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Mesenchymal Stem/Stromal Cells in Liver Fibrosis: Recent Findings, Old/New Caveats and Future Perspectives

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Abstract Mesenchymal stem/stromal cells (MSCs) are progenitors which share plastic-adherence capacity and cell surface markers but have different properties according to their cell and tissue sources and to culture conditions applied. Many recent publications suggest that MSCs can differentiate into hepatic-like cells, which can be a consequence of either a positive selection of rare in vivo pluripotent cells or of the original plasticity of some cells contributing to MSC cultures. A possible role of MSCs in hereditary transmission of obesity and/or diabetes as well as properties of MSCs regarding immunomodulation, cell fusion and exosome release capacities are discussed according to recent literature. Limitations in methods used to track MSCs in vivo especially in the context of liver cirrhosis are addressed as well as strategies explored to enhance their migratory, survival and proliferation properties, which are known to be relevant for their future clinical use. Current knowledge regarding mechanisms involved in liver cirrhosis amelioration mediated by naïve and genetically modified MSCs as well as the effects of applying preconditioning and combined strategies to improve their therapeutic effects are evaluated. Finally, first reports of GMP guidelines and biosafety issues in MSCs applications are discussed.

Keywords Cirrhosis · Mesenchymal stem cells · Neural crest · Hepatocyte-like cells · IGF-I · Biosafety · Cell source · Biodistribution · Mechanisms

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Liver fibrosis is a chronic disease with differences in pathogenesis according to the underlying etiology [1]. It evolves through cycles of subsequent hepatocyte/biliary duct cell apoptotic, inflammation and scarring processes, resulting in excesive extracellular matrix deposition. If parenchymal cell mass is significantly reduced, liver regeneration can be induced, thus resulting in the most advanced stage of fibrosis (cirrhosis). In case of hepatotoxic injuries most of myofibroblastic cells, responsible for collagen fibers accumulation, are originated from activated hepatic stellate cells (aHeSCs), while in cholestatic injuries they are of activated portal fibroblasts origin [2]. Different strategies were shown to reverse the degree of liver fibrosis [1, 3], including the extensive experimental application of mesenchymal stromal cell therapies [4, 5], a subject that was recently reviewed by us and others [6, 7].

According to current knowledge, mesenchymal stromal cells (MSCs; also known as mesenchymal stem cells or multipotent stromal cells) would be better described as in vitro progenitors with a fibroblastic-like morphology and plastic adherence properties. They can likely be derived from almost all tissues, and should express certain cell surface markers, such as CD105, CD73 and CD90 and be negative for hematopoietic cell surface markers (as reviewed in [6]). Finally, they must be able to differentiate into osteoblasts, chondroblasts and adipoblasts [8].

The research field of MSCs is increasing steadily: 45 % of almost 25,000 publications available on this topic (Pubmed; key words: "mesenchymal stromal cells" OR "mesenchymal stem cells" OR "multipotent stromal cells") are dated in the last 3 years. The clinical application of MSCs in specific cases seems to be approaching, with two phase-2/3 and four phase-3 clinical trials been recently completed (www.clinicaltrials.gov; NIH; same key words as before). Nevertheless, several fundamental aspects of MSCs biology remain unknown and it is expected that broadening of this knowledge will improve

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their beneficial uses. For instance, the possibility of selecting MSCs types according to their phenotype (i.e., in vivo cell source or their cell membrane expression profile) and functional (i.e., anti-fibrotic) properties, and of pretreating them with compounds/factors or genetically modifying them to express antifibrotic proteins, would likely improve their therapeutic outcome.

In this review we aim to discuss most recent evidence, highlighting some unsolved questions and bringing suggestions to foster MSCs research. This work will be focused on the experimental therapeutic application of MSCs in the context of liver fibrosis/cirrhosis.

The Biology of MSCs: Cellular and Tissue Origins

MSCs were first derived from the bone marrow; however, cells with similar in vitro properties have been obtained from different adult tissues/organs, such as adipose tissue (AT), skeletal muscle, peripheral blood, lung, liver, pancreas, dental pulp, brain, synovium, spleen and thymus (reviewed by Li and Ikehara [9]). MSCs were also obtained from extraembryonic tissues, including amnion, amniotic fluid and umbilical cord (UC). The UC has been itself a source of MSCs with different properties according to whether they were isolated from whole sample, its blood (cord blood-MSCs, CB-MSCs), its primitive connective tissue (Wharton's jelly, WJ-MSCs) or the tissue surrounding its vasculature (human UC perivascular cells, HUCPVCs) (reviewed by Bayo et al. [10]).

