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COMPREHENSIVE REVIEW

New Emerging Potentials for Human Wharton's Jelly Mesenchymal Stem Cells: Immunological Features and Hepatocyte-Like Differentiative Capacity

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In recent years, human mesenchymal stem cells (MSC) have been extensively studied. Their key characteristics of long-term self-renewal and a capacity to differentiate into diverse mature tissues favor their use in regenerative medicine applications. Stem cells can be found in embryonic and extraembryonic tissues as well as in adult organs. Several reports indicate that cells of Wharton's jelly (WJ), the main component of umbilical cord extracellular matrix, are multipotent stem cells, expressing markers of bone marrow mesenchymal stem cells (BM-MSC), and giving rise to different cellular types of both connective and nervous tissues. Wharton's jelly mesenchymal stem cells (WJ-MSC) express markers previously characterized in embryonic stem cells (ESC), such as Nanog and Oct3/4A. WJ-MSC further emerge as promising hypoimmunogenic cells, due to the expression of molecules able to modulate NK cells and expand regulatory T-cell populations. Moreover, it is now accepted that the differentiative capacities of such cells span all the mesoderm-derived tissues, extending to neuroectodermal as well as endodermal lineages. In this review, we compare very recent data on the potential of WJ-MSC to undergo hepatocyte-like differentiation with the results obtained from other adult MSC populations. Data in the literature strongly suggest that WJ-MSC can differentiate into diverse cell types, showing a unique ability to cross lineage borders. This, together with their in vitro proliferative potential and their immunoregulatory features, renders these cells extremely promising for regenerative medicine applications in different pathological settings.

Introduction

Mesenchymal stem cells (MSC), first isolated from bone marrow (BM-MSC) as stromal cells supporting hematopoiesis, and early described as capable of differentiating toward adipocytes [1,2], are pluripotent cells capable of long-term self-renewal and able to give rise to connective tissues (cartilage, bone, tendons, ligaments, and adipose) and nervous tissue [3]. These cells are able to undergo ex vivo expansion, growing on plastic surfaces, and expressing a number of markers that are also shared by several differentiated phenotypes. In fact, analysis of the gene expression profile of MSC demonstrated the presence of transcripts typical of osteoblasts, chondrocytes, endothelial cells (EC), epithelial cells, and neurons [4].

Several authors have contributed to the definition of markers useful for BM-MSC immunophenotyping (see Table 1). Among the "core" markers, CD44, CD73, CD90, CD105, CD166 are reproducibly found expressed in different preparations of BM-MSC. Other markers for which a general consensus exists are: CD49e, CD51, CD54, CD59, CD71, while it is widely accepted that CD117 is not expressed in human BM-MSC, albeit being reported in other MSC populations [5–9].

As reported in Table 1, BM-MSC do not express typical markers of endothelial/hematopoietic cells such as CD31, CD14, CD34, CD45, CD79, CD86, and glycophorin A (CD235a) [7,8,10,11]. Moreover, apart from their differentiative abilities, BM-MSC have shown a supportive role in organ regeneration processes [12]. In addition, several studies suggest that use of MSC in vivo should be safer than that of embryonic stem cells (ESC), due to their higher chromosomal stability and lower tendency to form neoplasms in the recipient host [13,14]. Even if adult human bone marrow is the most

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Table 1. Comparison of Markers Expressed by BM-MSC and WJ-MSC

Markers	*BM-MSC*	*WJC*	*References*
CD10	+	+	[27,32,37,40,44]
CD13	+	+	[27,32,37,39,44,90]
CD14	−	−	[7,29,40]
CD29	+	+	[27,32,37,40,48]
CD31	−	−	[7,29,37,40,90]
CD33	−	−	[29,37,40,48]
CD34	−	−	[7,29,37,40,90]
CD44	+	+	[5,7,27,32,37,40,90]
CD45	−	−	[7,29,37,40,90]
CD49e	+	+	[5,29]
CD51	+	+	[5,40]
CD54	+	NA	[5,6]
CD56	+	−	[29,39,40]
CD59	+	NA	[5,8]
CD68	NA	+	[72]
CD71	+	NA	[5]
CD73	+	+	[6,37,42]
CD79	−	NA	[8]
CD80	−	+	[37,45]
CD86	−	−	[7,37,42,45]
CD90	+	+	[5,6,37,42,90]
CD105	+	+	[5,6,37,40,42,90]
CD117	−	+	[36,37]
CD163	NA	−	[72]
CD166	+	+	[5,43]
CD235a	−	NA	[11]
CK-7	NA	–	[37]
CK-8	NA	+	[32,37]
CK-18	+	+	[32,37,55]
CK-19	+	+	[37,55]
Connexin-43	+	+	[37,50]
GATA-4	+	+	[37,50]
GATA-5	NA	+	[37]
GATA-6	NA	+	[37]
GFAP	+	+	[17,36,37,48]
HLA-A	+	+	[11,37,42]
HLA-B	+	+	[11,40]
HLA-C	+	+	[11,40]
HLA-DR	−	−	[29,37,40]
HLA-G	+	+	[37,64,70]
HNF-4α	NA	+	[37]
Nanog	+	+	[29,37,53]
Nestin	+	+	[37,46,48,49]
NSE	+	+	[17,36,37,48]
Oct3/4A	+	+	[29,37,53]
α-SMA	+	+	[17,37,49]
Vimentin	+	+	[37, 49]

Abbreviation: NA, not applicable.

common MSC source, the number of cells useful for regenerative medicine applications is extremely low (~0.001% to 0.01%) [15]. Moreover, yield of MSC from bone marrow significantly decreases with donor age [16]. Many researchers have therefore searched for alternative sources of MSC in adult (eg, adipose tissue) and extraembryonic tissues such as placenta, amniotic membrane, and umbilical cord [17–20].

Mesenchymal Stem Cell Populations in the Human Umbilical Cord

The umbilical cord (UC) is an extraembryonic formation that constitutes the essential link between mother and fetus during pregnancy. It is layered by cubic epithelial cells forming the umbilical epithelium, an ectodermal derivative that

continues with amniotic epithelial cells and the tegumentary epithelium of the fetus [21,22]. Umbilical cord is structured in order to protect the vessels, 2 arteries and a vein, with a surrounding matrix of mucous connective tissue, enriched in fibroblast-like cells embedded in an amorphous substance, rich in proteoglycans and mainly hyaluronic acid (HA), known as Wharton's jelly cells (WJC), the main component of the UC extracellular matrix [23]. Different structural and functional studies have recognized at least 5 distinct zones in human umbilical cord: surface epithelium, subamniotic stroma, intervascular stroma, perivascular stroma, and vessels. Two main cellular types have been identified in WJC: fibroblast-like cells [24] and myofibroblasts [25,26].

