs:* Umbilical cord emerged in recent years as a promising source of multipotent stem cells, which may be isolated from all of the organ zones [135]. Very recent data confirmed that another reliable source of MSCs that can undergo hepatocyte differentiation is the umbilical cord matrix. A very recent work showed the capacity of WJ-MSC-derived hepatocyte-like cells to accelerate the resolution of acute liver injury [136]. WJ-MSCs were induced to differentiate towards HLCs, by seeding them on different supports (plastic, matrigel and human acellular matrix). After 1 to 4 weeks of differentiation, the expression of several hepatic markers was assessed (Table **1**). The authors analyzed the expression of albumin, AFP, microsomal trialcyglycerol transfer protein (MTP), tryptophan 2,3 dioxygenase (TDO). In addition, some functional assays were performed. The authors showed that UC-derived cells can differentiate into functional HLCs without any support. Moreover, the authors demonstrated that undifferentiated UC-MSCs, once transplanted in a murine model of acute liver injury (induced by $CCl_4$), homed specifically at the injury site and attenuated the inflammatory process, resulting in a lower infiltrate, lower pro-inflammatory cytokines levels (TNF-α, TGF-β) and increased levels of IL-10. In addition, UC-MSCs transplantation ameliorated hepatic damage by stimulating the activity of catalase, one of the main liver protection systems against ROS (reactive oxygen species) [136].

More recently, Li and co-workers [32] pointed out the role of exosomes derived from human umbilical cord MSCs, which were demonstrated to alleviate liver fibrosis in vivo. In particular, transplantation of the sole exosomes in mice with $CCl_4$-induced liver fibrosis resulted in reduced inflammation and collagen deposition. In addition, treated mice showed a recovery in serum AST levels. Interestingly, the authors showed also an inhibition of the EMT (epithelial-to-mesenchymal transition), with a reduction of vimentin-positive cells and increase of E-cadherin positive ones, with a positive effect on hepatocyte protection.

## Placenta-Derived Stem Cells

Placenta has been historically viewed as a source of cells and molecules with important anti-inflammatory and anti-fibrotic actions. This is particularly true for the application of the intact placenta structures (such as intact amniotic membrane) as a therapeutic approach for over 100 years [137].

Jung and co-workers [138] recently showed that placenta extracts may be used to promote liver regeneration in $CCl_4$-injured rats. The authors investigated the use of extracts both *in vitro* (on rat hepatic cells) and *in vivo*. Treated mice showed improved serum levels of AST/ALT. In addition, the reduced expression of collagen I and smooth muscle actin, and the increase in matrix metalloproteinase-9 expression

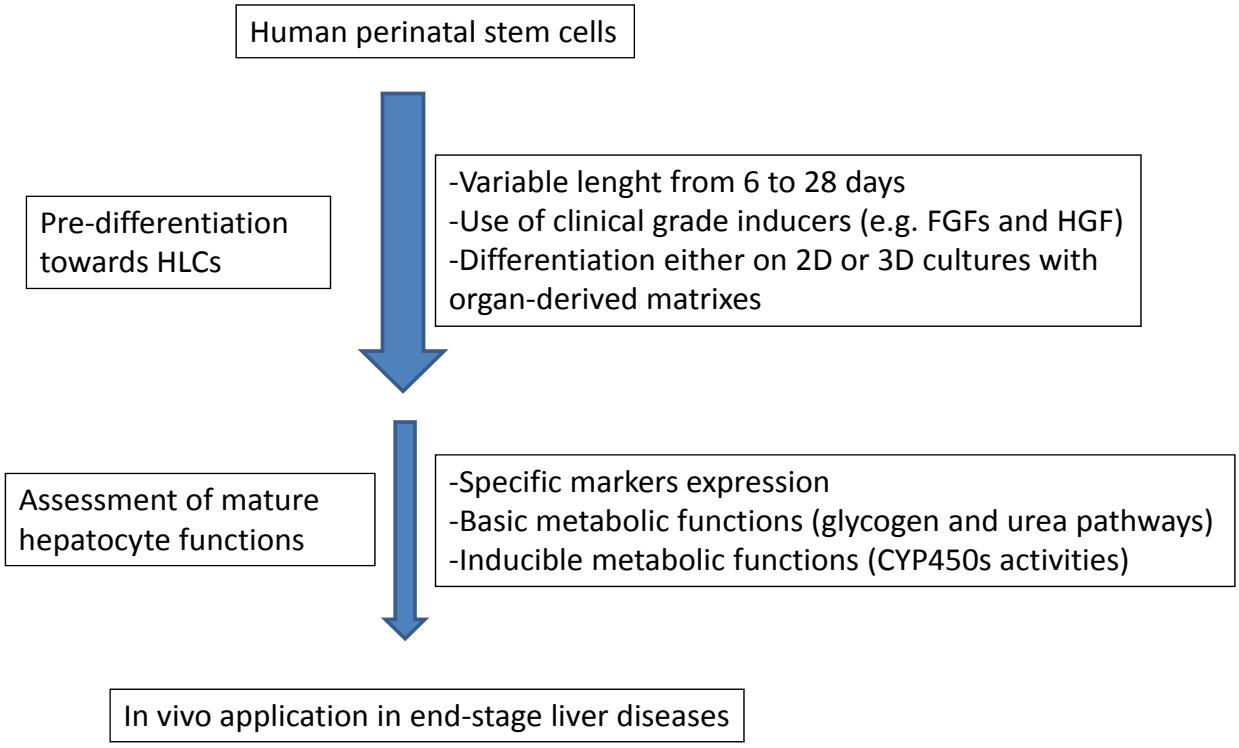


**Fig. (2).** Schematic diagram showing the proposed scheme for the use of perinatal stem cells in liver regenerative medicine applications. In vivo applications may also benefit from the immunomodulatory features of perinatal stem cells, which may avoid(or significantly limit) the use of immune suppressive drugs.

may justify the improvement in fibrosis score. In addition, placenta extract-treated mice showed an increase in anti-inflammatory cytokines, ad a decrease in pro-inflammatory ones, thus evidencing the multiple effects of placenta extracts.