In spite of the broad spectrum of MSCs tissue sources, in most of them it is still unknown whether MSCs are originated from only one in vivo cell type. In fact and at least for certain tissues/organs, it is believed that they originate from different cell types which might acquire/lose cell surface markers upon in vitro cultivation. Interestingly, Bakondi et al. [11] showed that human bone marrow (hBM)-MSCs with different biological properties can be obtained from $CD133^+$ and $P75^+$ BM enriched fractions. Moreover, a systemic application of MSCs obtained from these distinct subpopulations or of their culture media supernatant resulted in completely different physiological outcomes in a rat model of stroke. In this study, markers used for their specific isolation (CD133 or p75) were found downregulated and all cultures acquired almost identical MSCs cell surface epitopes as early as passage (P)2. Whether or not these features are induced by factors present in fetal bovine serum (FBS) remains to be elucidated [12, 13]. Furthermore, a contribution of neuroepithelial (neural crest) cells to BM-MSC cultures was also shown [13–15]. Mendez-Ferrer and collaborators [16] have also reported that perivascular $Nestin^+$-derived cells are the only BM cells capable of originating MSC cultures and that they are required for the homing and regulation of hematopoietic stem/progenitor cells (HSPCs). Interestingly, by using a Wnt1-Cre/Rosa 26R (R26R) double-transgenic mice model obtained from a C57Bl/6 mouse background Wislet-Gendebien et al. [13] showed that BM-MSCs originate in vivo from a mixture of neural crest and non neural crest-derived stromal populations. According to them, while at P0-P3, contribution of neural crest-derived cells (NCDCs) to MSC cultures was very limited, at P6 approximately 40 % of the total BM-MSC population is of this origin. However, considering that the recombination frequency of the Cre allele was not analyzed in this study, contribution of NCDCs to BM-MSC cultures might likely be higher [17]. A neural crest contribution with neonatal BM-MSCs was also supported in an in vivo lineage tracing study using Nestin-Cre mice [18]. Finally, considering our finding that myelinating Schwann cells can de-differentiate into a Schwann cell precursor-like state [17], the possible contribution of Schwann cells to BM-MSC cultures remains to be addressed (Fig. 1). Interesingly, in a rigourous lineage tracing study, dental MSCs has recently been shown to derive from Schwann cell precursors [19]. Thus, evidences previously discussed support a contribution of NCDCs to BM and dental pulp MSC cultures. Whether or not they could also contribute to MSCs cultures established using other tissues/organs, and to which extent, remains to be addressed.

Although the in vivo cellular sources of MSCs requires further elucidation, it is now accepted by the scientific community that they are located in perivascular areas [20]. Moreover, it was recently shown in human AT and in fetal BM samples that $CD146^+$ perivascular cells, also expressing Nestin, CXCL12 and/or leptin receptor (Lep-R), are able to sustain multilineage hematopoiesis in vitro [16, 21, 22]. Nevertheless, Corselli et al. [21] found that P3-10 $CD146^+$-derived MSCs lose the ability to sustain the full differentiation capacity of hematopoietic stem and progenitor cells. Therefore, significant differences can likely be found in cultured MSCs when compared to their in vivo cell sources, due to in vitro incubation and expansion. It is worth noting that bone marrow isolated $CD133^+$ cells are also able to establish ectopic locations of hematopoiesis when transplanted in vivo into athymic mice [23]. According to their in vivo morphology and frequency, other markers likely expressed by cells contributing to BM-MSC cultures could eventually be CD10 and CD73 [24] (Fig. 1).

Certain culture conditions might be able to maintain the "pluripotency" (capacity to differentiate into all body cells) of certain bone marrow subpopulations which can contribute with MSCs [6, 25, 26]. Alternatively, such conditions might induce this property through in vitro reprogramming and/or maintenance of the original phenotypic plasticity of certain cells which might be able to contribute to cells of all different germ layers without being truly pluripotent (Fig. 1).

After much research effort invested in this field, it is now clear that although many tissues/organs contain cells able to originate cultures with MSC properties, the identity of these