Several features of BM-MSC are recapitulated, albeit with some differences, in UC-derived MSC populations. WJC support ex vivo hematopoietic expansion [27] and in vivo engraftment of hematopoietic stem cells [28]. Weiss and collaborators demonstrated that WJC express osteopontin [29], a protein constituting a major component of the hematopoietic stem cell niche and a regulator of hematopoietic progenitor cells [30]. Another component of the hematopoietic stem cell niche is HA, and WJC are a likely source of HA [31]. Therefore, both the expression of the osteopontin gene and the presence of HA confirm at molecular level the observed role of WJC as a facilitator of hematopoietic expansion.

WJC secrete a range of cytokines partly similar to those of BM-MSC. Interestingly, and in a different way with respect to BM-MSC, WJC synthesize granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) [27]. Recent data have further extended the key differences in the basic biology of WJC compared to BM-MSC, documenting that WJC are slower in the differentiation toward adipocytes [32], have a higher frequency of CFU-F [33], feature a shorter doubling time [34], and can be isolated with 100% success (making them more promising for in vivo applications). Moreover, Saito et al. demonstrated that WJC support growth of ESC and embryonic-like stem cells [35].

Phenotypical Characterization of Wharton's Jelly Cells and Features in Common With BM-MSC

WJC are multipotent stem cells, they are plastic adherent, grow robustly, can be deep-frozen for long-term storage, and can be engineered to express exogenous proteins. Studies have demonstrated that WJC have faster and greater ex vivo expansion capabilities than BM-MSC. This may in part be due to the expression of telomerase by WJC [36], paralleled by the maintenance of long telomeres by cultured cells at high passages [37,38].

As depicted in Table 1, there is a significant overlap between the markers expressed by BM-MSC and Wharton's jelly mesenchymal stem cells (WJ-MSC). Immunocytochemistry experiments have shown that WJ-MSC (as well as BM-MSC) lack expression of CD14, CD31, CD33, CD34, CD45. CD56 is expressed by BM-MSC [39], but not by WJ-MSC. Moreover, both BM-derived and WJ-derived MSC lack expression of HLA-DR [11,27,29,40,41]. On the other hand, WJ-MSC express at protein level: CD73, CD90, CD105, HLA class I [42], as well as CD10, CD13, CD29, CD44, CD49e, and CD166 [5,32,37,43]; all of them were also characterized as BM-MSC markers [29,44,45]. WJ-MSC express mesodermal markers such as vimentin and α-smooth muscle actin and a typical marker of the hematopoietic stem cell lineage, CD117, the receptor for the stem cell factor (SCF); moreover, a subset of WJ-MSC expresses nestin [37]. This intermediate filament of the neuroectodermal cellular lineage is expressed as a precursor of neurofilaments, but it has also been observed in pancreatic progenitors capable of differentiating toward β cells, as well as in human and rat BM-MSC populations [46–49].

Recent Advances in the Characterization of WJ-MSC by Detection of New Potential Markers

Several recent reports have extended the range of prospective markers that help characterize and identify MSC from different sources. In particular, WJ-derived MSC have undergone an unprecedented characterization process further extending the similarities (and differences) with respect to other MSC populations (summarized in Table 1).

We recently showed that WJ-derived MSC (named HEMSC, human extraembryonic mesoderm stem cells) express at mRNA and protein levels the key transcription factors GATA-4, GATA-5, GATA-6 [37]. All of these transcription factors are involved in different developmental pathways of mesoderm- and endoderm-derived organs. For BM-MSC, only GATA-4 expression had been previously reported [50]. We also demonstrated that HEMSC express connexin-43 [37]. This molecule, which is typically expressed in embryonic and myocardial cells, is responsible for the formation of intercellular gap junctions. Recent reports indicate that Cx-43 expression along the myocardial differentiation pathway increases in a stage-related manner, and is correlated to proliferation arrest and acquisition of a mature phenotype [51]. Another key feature of MSC is the capability to undergo self-renewal, that is, maintaining their replicative potential together with their undifferentiated state. In this respect, the expression of Nanog and Oct3/4A, factors responsible for maintaining long-term self-renewal and the undifferentiated state also in ESC, is a key property that has been found in other prospective MSC populations [29,52,53] but that we have demonstrated for the first time in cells extracted from WJC, and it constitutes a molecular confirmation of the clonogenicity experiments previously performed [29,37].

Moreover, we have described for the first time in WJC isolated by nonenzymatic methods the expression of a subset of epithelial cytokeratins (CK) [54]. In particular, we demonstrated the expression of CK-8, CK-18, CK-19, while CK-7 was not detected [37]. These findings confirm that WJ-MSC maintain in vitro the expression of a subset of molecules that have been demonstrated in vivo in earlier reports [32]. On the other hand, only CK-18 and CK-19 expression have been demonstrated in BM-MSC [55]. In addition, we confirmed the expression of neuroectodermal markers such as glial fibrillar acidic protein (GFAP) and neuron-specific enolase (NSE) also in undifferentiated cells, as described in earlier reports in WJ-MSC and in BM-MSC [17,36,48,49].

As discussed in detail below, the potential of umbilical cord-derived cells to differentiate toward endoderm-derived organs is currently a key task for regenerative medicine applications. In this view, our data reporting hepatocyte nuclear factor 4α (HNF-4α) expression by HEMSC are promising as regards the use of these cells for regeneration of key

cell types such as hepatocytes and pancreatic endocrine cells [37]. In particular, HNF-4α has been demonstrated to be dispensable for early endodermal specification, but essential for maintaining the differentiated hepatocyte expression pattern [56].

Immunological Features of WJ-MSC: Extending the Immune Privilege of BM-MSC

When transplanted in vivo, whether differentiated or undifferentiated, stem cells must be able to successfully engraft without provoking an immune response from the host. Numerous data indicate hypoimmunogenicity as a key feature of MSC [57,58]. It is not yet clear if cell–cell contacts or production of diffusible factors are responsible for this process. There is general agreement that these cells express normal class I HLA molecules (HLA-A, HLA-B, HLA-C), and lack class II HLA molecules (as HLA-DR) [37,59,60]. Nevertheless, in some cases, promising MSC lines (eg, amniotic fluid cells) [52] lacked an in vivo ability to home to the diseased organ without provoking an immune response, even in immunocompromised hosts [61]. Therefore, it is becoming accepted that, apart from establishing the classical type I and II HLA setting of MSC, the expression of additional markers needs to be assessed to ensure the ability of these cells to evade the immune response and induce host tolerance.