As an additional benefit, MSCs derived from human placenta are free from ethical issues [139,140]. A recent study showed that human placenta mesenchymal stem cells (hPMSCs) may be differentiated towards HLCs when grown in specific media. HLCs prolonged the survival time of pigs in an *in vivo* model of acute liver failure. In particular, regarding *in vitro* experiments, hepatocyte-differentiated hPMSCs expressed specific markers such as CK-18, CK-19, AFP and albumin, stored glycogen, synthesized urea, and featured inducible CYP450 enzymatic activity. Moreover, after transplantation in Chinese miniature pigs with D-Galactosamine-induced liver failure, the hPMSCs improved liver functions: after infusion, the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), cholin esterase (CHE), total bile acids (TBA) and total bilirubin (TBL) returned to normal values. Inflammatory processes and necrosis were also reduced. One of the important points highlighted by the authors was the comparative analysis between two different ways for cellular administration: jugular vein and portal vein. The authors clearly showed that infusion through portal vein resulted in better clinical parameters than jugular vein one [141].

More recently, Lee and co-workers [142] performed a comparison of the in vitro hepatogenic differentiation potential of various placenta-derived stem cells and other adult stem cells. The authors comprehensively analyzed the acquisition of mature hepatocyte features in differentiated cells: placenta-derived cells showed a higher proliferation rate and higher expression of hepatocyte markers, as well as an increased production of HGF, which was not demonstrated for differentiated adult MSCs populations.

In another report, Jung and colleagues [143] demonstrated that chorionic plate-derived mesenchymal stem cells (CP-MSCs) isolated from placenta promoted liver regeneration *via* an autophagy-mediated mechanism. The expression levels of hypoxia inducible factor-1α (HIF-1α) and factors for autophagy, survival and regeneration, were significantly increased after CP-MSCs transplantation. A significant decrease of necrotic cells was observed as well as a significant increase of autophagic signals in $CCl_4$-treated primary rat hepatocytes during in vitro co-culture with CP-MSCs.

## CONCLUSIONS AND FUTURE PERSPECTIVES

Stem cell therapy for liver diseases is viewed as a promising option in both acute and chronic settings, for the possibility to find a reliable alternative to orthotopic transplantation, or a bridge to transplant. Liver function may be functionally restored if transplanted cells may account for the 5% of the total organ mass. Albeit it can be viewed as a small figure, the cell numbers involved are higher than those achievable with other techniques, as the hepatocyte transplantation. Therefore the possibility to use cellular populations which may be infused in high numbers and bear functional hepatocyte features and/or immunomodulatory ones is of high interest (Fig. **2**). In particular, fibrotic and inflammatory liver diseases treatments would benefit from the administration of cells which may, *via* their secreted

molecules, both reduce modifications to the tri-dimensional organ network, and dampen the phenomena at the basis of the disease, therefore allowing resident progenitors to replenish (and actually regenerate) the organ.

To this regard, research on placental stem cells increased in the last ten years allowing to determine new potential applications for the perinatal tissues, cells, extracts and soluble molecules. In the light of the ease of sourcing and culturing, inexhaustible availability and lack of ethical issues of these cells, their potential application as off the shelf therapy is increasingly favouringly viewed.

Recent research directions are concentrated on the clarification of the finer molecular mechanisms behind the positive effects of these cells. Immunomodulation is increasingly viewed as one of the main processes featured by perinatal stem cells both *in vitro* and *in vivo*, and its intrinsic importance in allogeneic transplant should boost the use of these cells in regenerative medicine applications. In particular for the treatment of liver end stage diseases, the possibility to have additional sources of extrahepatic progenitors is clearly envisioned. Current treatments (including the standard orthotopic transplantation of the entire organ, or the infusion of primary hepatocytes) are limited by the occurrence of side effects and the lack of a sufficient number of donors. Recent literature data support, for pretty all the perinatal stem cells populations examined, the possibility to acquire a HLC phenotype after *in vitro* differentiation. In addition, seminal recent works strongly suggested that differentiated HLCs do maintain the expression of immunomodulatory molecules similarly to the parental undifferentiated cells, therefore suggesting the possibility to be infused in vivo without a concurrent standard immunosuppression protocol or with a reduced-dosage one. Moreover, such cells would be able to perform a double action in the diseased organ, since capable to provide a functional replacement to resident hepatocytes, and favour the re-activation of liver self-reparative mechanisms and progenitor cells.

Overall these results are extremely encouraging and we expect a further expansion of the basic and applied research advancements aimed to the use of these cellular populations in regenerative medicine applications for liver diseases.

## CONFLICT OF INTEREST

Dr. La Rocca is a member of the Scientific Board of Auxocell Laboratories, Inc. The funders had no role in article design, data collection, decision to publish, or preparation of the manuscript

## ACKNOWLEDGEMENTS

Author's work referred to in the present paper has been supported by University of Palermo (to RA and GLR) funding and IEMEST funds (to GLR).

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Received: September 29, 2013 Revised: October 05, 2013 Accepted: October 05, 2013