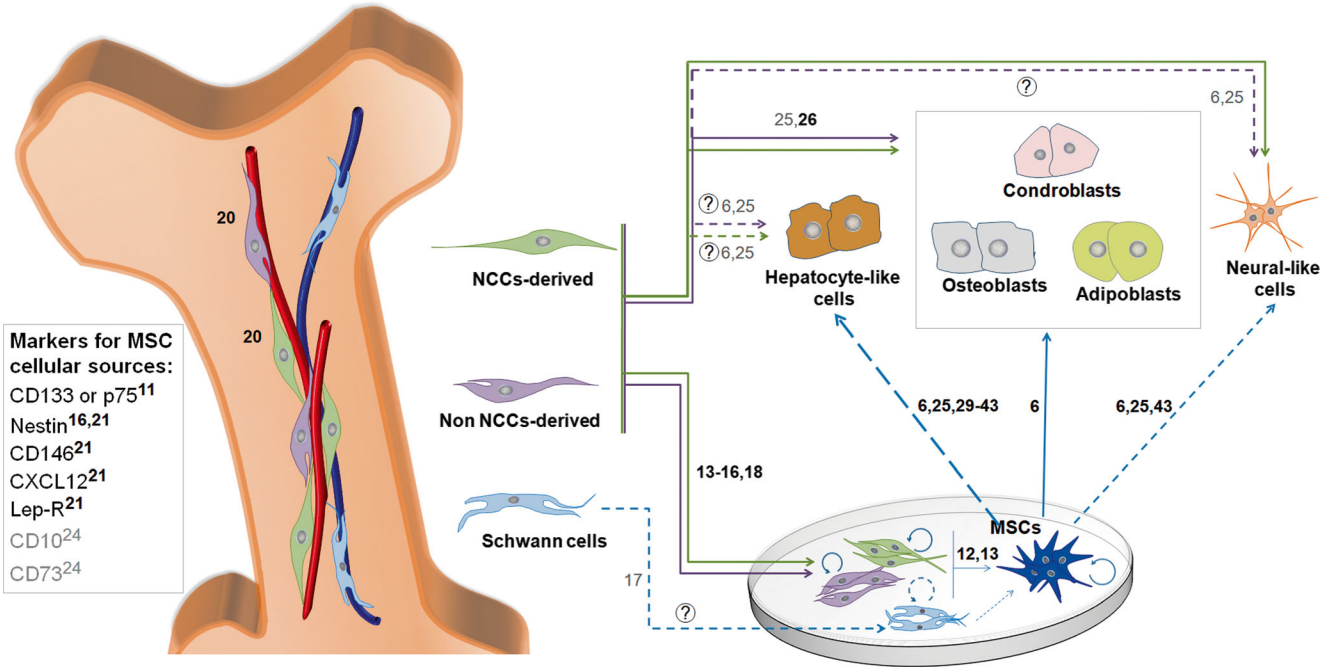

Fig. 1 In vivo cells contributing to mesenchymal stromal cell cultures. Different subtypes of perivascular cells, of neural crest and non-neural crest origin, likely contribute with cells to MSC cultures. Note that evidences regarding differentiation potential required for MSCs are mostly in vitro and there is lack of knowledge with respect to the capacity of in vivo cells to originate MSC derivatives. *Numbers* shown correspond to publications as listed in the Reference section. *Dashed lines*: hypothetical features. *Large dashed lines*: many reports support such hypothetical feature so that it can be considered as close to established knowledge. *Bold black numbers*: articles provide solid evidence addressing the specific feature indicated in the figure. *Grey numbers and characters*: articles provide some clues in support of this hypothetical feature. Within the bone: *red*, capillaries; *blue*, nerve fibers

native in vivo cells varies according to their location in the body and developmental origin, and their relative contribution to MSC cultures would also depend on technical procedures applied to isolate and expand them in vitro (i.e., methods of cell source selection, culture medium composition and degree of in vitro expansion) [27, 28].

Plasticity: MSCs-Derived Hepatocyte-Like Cells

MSCs give rise to cells of mesodermal lineages including osteoblasts, adipoblasts and chondroblasts. Non-canonical contribution of MSCs might include some ectodermal (neurons), endodermal (hepatocytes) as well as other mesodermal lineages (cardiomyocytes), which remains controversial [25] (Fig. 1).

New evidences of a putative differentiation of MSCs into hepatocyte-like cells have been shown in many recent publications [29] (Fig. 1). They have been obtained from MSCs which were originated from BM [30–33], AT [34–36], UC and/or WJ [37–39], and decidua [40] samples. An increased efficiency of this process was achieved in P3-P5 human MSCs by different research groups who have applied defined sequential protocols [30, 32, 38, 39], as reviewed by Volarevic et al. [41]. A shortage and/or improvement in this differentiation process can be achieved by culturing cells in hypoxic conditions [39] or on extracellular matrix [32]. Differentiated cells were found to: express hepatocyte markers (such as AAT, AFP, albumin, CK18, CX32, CYP1A1, CYP3A4, CYP7A1, G6P, HepPar, HGFR and/or HNF-4a) [35, 38, 39], store glycogen and/or uptake/secrete indocyanine green (ICG) [40]. In addition, they were shown to clear ammonia/produce urea [30, 34, 35], secrete albumin and/or uptake low density lipoprotein (LDL) [32, 35, 39]. From previous data, it seems now reasonable to conclude that some MSCs have the capacity to differentiate into hepatocyte-like cells [42]. It might be crucial then to clarify if this is a rare in vitro event or if any of the cellular sources of MSCs could also be able to originate hepatocyte-like cells in vivo or after minimal in vitro expansion (Fig. 1). Finally, by addressing previous questions and if the availability of certain cell sources of MSCs with the potential to differentiate in vivo into neural, mesodermal and hepatocyte-like cells is demonstrated, then the biology of subpopulations of pluripotent-like cells [43, 44] able to generate MSCs will be better understood.