Recent data indicate that non-classical HLA molecules (as HLA-E, HLA-G, HLA-F) should be expressed by MSC cell lines. These molecules have been implicated in the induction of tolerance of NK cells toward self-cells, as well as in the process of tolerance of the mother's immune system toward the semi-allogeneic embryo, which is a key feature of the embryo implantation process [62,63]. In particular, HLA-G expression by BM-MSC has been reported to suppress T lymphocytes as well as natural killer cells function, inducing also $CD4^+CD25^+FOXP3^+$ regulatory T cells [64]. Very few data are available on the expression of non-classical HLAs by MSC populations. In particular, our group recently demonstrated that HEMSC isolated by non-enzymatic methods from the UC matrix showed the expression of a set of molecules that support their ability to potentially induce immune tolerance [37]. In fact, we showed that HEMSC bear normal type I MHC (HLA-A), while lacking type II MHC (HLA-DR). Moreover, we demonstrated for the first time in these cells the expression of HLA-G, one of the main molecules responsible for inducing tolerance of the mother toward the fetus [65], thus suggesting an immunosuppressive role for this WJC population [37]. These first observations were further supported by the demonstration of HLA-G expression in human BM-MSC [66]. Moreover, even if the general consensus is that MSC are negative for B7 co-stimulatory molecules [67], HEMSC showed a particularly favorable combination of B7 co-stimulatory molecules ($CD80^+$, $CD86^-$) indicating potential tolerance-inducing properties that should act synergistically with those given by HLA-G expression [68,69]. In a parallel report, other authors [70] showed the expression of HLA-G at RNA level in MSC isolated by enzymatic dissociation methods from the umbilical cord matrix, thus strengthening the observation from our group. Taken together, these data indicate that a proper immune characterization of MSC lines should be based on a more extended panel of markers, spanning from HLA typing to co-stimulatory molecule expression, in order to predict the extent of interactions between transplanted cells and the host immune system. The potential usefulness of MSC as tolerance inducers is consistently acknowledged [71].

Finally, we demonstrated very recently for the first time that WJ-MSC can express the CD68 molecule when kept undifferentiated, both in protein and mRNA, at levels comparable to those evidenced in the HL-60 promyelocytic cell line [72]. This reinforces the emerging concept of the non-macrophage-restricted expression of CD68 antigen, whose presence has been demonstrated in several primary and stable cell lines of multiple origins by other authors [73,74]. On the contrary, a more specific macrophage marker, CD163 [75], was not detectable in HEMSC [72]. Therefore, these novel findings reinforce the need for a more extended characterization of MSC in terms of surface molecule expression, which should further disclose the potential of these cells in interacting with immune system cells, while preventing hidden pitfalls when in vivo transplantation is attempted.

Multilineage Differentiation Capability of WJ-MSC

Several reports indicate that WJ-MSC are multipotent cells, therefore capable of giving rise to different mature cellular types. Most studies agree that WJ-MSC can be successfully induced toward connective tissue phenotypes (osteoblasts, adipocytes, and chondrocytes), thus opening new paths in regenerative medicine applications to the musculoskeletal system. This trilineage differentiation potential of WJ-MSC fulfills the minimal criteria stated in 2006 to uniformly define MSC properties [76]. Phenotypical and morphological criteria can be used to define the effectiveness of the differentiation of MSC toward the mature cytotypes of these connective tissues.

The standardized protocols to obtain osteogenic differentiation of MSC [77–79] result in the acquisition of a differentiated phenotype that may be confirmed by specific histological stains for extracellular calcium, such as Alizarin Red S and Von Kossa [37,80]. Moreover, differentiated MSC should express specific proteins, such as osteonectin, osteocalcin, periostin, runx2 [81].

After adipogenic differentiation protocols [82–84], differentiated adipocytes should be demonstrated by lipid-specific histological stains such as Oil Red O [37,84]. In addition, newly differentiated adipocytes should express specific proteins such as adiponectin, leptin, and PPAR-γ.

When chondrogenic differentiation of MSC is performed by standardized methods [85–87], the differentiated cells can be specifically stained by Alcian blue or Safranin O-Fast Green [88]. In addition, the acquisition of the chondrocyte phenotype can be demonstrated by the expression of specific proteins such as collagen type II, cartilage oligomeric matrix protein (COMP), and aggrecan [89].

Neurogenic differentiation. WJC cultured in medium supplemented with basic fibroblast growth factor (bFGF), butylated hydroxyanisole, and dimethyl sulfoxide (DMSO), with low serum percentages, have been successfully induced to differentiate into glial cells and neurons [36]. The authors described the expression of neural markers (as NSE and GFAP) also by undifferentiated cells, while differentiated neurons and glial cells overexpressed these molecules and began expressing more specific markers for

catecholaminergic neurons. More recently, Weiss and colleagues confirmed these data on human umbilical cord matrix stem cells, extending their relevance by transplantation of cells in vivo in a hemiparkinsonian rat model [29].

Myocardiocyte differentiation. Myocardial repair via heterologous stem cells is a fascinating area of stem cells research. Besides other MSC populations, recent experiments suggest that also WJ-derived cells can play a role in myocardial regeneration. The first report on the possibility that WJC can differentiate into myocardiocytes came from Wang et al. After being treated with 5-azacytidine for 3 weeks, WJC expressed typical myocardial markers such as cardiac troponin I, connexin-43, and desmin, and exhibited myocardial morphology [40]. While 5-azacytidine treatment is based on demethylation of DNA, being therefore an unspecific differentiation signal to cells, these experiments suggested that WJC should also be of prospective utility for regenerative medicine application in heart diseases. More recently, Wu et al. reported a differentiation protocol of WJ-derived stem cells in which an induction phase with 5-azacytidine treatment (24 h) was followed by 4 weeks culture in medium supplemented with bFGF and platelet-derived growth factor (PDGF) [90]. The authors showed that differentiated cells expressed in vitro cardiac myosin and cardiac troponin T (cTnT). In vivo experiments showed that stem cells, injected into the viable myocardium bordering an experimental infarcted area, were incorporated in the vasculature and occasionally were positive for cTnT [90]. Other reports claimed a supportive role for several MSC populations in terms of suppression of inflammation in acute myocardial infarction models, microenvironment-driven direct differentiation, as well as paracrine effects on the repairing myocardium [91–93].

Skeletal muscle differentiation. Conconi and colleagues demonstrated that WJC are able to give rise to skeletal muscle cells. When cultured in myogenic medium, WJC expressed myogenic factor-5 (Myf-5) from day 7 and myogenic differentiation (MyoD) from day 11 [94].

Endothelial differentiation. As demonstrated previously for human ESC [95], human WJC can be differentiated into EC after culturing in low serum medium supplemented with vascular endothelial growth factor (VEGF) and bFGF [84]. In the evaluation of successful differentiation toward EC, phenotypical and morphological characterization criteria should include typical markers of endothelial phenotype such as CD31, vWF, eNOS [95–97]. Indeed, the success of differentiation was confirmed by the expression of CD34 and CD31, as well as by demonstrating acetylated low-density lipoprotein (Ac-LDL) uptake. Moreover, in vivo experiments confirmed that UC-derived cells differentiated toward EC in an ischemia/reperfusion model [84]. More recently, Chen and colleagues [98] comparatively analyzed the differentiation potential of MSC isolated from umbilical cord matrix and bone marrow. These experiments provided evidence that UC-MSC responded to the inductive stimuli expressing vascular-specific molecules at higher levels compared to BM-MSC. Moreover, in vitro angiogenesis assays demonstrated that mean tubule length, area, and diameter were higher in UC-MSC than BM-MSC, leading the authors to hypothesize that WJ-derived cells are more effective in endothelial differentiation than bone marrow-derived cells.