Organotins and Obesity: ¿a Role for MSCs?

Metabolic diseases, such as metabolic syndrome, obesity and diabetes, could result in the development of non-alcoholic steatohepatitis (NASH), a chronic liver disease able to induce

liver fibrosis that is growing in prevalence worldwide [45]. We have recently shown in a mouse model of NASH that the use of pan-caspase inhibitors is able to block hepatocyte apoptosis and therefore to ameliorate liver fibrosis [46]. It could be of relevance to uncover new causes of such metabolic diseases and to find new therapeutic avenues in order to prevent the liver fibrosis which develops from this etiology.

Organotins or obesogens are common environmental contaminants which are agonists of peroxisome proliferator activated receptor (PPAR)-γ and of its heterodimerization partner retinoid X receptor (RXR), and were found to be potent inducers of bone marrow adipogenesis [47, 48]. They might then influence the behaviour of in vivo cells contributing to MSCs. These compounds are spreadily used as antifouling agents and in agricultural pesticides, wood preservatives, and plastics manufacturing. Some levels of them can be measured in house dust [48]. Exposure to organotins is now considered as possible contributing factor to diabetes and obesity epidemics and scientific evidence is considered as suggestive-to-strong [49].

Interestingly, Yanik et al. [48] showed that the organotins dibutyltin, tributyltin (TBT) and triphenyltin are able to potently estimulate the differentiation of BM-MSCs into adipocytes. In addition, TBT which likely acts through several nuclear receptor pathways was found to induce the expression and to efficiently activate PPAR-γ in MSCs, a nuclear receptor involved in their adipogenic differentiation [48]. Prenatal exposure of mice to TBT was shown to increase the expression and/or to induce the hypomethylation of the promoter/enhancer region of PPARγ target genes, such as the fatty acid-binding protein 4, in human (h)AT-MSCs [47]. Furthermore, the effects of TBT mediated by epigenetic modifications on master metabolic-regulatory genes were found to be heritable at least up to the third mice generation [50]. Whether or not cell sources of MSCs play a role in maintaining the observed epigenetic modifications as well as in the transgenerational predisposition to overweight/obesity requires to be addressed. Previously discussed evidences suggest that the use of organotins might influence the increase in the prevalence of NASH, and of liver fibrosis in association with it.

Immunomodulatory Properties

As previously discussed, liver fibrogenesis evolves through repeated cycles of hepatocyte/biliary duct cell apoptosis-inflammation-scar processes [1]. During the inflammatory phase of the cycle, Kupffer cells and infiltrating bone marrow-derived macrophages release reactive oxygen species (ROS) and cytokines that activate HeSC and/or fibroblasts to produce collagen. At the same time, these factors cause an unbalance in between liver resident immune cell populations and induce an immune cell infiltration, which promote liver injury and fibrosis [51]. Thus, therapeutic avenues/strategies able to protect hepatocytes/biliary duct cells from insults and/or to modulate inflammation could prevent the development of liver fibrosis/cirrhosis. It is worth noting that MSCs are immune-privileged cells able to inhibit or modulate immune responses through different and complex mechanisms, some of them causing a reduction in the amplification of pro-inflammatory signals [52–55], which were the subject of recent and detailed reviews [51, 56–58] and are briefly summarized in Fig. 2. Thus, the immunomodulatory properties of MSCs are thought to largely contribute to their anti-fibrogenic effect in the context of liver fibrosis/cirrhosis (see below and Fig. 2). In vivo mechanisms mediated by MSCs likely involve impairment of Toll-like receptor (TLR)-4 induced activation of dendritic cells, leading to their reduced capacity to migrate to lymph nodes and to prime T-cells [59] (Fig. 2).

Finally, MSCs were suggested to behave in some cases as antigen-presenting cells and/or to trigger immune responses [60, 61]. Interestingly, Sánchez-Abarca et al. [60] showed that P3 hBM-MSCs are capable of capturing and releasing antigens, although at much lower levels than DCs, with possible involvement of TLRs. Taking into consideration that preclinical studies are not always homogeneous and/or unequivocal regarding MSCs immunomodulatory and/or their immunogenicity properties and in order to be able to build knowledge on consistent and solid data, the MSC Committee of the International Society for Cellular Therapy published a "working proposal for a standardized approach based on a critical view of literature data" [62].

MSCs Fusion and Exosome Release Properties

Evidence from some recent reports suggests that MSCs may fuse with postmitotic neighbor cells [63]. Acquistapace et al. [63] showed events of partial and transitory heterologous fusion of hAT-MSCs with differentiated cardiomyocytes. This was found to result in the reprogramming of the latter cell type back to a progenitor-like phenotype. Heterotypic cell fusion was also observed in vivo between myeloid/lymphoid cells and non-haematopoietic cell lineages (including hepatocytes, cardiomyocytes and cerebellum Purkinje cells) after organ-specific injuries or irradiation events, a feature found to be significantly enhanced in case of chronic inflammation [64]. Although no such mechanisms have been reported in vivo for MSCs they might be relevant when applied after regional hepatic irradiation in the context of liver fibrosis [65] (Fig. 2).

In addition, MSCs were found to release microparticles or exosomes which then circulate through the blood stream [66] and might eventually influence cells under stress or with high metabolic rates [67, 68]. Interestingly, MSC-derived microparticles likely transfer RNA to target cells a feature which

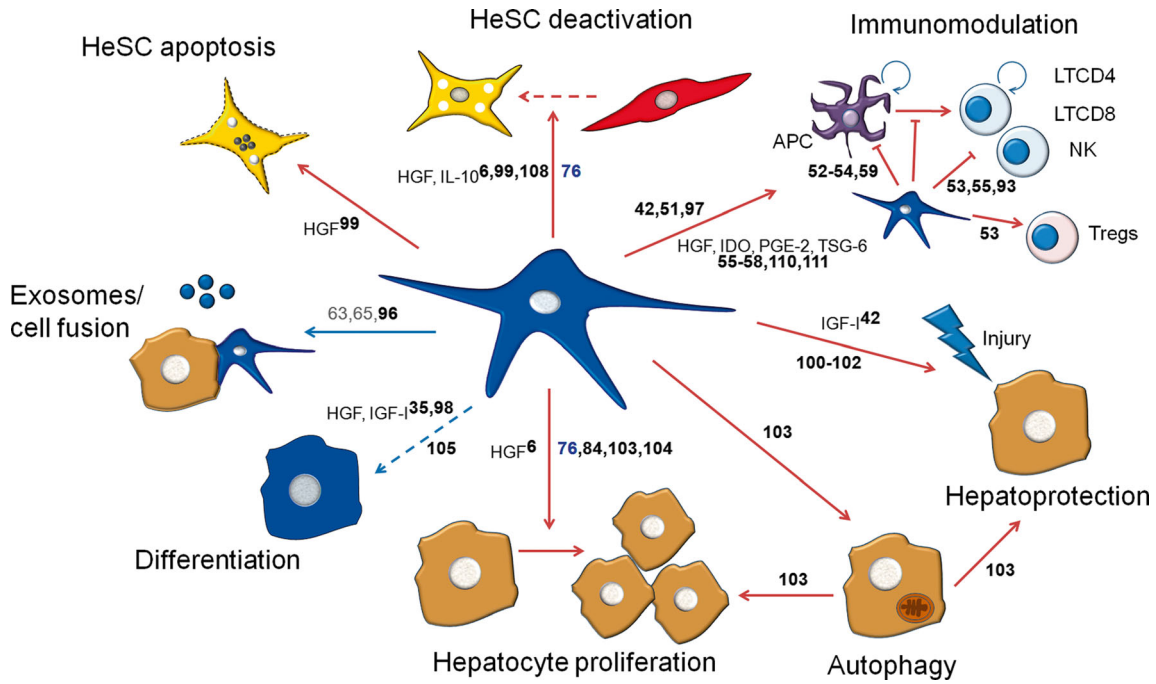

Fig. 2 Mechanisms induced by MSCs contributing to liver fibrosis/cirrhosis amelioration. According to available reports, in vitro MSCs likely benefit liver fibrosis mainly through paracrine mechanisms (*red arrows*); nevertheless, contribution of cell fusion/exosomes release and/or hepatic cell differentiation of MSCs (*blue arrows*) have also been suggested. Soluble molecules (and among them several growth factors) likely mediate paracrine mechanisms to a large extent; including: modulation of immune responses (i.e., inhibition of antigen-presenting cells -APC- maturation, proliferation, activation and/or T cell priming activity; reduction of lymphocytes and/or NK cells proliferation, maturation or activity, and induction of regulatory dendritic cells), enhanced hepatoprotection and hepatic cell proliferation. *Numbers* shown correspond to publications as listed in the Reference section. *Grey characters*: hypothesized mechanism. *Blue bold numbers*: results from early events induced by MSC transplantation. *Dashed line in blue*: contribution to this feature might be irrelevant for improvements observed in liver fibrosis. *Dashed line in red*: it is unknown whether these cells can be restored to the quiescent and vitamin A reservoire phenotype

is suggested to be required for their beneficial effect [69, 70]. Nevertheless, the in vivo significance of such events in a fibrotic liver requires to be further elucidated. On the other hand it will be of interest to know if exosomes derived from hepatocytes or immune cells might be relevant in modulating MSCs gene expression profile.