Molecular Basis for the Differentiation of Extrahepatic Stem Cells Into Hepatocyte-Like Cells and Their Characterization

Several acute and chronic liver pathologies should benefit from cell-mediated liver repopulation strategies, which can restore liver functions when self-repopulation is compromised and prospectively avoid whole organ transplantation. Hepatocytes and liver progenitor cells normally respond to variations in the microenvironment by changing the gene expression and re-entering the cell cycle, thus providing reserve cells to replace damaged ones [99,100]. Independently from the starting stem cells population used, some minimal criteria must be fulfilled to ensure therapeutic success: in vitro expandability, extensive expression of hepatocyte functions, and minimal or absent immunogenicity and tumorigenicity in the recipient host [101,102].

A number of recent studies show that extrahepatic mesenchymal stem cells can differentiate into endoderm-derived cellular lineages such as hepatocytes. Several hepatic differentiation protocols of MSC have been published in recent years, based on cellular stimulation with exogenous cytokines/growth factors, co-culture with fetal or adult hepatocytes, challenging with conditioned media from cultured hepatocytes, 2- or 3-dimensional matrices to favor differentiation.

The hepatocyte differentiation protocols reported in the literature are based on the administration, to cultured cells, of a mixture of inducer agents, in order to recapitulate the developmental sequence of processes involved in the specification and differentiation of mature hepatocytes. Most used factors are hepatocyte growth factor (HGF), fibroblast growth factor (FGFs; eg, FGF-2 and FGF-4), usually needed for the first inductive phase, and oncostatin M (OSM), involved in the final differentiation phase [103]. Differentiation protocols should be based on the parallel administration of these factors [104], or follow a stepwise process [105]. Further supplements used in the differentiation protocols are insulin–transferrin–sodium selenite (ITS), dexamethasone at submicromolar concentrations, and epidermal growth factor (EGF). These factors should be applied to cells growing in a monolayer culture [104], in 3D scaffolds [106], or in co-culture systems with fetal or adult hepatocytes [107]. Most differentiation experiments have been performed using low (1%) serum culture medium.

The panel of markers used to characterize the extent of differentiation of MSC to hepatocyte-like cells is extremely wide. While some studies refer to one marker alone, or a small number of them [104], most published data refer to multiple markers, whose expression is assessed at both the protein and mRNA levels [108]. One of the most widely used markers is albumin secretion, together with the evaluation of α-fetoprotein (AFP), metabolic enzymes, and cytoskeletal proteins. In particular, regarding the latter group, a "cytokeratin switch" can be observed as a later process in the maturation of hepatocytes from bipotential progenitors. In fact, the bipotential hepatoblasts express both CK-18 and CK-19, while mature hepatocytes feature CK-18 alone, and CK-19 specifically identifies colangiocytes populations [54,100,109,110]. It is important to note that most of these "in vitro" markers are useful for characterizing differentiated cells, but cannot constitute reliable evidence on their own. In fact, AFP and

transthyretin (TTR) are expressed not only in liver, but also by extraembryonic cells in the yolk sac [100]. In addition, in a very recent study, Zemel and collaborators [111] evidenced that naïve MSC from adipose tissue expressed some of the "hepato-specific" markers, for example AFP, CK-18, CK-19, and HNF-4α, all known as early-expressed genes in the liver. This confirms earlier observations by our group on WJC-derived MSC [37]. In fact, we demonstrated that HEMSC express, when kept undifferentiated, CK-18, CK-19, and HNF-4α. Taken together, these recent data support the notion that, while the markers used are actually expressed in the mature liver or during development, their expressional pattern is far from stringent, and cannot be used as the sole proof of a successful differentiation. Nevertheless, the presence and activity of key liver-specific transcription factors (eg, HNF-4α, HNF-3γ, HNF-6, GATA-6) needs to be consistently checked in differentiation protocols, to prove that a genetic reprogramming of cells is actually occurring, rather than simply cellular mimicry [56,112,113].

There is growing evidence that, apart from expressing specific markers, differentiated cells should carry out the functional activities of mature hepatocytes, which will be determinant in the supportive functions needed for regenerative medicine applications. These enzymatic functions should also be considered as more reliable "markers" of the successful differentiation of MSC. Basic metabolic activities of hepatocytes, investigated in different works, include: glycogen storage (eg, visualized by PAS staining procedure) [104,114]; ammonia metabolization and urea production (determined by colorimetric or fluorometric assays) [108,115,116]; selective uptake of vital stains (eg, indocyanine green, which is uptaken exclusively by hepatocytes) [117]; secretion of plasma proteins (eg, albumin, determined by ELISA) [118,119]. Several works have shown the use of one or more of these assays as the formal proof of differentiation. Indeed, further metabolic functions can be evaluated, as shown for different cytochrome 450 (CYP450)-dependent activities in response to chemical inducers. Recent data from Campard and colleagues [108] showed that WJ-derived MSC, differentiated with a multistep protocol, express functional inducible CYP3A4. Further CYP450 activities (as the more stringently hepato-specific CYP7A1, 1A2, 2B6) should also be evaluated in order to correctly assess the extent of functional maturity of differentiated cells [120].

Comparative Analysis of the Differentiative Ability of WJ-MSC Toward Hepatocyte-Like Cells With Respect to Other MSC Populations

Among the different extrahepatic sources of differentiated cells to be used for regenerative medicine applications (reviewed in ref. [100]), MSC are emerging as a useful cytotype, as different reports published in the last few years have indicated. Table 2 shows the sum of the data used for this review. We here analyze in detail the results obtained using different MSC populations for hepatocyte differentiation protocols, as well as the markers expressed and the in vitro and in vivo controls applied.

Bone marrow mesenchymal stem cells. Lee and colleagues, for the first time, comparatively showed that MSC derived from different sources such as bone marrow and umbilical cord blood (UCB) can differentiate into hepatocyte-like cells, when cultured with appropriate inductive factors. The differentiated cells showed functional characteristics of liver cells including albumin production, glycogen storage, urea secretion, LDL uptake, and phenobarbital-inducible cytochrome P450 activity [121].

More recently, Lange et al. demonstrated that rat BM-MSC, co-cultured with fetal liver cells, differentiated toward hepatocyte-like cells. This study suggested also that the presence of MSC in co-culture generated an optimal microenvironment for the expansion and differentiation of fetal liver cells. Interestingly, differentiated rat BM-MSC expressed liver-specific genes like albumin, AFP, and CK-18 only over the first 2 weeks of co-culture, while in the subsequent culture period these cells lost hepatocyte-specific gene expression [122].