In Vivo Tracking

The extent of in vivo contribution of MSCs remains largely controversial due to unspecificity and/or unreliability of methods/trackers/reagents frequently used for the identification of transplanted cells or of the phenotype they might acquire (as reviewed in [71, 72]). Regarding common trackers used, when Hoechst-stained BM-MSCs were intraneurally applied virtually all cells within a dorsal root ganglion were labeled suggesting that the dye was uptaken by most cells in the tissue [73]. Indeed, as soon as 30 min after intrahepatic application of Hoechst-stained BM-MSCs, we were able to find that the nuclei of all cells surrounding the injection site were already labeled (our unpublished results). Other trackers, such as magnetic nanoparticles, quantum dots or DiI/DiR can be uptaken in vivo by macrophages [72]. In different liver fibrosis/cirrhosis in vivo models, abundant highly autofluorescent ceroid-ladden macrophage cells are found in proximity to portal areas and fibrotic bridges [74, 75], which makes difficult to truly identify cells under the fluorescence microscope. By mean of chromogenic immunostaining techniques and using green fluorescent protein (GFP) as marker we were able to show that significant numbers of MSCs are present within liver fibrotic parenchyma at least during the first 7 days after their systemic application [76].

Strategies to Increase MSCs Migratory, Survival and Proliferation Properties

When MSCs are systemically applied, they are thought to be mobilized towards injured or highly remodeled areas [10, 77]. For this reason, some recent publications have investigated the mechanisms involved in their migratory capacity and/or have aimed at modulating them in order to increase their recruitment capacity towards such areas. Novo et al. [78] showed that intracellular reactive-oxygen species (ROS) are required for MSCs to migrate in the presence of different growth

factors, chemokines or extracellular ROS. New studies took advantage of previous knowledge on the involvement of the stromal cell-derived factor-1a (SDF-1a)-C-X-C chemokine receptor type 4 (CXCR4) signaling axis in MSC migration towards injury sites [6, 79–82].

As previously discussed, MSCs in vivo survival after their systemic application remains controversial, with some reports suggesting that they are only recruited to the lung but not to the liver [83], and others showing that they might survive within the liver for more than a month [84, 85]. Bianco et al. [20] postulated a Yin-Yang dualistic view of MSCs according to the way scientists might eventually consider them: some might see MSCs as skeletal stem cells, being only located within the bone marrow, which when transplanted without in vitro expansion are able to form bone tissue and to survive decades, and others might consider them as in vitro expanded cells derived from cells ubiquitously located in the body, and which, after being infused, can function as drugstores, embolize and die in a few hours. Despite the lack of enough in vivo tracking evidence to support any of those views, some articles showed new strategies aiming at prolonging the survival of MSCs in an adverse environment. P2 hBM-MSCs genetically-modified to transiently express cytoprotective factors (i.e., heme oxygenase-1 or nuclear factor erythroid-2 related factor 2), by using adenoviral vectors, were able to survive better in hypoxic and oxidative-stress conditions [86, 87].

Another limiting factor for achieving therapeutic benefits is the production of enough numbers of MSCs without excessive in vitro expansion [38]. Interestingly, differences in proliferation rate were seen among MSCs obtained from diverse tissues [88]. In order to reach sufficient amount of cells at early passages, new strategies have recently been applied. For instance, MSCs proliferation can be enhanced by adding extracellular matrix components to culture medium composition, without the need of using growth factors affecting MSC differentiation potential [89]. Estrada et al. [90] showed that MSCs cultured at low oxygen tension (3 %) are more genetically stable and grow faster than in normoxic conditions (20 % oxygen). By preconditioning rat BM-MSCs with S-nitroso N-acetyl penicillamine (SNAP), Masoud et al. [91] were able to increase the survival and proliferation of MSCs, likely through the upregulation of key genes (such as IGF-I, AKT, BCL-1 and PCNA); nevertheless, whether or not this treatment might increase their migratory capacity remains to be elucidated. Finally, Ahmadbeigi et al. [92] proposed to use, as source of MSCs, the cell aggregates which appear at the upper fraction after ficoll-paque centrifugation or are left after filtering the suspension of extruded bone marrow samples. Interestingly, this fraction seems to be more enriched in cells able to originate MSCs when compared to those derived using standard procedures.

Mechanisms Involved in MSC-Mediated Liver Fibrosis/Cirrhosis Amelioration

Application of MSCs in in vivo models of liver fibrosis/cirrhosis and eventually in patients ameliorates the development of the disease [6, 93–95]. Similar results were obtained when MSCs conditioned-media (CM) or exosomes were applied instead [29, 96] suggesting that MSCs long-term survival might not be necessary for their beneficial effects. Thus, crucial events triggered by MSCs might be induced early after their application in vivo. In fact, we have recently shown that as soon as 1 day after MSCs systemic application a peak in insulin growth factor like-I (IGF-I) and hepatocyte growth factor (HGF) is observed in the fibrotic liver, subsequently followed by a significant downregulation of transforming growth factor-beta 1 (TGF-β1), alpha smooth muscle actin (α-SMA) and pro-collagen 1A2 expression levels [76] (Fig. 2).

In spite of these evidences, most of in vivo data suggesting mechanisms likely involved in MSCs antifibrotic effect results from analyses made at 2 to 4 weeks after cellular application and might therefore be likely indirect (Fig. 2). From those studies multiple mechanisms have been suggested to play a role in in vivo liver amelioration [97], such as: immunomodulation [56, 98] (as discussed above); apoptosis of HeSCs [99]; inhibition of HeSC activation and/or their deactivation [76, 100]; protective effects on hepatic cells [100–103] (see above), and restoration or induction of hepatic cell proliferation [76, 84, 103, 104] (Fig. 2). In vivo differentiation of MSCs into hepatocytes or cell fusion with resident cells in the context of liver fibrosis remains controversial and it seems irrelevant regarding MSCs antifibrotic effects [105] (Fig. 2). Interestingly, Jung et al. [103] showed in vitro data suggesting that MSCs might exert autophagic-inducing mechanisms on hepatic cells, resulting in damaged-cell clearance or in their protection (Fig. 2). Whether or not MSCs application is able to induce changes in the phenotype of macrophages infiltrating liver tissue requires to be addressed [106, 107].

Based on these evidences, the available literature strongly suggests that MSCs might mainly exert their antifibrotic effects through paracrine mechanisms (Fig. 2). With this regard, trophic factors expressed by MSCs, including the protective HGF and IGF-1, were linked to therapeutic benefits and with the mechanisms previously discussed [6, 30, 42] (Fig. 2). For instance, antifibrotic [99, 108, 109] and immunomodulatory properties [110, 111] were found for HGF as well as for IGF-1 (Fig. 2). These proteins might eventually counteract TGF-β1, a liver profibrogenic cytokine found to be upregulated after injury in the liver [1, 112, 113]. Moreover, proinflammatory factors were shown to induce HGF expression levels in MSCs [114]. Forced expression of HGF in MSCs was able to further ameliorate liver fibrosis when compared to MSCs-alone treatment in a rat dimethylnitrosamine model [115].

Interestingly, the application of recombinant IGF-1 has been shown to increase serum albumin and HGF levels in the context of liver fibrosis [116]. Furthermore, our own results suggest that an upregulation in the expression of HGF and/or IGF-1 by MSCs, as well as their de novo expression by hepatocytes, early after MSCs transplantation, are likely involved in their therapeutic effect [76] (Fig. 2). Finally and considering that IGF-I expression levels are downregulated in a fibrotic liver [113], it is worth noting that by applying multiple doses of IGF-I-transduced MSCs in the context of liver fibrosis into immunocompetent mice we were able to significantly improve the therapeutic effect of a single MSC dose of same treatment [76].

MSC Pre-Conditioning and Combined Treatments for Liver Fibrosis

Some recent studies suggest that MSCs pre-conditioning might be able to enhance the therapeutic effects of these cells in the context of liver fibrosis. Ali et al. [117] showed that when a mouse model of carbon tetrachloride (CCl4) was treated with sodium nitroprusside (SNP) before MSCs transplantation, liver fibrosis was further ameliorated when compared to control MSCs-treated group suggesting a positive role of nitric oxide. Interestingly, pre-incubation of MSCs with interferon-gamma (INF-γ) was shown to induce the expression of well known immunomodulatory factors such as indoleamine 2,3-dioxygenase (IDO), HGF and cyclooxygenase-2 (COX-2), likely playing a role in liver fibrosis amelioration mediated by MSCs [114].

Nasir et al. [102] showed that application of several doses of IL-6 prior to a single application of MSCs enhanced the anti-fibrotic mechanisms mediated by MSCs with higher induction of glycogen storage and of pro-survival mechanisms. Wang et al. [109] have reported that HGF and direct MSC cell contact are able to synergize at inducing enhanced inhibition of HeSC activation. We have recently shown that the antifibrotic effect resulting from combining daily applications of recombinant IGF-I protein with MSCs could be achieved or even overpassed by a single dose of IGF-I overexpressing MSCs [76].

Appart from suggesting new strategies to improve the therapeutic outcome of MSCs application in the context of liver fibrosis/cirrhosis, these findings further support a main role of MSC immunomodulatory properties in their anti-fibrotic effect which seems also linked to hepatocyte survival and to a reduction in HeSC activation (Fig. 2).

Biosafety: New Scenes and Strategies

Even though fundamental questions regarding MSCs biology remain unknown, the beneficial effects of MSCs in preclinical studies, the lack from serious adverse effects and the positive short-term results from some early clinical phase studies [42, 94, 118–122], prompted the development of more advanced clinical trials and fostered the application of good manufactuting practices (GMP) and of safer techniques to avoid potential undesirable effects [123].