Deng and coworkers suggested a possible role of liver stellate cells (LSC) in the differentiation of BM-MSC toward hepatocyte-like cells. This study showed that Kupffer cell-activated LSC could induce the differentiation of BM-MSC in hepatocyte-like cells. The authors demonstrated that differentiation of BM-MSC was triggered by HGF secretion by activated LSC, rather than by direct cell–cell contact [123].

Lysy and colleagues further investigated, with in vitro and in vivo experiments, the hepatocyte differentiation ability of BM-MSC, evaluating the expression of hepato-specific markers and mature hepatic functions. The authors observed that in vitro the cells presented a chimerical phenotype after hepatocyte differentiation of BM-MSC, bearing both mesenchymal and hepatic markers. Interestingly, in vivo MSC-derived hepatocyte-like cells lost the chimerical phenotype, maintaining the expression of hepatic markers [124]. These data reinforced the concept that the liver microenvironment triggers a definite differentiation pathway toward hepatocytes.

More recently, Kazemnejad and colleagues reported the use of a 3-dimensional biocompatible nanofibrous scaffold to enhance hepatocyte differentiation of BM-MSC. The cells, grown in 2D and 3D conditions, were stimulated to differentiate by HGF, dexamethasone, and OSM for 3 weeks. The differentiated cells grown on 3D matrix showed increased expression of albumin, as well as transferrin, urea, glutamic oxaloacetic transaminase (GOT), and glutamic pyruvate transaminase (GPT) with respect to cells differentiated on a 2D culture system, therefore evidencing the influence of a biomimetic microenvironment in the differentiation process of MSC toward hepatocyte-like cells [119].

Bone marrow hematopoietic stem cells. Interestingly, contrasting reports indicate that bone marrow-derived adult hematopoietic stem cells (BM-HSC) can also undergo differentiation toward hepatocytes in rodents. In particular, Khurana and Mukhopadhyay demonstrated that HSC derived from bone marrow differentiated into hepatocytes when cultured in the presence of sera from mice with damaged liver [125]. In a contrasting report, Cho and colleagues recently compared the potential for regeneration of injured liver of both BM-MSC and BM-HSC. The authors demonstrated that only BM-MSC expressed AFP and CK-19, and showed migratory specificity toward CCl4-injured livers [126]. The shortness of differentiation protocol applied and the evaluation of few markers of hepatic differentiation suggest caution in interpreting the results and call attention to the need for further experiments to evaluate the ability of BM-HSC to exert liver-regenerating effects.

Adipose tissue-derived MSC. Several factors, such as ease of sourcing, the possibility to grow autologous cells for

transplant, and the differentiative abilities in vitro, favor the development of research on adipose tissue-derived MSC. Apart from the classical mesoderm-derived tissues (bone, cartilage, fat), adipose tissue-derived MSC have been shown to be able to differentiate toward both ectoderm-derived and endoderm-derived tissues [110,127].

Interestingly, many articles pointed out that MSC from adipose tissue present a differentiation potential similar to what is observed for BM-MSC. In particular, Seo and coworkers demonstrated that human adipose tissue-derived stem cells (hADSC) could differentiate into hepatocyte-like cells by exposing cells to various cytokines. In vitro, differentiated cells become able to uptake LDL and produce urea, functions typical of hepatocytes. In vivo experiments further demonstrated that after transplantation of differentiated hADSC in SCID mice, the number of albumin-expressing cells was higher than that achieved with undifferentiated cells [116].

More recently, Taléns-Visconti and collaborators carried out a study comparing BM-MSC and hADSC [105]. They highlighted that BM-MSC and ADSC showed a similar expression pattern of surface protein marker, as well as comparable hepatic differentiation potential. They also demonstrated for the first time that differentiated ADSC expressed drug-metabolizing enzymes such as CYP2E1 and CYP3A4.

In a recent report, Banas and colleagues showed that ADSC can be differentiated in vitro toward hepatocyte-like cells by a very short induction protocol, ameliorating liver functions when transplanted in vivo [110].

In a more recent work, Aurich and coworkers performed experiments in which adipose tissue-derived MSC (AT-MSC) were transplanted into liver of immunodeficient Pfp/Rag2$^{-/-}$ mice with versus without prior in vitro hepatocyte differentiation. The results demonstrated that human cells expressed albumin and HepPar1. Moreover, the authors showed that pre-differentiated AT-MSC underwent a more efficient engraftment of cells with respect to undifferentiated cells [128].

Umbilical cord blood-derived MSC. Kakinuma and coworkers demonstrated that UCB cells can be a source of transplantable hepatocyte-like cells. When investigating the phenotypical changes occurring in differentiated cells in vivo, the authors demonstrated the presence of human albumin in the sera of recipient mice [129].

More recently, Hong and colleagues showed further supportive in vitro experimental data of the hepatic differentiation potential of human UCB-derived mesenchymal stem cells (UCB-MSC). The functional properties of differentiated UCB-MSC were evaluated in terms of their ability to uptake low-density lipoprotein (LDL), while the expression of some hepatocyte-specific markers was assessed by RT-PCR and, at the protein level, by western blotting and immunofluorescence [130].

In addition, Kang and coworkers demonstrated that UCB-MSC should be differentiated toward urea-producing hepatocyte-like cells that were morphologically similar to the differentiated cells and were also able to store glycogen [104].

In a recent report, Jung and colleagues provided in vivo data on the effects of UCB-MSC transplantation in a cirrhotic rat model. The authors demonstrated that undifferentiated UCB-MSC, infused into CCl4-injured rats, homed to injured livers, expressed human albumin and AFP within 4 weeks after transplantation, and favored the recovery of liver function as demonstrated by the decrease of serum cirrhosis markers alanine aminotransferase (ALT) and aspartate aminotransferase (AST), as well as by the increase in serum total proteins and albumin. Moreover, both markers (α-smooth muscle actin and transforming growth factor β1) and extent of fibrosis were reduced in animals that received the MSC infusion [131].

Taken collectively, these data suggest that UCB-MSC could be a source of cells to be used in regenerative medicine applications for liver diseases, though more in vivo studies are required confirming the integration of differentiated cells in recipient organs, as well as the maintenance of hepatocyte-specific gene expression in vivo.

Extraembryonic tissue-derived MSC: Wharton's jelly. As stated earlier, bone marrow is the most acknowledged source of MSC, but it has been demonstrated that the number of MSC decrease depending on several donor-related parameters. Therefore, researchers have sought alternative sources such as extraembryonic tissues (amniotic membrane, umbilical cord, placenta). These constitute a practically unlimited source of rapidly dividing and easily expandable cells, without ethical issues, and with the possibility to reach a higher rate of compatibility between donor and recipient. Also in the field of hepatic regeneration, enormous progress has been made, rendering these cellular populations a promising candidate for liver-targeted regenerative medicine.