No reports were published so far suggesting cellular transformation potential and/or tumorigenesis risk of hMSCs at early-passages/relatively low-population-doubling levels [124–126]. Nonetheless, Barkholt et al. [124] made several authorized recommendations. For instance, cytogenetic testing was recommended when MSCs would be incubated in specific culture media with components/procedures able to induce chromosomal abnormalities. It is worth noting that Tarte et al. [127] reported some in vitro transient and donor-dependent levels of aneuploidy in P1-P2 hBM-MSCs, likely independent of culture conditions (such as addition or not of FGF-2 and/or platelet lysate to culture composition); nevertheless, in all such cases MSCs entered senescence and did not undergo cellular transformation. The application of an in vitro senescence test was recently suggested to be a sufficient method for addressing whether certain cells are or not unlikely to produce tumors or malignancies in patients [128]. Moreover, senescence was recently found to be closely linked to chromosome aneuploidy in hMSCs [129]. Binato et al. [27] found chromosomal variability in hBM-MSCs after P4, while others were not able to find such abnormalities in MSCs even at higher passages [27, 124].

Regarding manufacturing of BM-MSCs for phase I clinical trials, Hanley et al. [130] published the following recommendations: 1) limit expansions to four passages and to less than 30 cell doublings; 2) replace FBS by platelets lysate; 3) perform cell culture manipulations, quality and release testing as well as flow cytometry in GMP facilities; 4) send BM aliquots for phenotyping, cytogenetics and sterility testing, and 5) send isolated mononuclear cells for phenotyping, sterility and viability count. In addition, they reported a list of the reagents they use in laboratory routines as part of GMP procedures.

Animal components might lead to immunologic responses and virus/prion/zoonoses transmission to patients. Thus, with the aim of developing defined serum-free and xeno-free culture media (SFM-XF), Chase et al. [131] were able to expand P5 hBM-MSCs in SFM-XF when medium was supplemented with platelet-derived growth factor-BB (PDGF-BB) and/or basic fibroblast growth factor (bFGF) and/or TGF-β1. They were also able to expand hAT-MSCs for several passages and they found no chromosome alterations after gross-karyotype analyses. Chieregato et al. [132] followed a xero-free culture protocol and showed that addition of epidermal growth factor

(EGF), bFGF and PDGF-BB to a medium supplemented with human platelet-poor plasma (hPPP) was able to expand hAT-MSCs better than FBS, without modifying the MSCs phenotype but enhancing their adipogenic and osteogenic differentiation potential. De Lima Prata et al. [133] showed that P5-P7 hUC-MSCs can be cryopreserved in xeno-free dimethyl sulfoxide without significant reduction in viability while keeping MSCs immunomodulatory and karyotype properties. Julavijitphong et al. [134] showed evidences supporting that human serum from umbilical cord blood (hUCS) could replace fetal bovine serum as culture reagent for hWJ-MSCs. In this work, culture medium was supplemented with bFGF and no gross chromosome abnormalities were found.

Several reports suggest that genetically-modified MSCs can achieve better therapeutic results when compared to naïve cells or control (see below). However, to reach high gene transfer rate viral vectors are frequently used as technical approach. While retrovirus and lentivirus vectors integrate their genetic material into the host cell genome [135], recombinant adenoviruses would not raise such safety concern; nevertheless, their direct systemic use can trigger potent immune responses against their own proteins instead [72, 136]. Interestingly, Treacy et al. [137] showed that adenoviral transduction of MSCs did not induce immune responses in vitro or after a single application in vivo. Furthermore, the expression levels of markers related to immune responses were not greatly modified when compared to using naïve cells. We recently showed that multiple applications of adenovirally transduced BM-MSCs, overexpressing IGF-I or GFP (as control), increased the antifibrotic effect of respective single application without resulting in the development of immunogenicity against adenoviral antigens [76].

Summary and Future Perspectives

Knowledge regarding MSCs biology and their application in liver fibrosis has significantly increased during the last years. Nevertheless, fundamental questions remain to be addressed such as: a) identification of in vivo cell sources contributing to MSC cultures and knowledge of their differentiation potential; b) differences among tissue sources; c) their role in hereditary parental transmition of obesity and/or diabetes; d) mechanisms influencing their immunomodulatory behavior; e) significance of MSC fusion and exosome production in the context of liver fibrosis; f) early mechanisms involved in MSCs therapeutic effects; g) the outcome of repeated vs. single applications of naïve or genetically modified MSCs as well as of combined pharmacological and genetic strategies, and h) safety issues regarding culture management conditions. Progress in these areas would allow the design of optimal conditions for the enhancement of the therapeutic activity of MSCs.

It is worth noting that although adverse outcomes after MSCs administration could not be completely ruled out, for the moment no major negative consequences were reported so far after the completion of many clinical trials. Thus, there is hope for establishing new and relatively safe MSC-based treatments in the near future to improve the condition of patients suffering from illnesses such as liver cirrhosis.

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