Very recent data showed that another source of MSC that can undergo hepatocyte differentiation is umbilical cord matrix. Campard and coworkers showed that umbilical cord matrix stem cell (UC-MSC) can differentiate toward endodermal cellular lineages. UC-MSC were cultured in a medium supplemented with factors promoting hepatic differentiation [108]. The initial population of UC-MSC expressed CK-8, CK-18, CK-19, was negative for CK-7, expressed G6Pase, PEPCK, α-1-antitrypsin (α-1AT), tryptophan 2,3-dioxygenase (TDO), and lacked HepPar1 positivity and HNF-4α or CYP3A4 expression. The UC-MSC-derived hepatocyte-like cells increasingly expressed markers such as tyrosine aminotransferase (TAT) and TDO, but remained negative for HNF-4 and HepPar1 antibody, therefore questioning the attainment of a mature hepatocyte phenotype, and leaving room for further functional improvements of the protocol [120]. Nevertheless, functional assays showed that differentiated cells responded well to the differentiative stimulus, being able to store glycogen, producing urea and possessing active hepato-specific enzymes (CYP3A4, G6Pase). Moreover, in vivo experiments showed that after transplantation of undifferentiated UC-MSC in liver of SCID mice with partial hepatectomy, the engrafted cells expressed human hepatic markers such as albumin and AFP, after 2, 4, and 6 weeks following transplantation. These data strongly suggest that also umbilical cord matrix-derived cells could be of great interest for the regenerative medicine approaches in liver diseases [108]. Interestingly, more recent data suggest a supportive role of undifferentiated human umbilical mesenchymal stem cells (HUMSC) in rescuing injured liver functions and reducing fibrosis in vivo. Tsai and coworkers infused undifferentiated HUMSC to rats which underwent CCl4 liver injury for 4 weeks. Then the rats were administrated with CCl4 for 2 more weeks. Compared with control group, HUMSC-infused rats showed lower levels of serum GOT and GPT, as well as a reduction of α-SMA and TGFβ1 in the injured livers, which correlated with an overall reduction of liver fibrosis

Table 2. Summary of Literature Reports Indicating the Differentiative Ability of Several MSC Populations Toward Hepatocyte-Like Cells

MSC population	*Hepatic differentiation protocol*	*Analysis of markers expression*	*Functional assays*	*References*
Human bone marrow-derived mesenchymal stem cells	Two steps—7 weeks protocol: Pre-conditioning: DMEM plus EGF and bFGF for 2 days	I. AFP, albumin, G6Pase, TO, CK-18, TAT, CYP2B6, HNF-4α by RT-PCR II. Positivity to monoclonal antibody 9B2 by FC and IF III. Albumin expression by IF	In vitro: LDL uptake analysis PROD Assay PAS stain for storage glycogen Urea production assay	[121]
Umbilical cord blood-derived mesenchymal stem cells	Serum-free IMDM, supplemented with HGF, bFGF, nicotinamide for 7 days Serum-free IMDM plus OSM, dexamethasone, ITS, thereafter	I. CYP2B6, HNF-4α by RT-PCR II. Albumin by IF	In vitro: LDL uptake analysis PROD Assay PAS stain for storage glycogen Urea production assay	
Adult BM-derived rat mesenchymal stem cells (BM-MSC)	Co-culture of rat BM-MSC with fetal rat liver cells Fibronectin matrix-coated plates One step—3 weeks protocol: Stem span SFEM medium, plus dexamethasone, SCF, HGF, EGF, FGF-4	Albumin, AFP, and CK-18 by RT-PCR	None	[122]
Rat bone marrow-derived mesenchymal stem cells	Co-culture of MSC with liver stellate cell (LSC) One step—3 weeks protocol: DMEM-LG, 10% FBS	I. Albumin, AFP, CK-18, GS, TAT, PEPCK, G6PD by RT-PCR II. Albumin, AFP, CK-18, PEPCK by real-time PCR III. Albumin, AFP, CK-18, CK-19, α-SMA by IF IV. Expression of HGF in LSC by RT and real-time PCR	In vitro: PAS stain for glycogen	[123]
Human bone marrow-derived mesenchymal stem cells	Collagen type I-coated dishes Two steps—4 weeks protocol: IMDM plus HGF, FGF-4, ITS, nicotinamide for 10 days IMDM plus OSM, dexamethasone, ITS for 20 days	I. Albumin, AFP, DPPIV, E-cadherin, connexin-32 by ICC II. Albumin by FC and ELISA III: Albumin, CK-8, G6Pase, AFP, α1-AT, PEPCK, TAT, TDO, c-met, vimentin, α-SMA, fibronectin by RT-PCR	In vitro: PAS stain for glycogen G6Pase activity assay Urea assay Gluconeogenesis assay In vivo: MSC transplanted in SCID mice. Expression of albumin, AFP, vimentin, fibronectin, CK-18	[124]
Human BM-MSC	2D or 3D culture conditions (biomimetic scaffold) Two steps—3 weeks protocol: One week plus DMEM-LG, 15% FBS, HGF, dexamethasone Two weeks with addition of OSM to differentiation medium	I. Albumin, AFP, CK-19, CK-18, CYP3A4 after both by RT-PCR II. Albumin and transferrin by ELISA; AFP by IF	In vitro: Evaluation of GOT, GPT, and urea synthesis	[119]
Adult hematopoietic stem cells (HSC)	Laminin, gelatin, and hyaluronic acid-coated plates One step—1 week protocol IMDM plus 10% serum of liver damaged mice	I. Albumin, CK-18 by ICC II. Albumin, HNF-3β, HNF-1α, HNF-4α, TDO, TAT, c-met, SCF, IL-6, Flt-3, OSM, HGF, EGF, FGF, TGF-α VEGF-α by RT-PCR III: IL-6, HGF, OSM by ELISA	In vitro: PROD assay In vivo: $Lin^{-}OSMR\beta^{+}$ cells or differentiated hepatic cells transplanted in FVB/J mouse. Expression of albumin and CK-18	[125]

Table 2. (Continued)

MSC population	*Hepatic differentiation protocol*	*Analysis of markers expression*	*Functional assays*	*References*
Human adipose tissue-derived stem cells (hADSC)	Fibronectin-coated dishes One step—4 weeks protocol DMEM-LG plus HGF, OSM, DMSO	I. Albumin, AFP by RT-PCR II. Albumin by ICC and IHC	In vitro: LDL uptake Urea assay In vivo: Transplantation of hADSC into NOD/SCID mice after CCl4 treatment. Albumin expression	[116]
Adipose tissue-derived stem cells (ADSC) Bone marrow-derived mesenchymal stem cells	Two steps—3 weeks protocol Pre-conditioning: DMEM plus EGF and bFGF for 2 days DMEM plus HGF, bFGF, nicotinamide for 7 days DMEM plus OSM, dexamethasone, ITS up to 21 days	I. Albumin, Thy-1, AFP, CYP3A4, CYP2E1, CK-18, CK-19, HNF-4α, C/EBPβ by RT-PCR II. Albumin and AFP by IHC	None	[105]
Adipose-derived stem cells (ADSC)	Collagen type I-coated dishes Three steps—3 weeks protocol: DMEM plus activin A and FGF-4 for 3 days HCM plus HGF, FGF-1, FGF-4, OSM, ITS, dexamethasone, DMSO and nicotinamide for 10 days HCM plus nicotinamide and dexamethasone for few days	I. Albumin, TDO, GAPDH, FOXA2 by real-time PCR II. Albumin by IF and ELISA	In vivo: ADSC-derived hepatocytes transplantation into mice with CCl4-induced injury Evaluation of ALT, AST, UA, and ammonia	[110]
Human umbilical cord blood cells (UCB cells)	One step—3 weeks protocol: Gelatin-coated tissue culture dishes DMEM plus 15% FBS, HEPES, monothioglycerol, FGF-1, FGF-2, LIF, SCF, HGF, OSM	I. Albumin, AFP, GS, CK-18, GAPDH by RT-PCR analysis II. Albumin, CK-18, CK-19, PCNA by immunofluorescent staining analysis III. Expression of albumin by immunohistological analysis	In vivo: UCB cells transplanted in liver-injured SCID mice. Albumin expression	[129]
Human umbilical cord blood-derived mesenchymal stem cells	Two steps—4 weeks protocol: IMDM plus 10% FBS, dexamethasone, ITS, HGF for 2 weeks IMDM plus 10% FBS, dexamethasone, ITS, OSM for subsequent 2 weeks	I. Albumin. AFP, CK-18, GS, TAT, HGF, c-met, PEPCK by RT-PCR II. Albumin, AFP, CK-18, and CK-19 by WB and IF III. Albumin by FISH and IHC	In vitro: LDL uptake analysis	[130]
Human umbilical cord blood-derived mesenchymal stem cells	One step—4 weeks protocol IMDM supplemented with 10% FBS, HGF, FGF-4	I. AFP and albumin expression by radioimmunoassay II. CK-18 by immunocytochemistry III. AFP, albumin, CK-18 expression by RT-PCR	In vitro: Urea production assay PAS staining for glycogen	[104]

(*Continued*)

Table 2. (Continued)

MSC population	*Hepatic differentiation protocol*	*Analysis of markers expression*	*Functional assays*	*References*
Umbilical cord matrix stem cells (UC-MSC)	Rat tail collagen type I-coated plates Three steps—3 weeks protocol: IMDM plus bFGF, EGF. for 2 days IMDM plus HGF, bFGF nicotinamide, ITS for 10 days IMDM plus OSM, dexamethasone, ITS for 10 days	I. Albumin, AFP, connexin-32, CK-8, CK-18, CK-19, DPPIV by FC II. Albumin by ELISA III. Albumin, α1-AT, AFP, connexin-32, CK-8, CK-18, CK-19, G6Pase, c-met, PEPCK, TDO by RT-PCR	In vitro: PAS stain for glycogen Glucose-6 phosphatase assay Urea assay Cytochrome P450 3A4 assay In vivo: UC-MSC transplanted in SCID mice after partial hepatectomy. Expression of albumin, AFP, fibronectin	[108]
Umbilical cord mesenchymal stromal cells (UC-MSC)	One step—3 weeks protocol: IMDM supplemented with 1% FBS, HGF and FGF-4	I. Albumin, AFP, CK-18 by IF II. Albumin, AFP, CK-18 by real-time RT-PCR III. Albumin, AFP, CK-18 by WB	In vitro: PAS stain for glycogen LDL uptake analysis	[135]
Umbilical cord mesenchymal stromal cells (UC-MSC)	Two steps—4 weeks protocol: DMEM/F-12 supplemented with HGF, bFGF, ITS+ premix for 16 days DMEM/F-12 supplemented with OSM, dexamethasone, ITS+ premix for 2 weeks	I. Albumin, AFP, CK-19 by IF II. Albumin, AFP, CK-19, G6P, TO by RT-PCR III. Albumin by ELISA	In vitro: LDL uptake analysis In vivo: UC-MSC transplanted in CCl4-injured NOD/SCID mice. Expression of human albumin by IHC	[136]
Human placenta-derived multipotent cells	Culture dishes untreated or coated with fibronectin or poly-L-lysine Two step—3 weeks protocol: 60% DMEM-LG, 40% MCDB201 plus ITS, linoleic acid, BSA, dexamethasone, ascorbic acid, EGF, PDGF-BB for 16 h Same medium plus HGF, FGF-4	I. Albumin, AFP, TAT, CYP3A4, GAPDH by RT-PCR II. Albumin, CK-18 by ICC III. AFP, positivity to anti-human hepatocyte by IHC IV. c-met, albumin, CK-18 by IF V. AFP by WB VI: CYP3A4 induction (RT-PCR) after rifampicin treatment	In vitro: LDL uptake PAS stain for glycogen	[137]
Human amniotic membrane-derived mesenchymal stem cells	Collagen type I-coated dishes One step—3 weeks protocol: α-MEM plus 10% FBS, hHGF, hFGF-2, OSM, dexamethasone	I. Albumin, AFP, CK-18, α1-AT, HNF-4α by RT-PCR II. Albumin, CK-18, AFP by ICC III. Albumin, AFP by IF	In vitro: PAS stain for glycogen	[138]
Human amniotic fluid-derived mesenchymal stem cells Human bone marrow-derived mesenchymal stem cells	Collagen gel type I-coated plates Three steps—3 weeks protocol: Days 0–2: basal medium plus FGF-4; Days 3–5: basal medium plus HGF; Days 6–18: basal medium plus HGF, ITS, dexamethasone, trichostatin A Phenobarbital was added 18 days after differentiation	I. Albumin, AFP, CK-18, HNF-1α, C/EBP, CYP1A1 by real-time PCR II. AFP, albumin, CK-18, HNF-1α, C/EBP, CYP1A1 by IF	In vitro: PAS stain for glycogen Urea assay	[114]

Table 2. (Continued)

MSC population	*Hepatic differentiation protocol*	*Analysis of markers expression*	*Functional assays*	*References*
Mesenchymal stem cells derived from human fetal lung	One step—3 weeks protocol: DMEM-F12 plus HGF, bFGF, EGF	I. AFP, albumin, CK-19, IL-6, M-SCF by RT-PCR II. Albumin, AFP, CK-19 by FC	None	[139]

Abbreviations: FGF, fibroblast growth factor; LIF, leukemia inhibitory factor; SCF, stem cell factor; HGF, hepatocyte growth factor; OSM, oncostatin M; AFP, α-fetoprotein; GS, glutamine synthetase; CK-18, cytokeratin-18; CK-19, cytokeratin-19; PCNA, proliferating cell nuclear antigen; ITS, insulin–transferrin–sodium selenite; TAT, tyrosine aminotransferase; c-met, HGF receptor; PEPCK, phosphoenolpyruvate carboxykinase; CPS, carbamoylphosphate synthase; α-SMA, α-smooth muscle actin; bFGF, basic fibroblast growth factor; TO or TDO, tryptophan-2,3-dioxygenase; CYP1A1, cytochrome P450 1A1 subunit; CYP2B6, cytochrome P450 2B6 subunit; CYP3A4, cytochrome P450 3A4 subunit; HNF-4α, hepatic nuclear factor-4α; Foxa2, Forkhead transcription factor a2; DPPIV, dipeptidylpeptidase-IV; α-1AT, α-1-antitrypsin; G6Pase, glucose-6-phosphatase; G6PD, glucose-6-phosphate dehydrogenase; C/EBP, CCAAT enhancer-binding protein; IL-6, interleukin-6; Flt-3, fetal liver tyrosine kinase-3; EGF, epidermal growth factor; FGF-4, fibroblast growth factor-4; TGF-α, transforming growth factor-α; VEGF-α, vascular endothelial growth factor-α; MSC, mesenchymal stem cells; BM-MSC, bone marrow mesenchymal stem cells.

[132]. These data support the hypothesis that, even in the absence of an actual transdifferentiation process, MSC from umbilical cord could exert a supportive action in increasing the functional recovery of recipient livers, perhaps stimulating the differentiation of endogenous parenchymal cells and promoting degradation of fibrous matrix [133].

In addition, recent data from another work by Yan and collaborators demonstrated in vivo engraftment of UC-derived MSC in livers of CCl4-injured mice by the expression of human AFP, TDO, and CK-18 14 and 21 days after cellular administration. Moreover, the authors demonstrated that engraftment of undifferentiated UC-MSC was followed by a decrease of hepatocytes apoptosis and an increase of hepatocytes proliferation, with respect to the control group [134].

More recently, Zhang and coworkers [135] reported the results of an in vitro study on UC-MSC differentiation by a one-step protocol using HGF and FGF-4. The differentiated cells expressed liver-specific markers (albumin and AFP), stored glycogen, and showed uptake of LDL, thus reinforcing the concept of their usefulness as cellular therapy tools for liver diseases.

In a very recent report, Zhao and collaborators [136] demonstrated that WJ-MSC maintain in vitro hypoimmunogenicity even after a hepatic differentiation protocol has been performed. In fact, differentiated hepatocyte-like cells, apart from expressing hepatocyte markers like G6P and TO in vitro, and albumin in vivo, did not express HLA-DR following 2 or 4 weeks differentiation in vitro, therefore demonstrating that the differentiative process did not exert any change on the immunological features of these cells. This datum is of key importance since it provides a molecular confirmation of the low-immunogenic phenotype of WJ-MSC in vivo.

Extraembryonic tissue-derived MSC: placenta. Chien et al. attempted to demonstrate that human placenta-derived multipotent cells (PDMC) can differentiate into endodermal hepatic lineage cells. After culture in medium supplemented with hepatic differentiation factors, PDMC switched their morphology from fibroblastoid to epitheliod, expressed albumin, CK-18, AFP, TAT, and acquired liver-specific bioactivities, including LDL uptake, glycogen storage, and rifampicin metabolization by CYP3A4. These interesting in vitro observations need to be followed by confirmative in vivo studies to characterize the engraftment ability as well as the maintenance of the differentiated phenotype in the diseased organ of animal model systems [137].

Extraembryonic tissue-derived MSC: amniotic membrane. Tamagawa et al. investigated by in vitro experiments the hepatic differentiation potential of mesenchymal cells derived from human amniotic membranes (MC-HAM). This study demonstrated that differentiated MC-HAM expressed albumin, AFP, CK-18, HNF-4α, and stored glycogen, but did not express G6Pase or ornithine transcarbamylase (OTC), markers of mature hepatocytes. Therefore, these data show that MC-HAM could differentiate into hepatocyte-like cells, but further studies should be carried out to analyze their hepatic function in vivo [138].

A recent comparative in vitro study between BM-MSC and human amniotic fluid-derived MSC (hAF-MSC) showed that the latter had higher proliferation capacity, greater hepatic differentiation potential, and were more genetically stable compared to the first ones. Functional assays showed that hAF-MSC-derived hepatocyte-like cells expressed liver-specific markers, produced urea and stored glycogen, all typical functions of mature hepatocytes [114].

Fetal tissue-derived MSC. In the last few years, greater attention has been focused on MSC derived from fetal lung. These cells were characterized as multipotent cells having even lower immunogenicity than adult MSC. Ling and coworkers demonstrated that fetal MSC derived from lung could differentiate into hepatocyte-like cells. In this study, fetal lung-derived MSC, in a specific differentiation medium, showed morphological features of mature hepatocytes and expressed markers as AFP and albumin [139]. Further studies should be carried out to better evaluate the functional features of differentiated cells as well as their engraftment success rate.

Concluding Remarks and Perspectives

The role of WJ-derived MSC as a promising tool for regenerative medicine applications has been suggested by a number of recent reports. While few doubts exist about their in vitro differentiation capabilities, more work is needed to translate into "in vivo" settings the results achieved.

Different methods of WJ-MSC isolation and different levels of the characterization procedure may underscore issues that can actually determine the success of a cellular therapy approach. The recent data that make it possible to identify the expression of tolerogenic molecules (as HLA-G) by WJ-MSC could constitute a reliable basis for obtaining cells that are able to evade the immune host response or can even induce immune tolerance in the host. Moreover, the identification of new markers of different embryo layers expressed by these cells opens new paths for regenerative medicine applications, for a number of organs throughout the body. In particular, we reported in this review that, similarly to other MSC populations, WJ-MSC show promising results in response to hepatic differentiation strategies, both in vitro and in vivo. Their ability to virtually cross all of the lineage borders separating the 3 germ layer derivatives further confirms the prospective reports on their usefulness in several cellular therapy applications. Even if more work is needed to fully understand the features of MSC (independently from their sourcing organ) in order to increase safety of future clinical applications, encouraging results are coming from the very recent experiments on hepatocyte-like differentiative ability of WJ-MSC. In fact, these cells are able to express a number of mature hepatocyte functions in vitro and contribute to ameliorating liver dysfunction when transplanted in recipient animals. Moreover, the global features of WJ-MSC, namely in vitro expandability, hypoimmunogenicity, and a success of isolation in nearly 100% of specimens, linked to the virtually unlimited availability of the starting tissue (which is still usually discarded at birth, with the exception of UCB) render the use of this cellular population of extreme interest in liver cellular therapy. As usual, caution should be applied in order to be sure about all the features of differentiated cells, in terms of achievement of a proper differentiation, absence of short-term as well as long-term side effects in the recipient host, and feasibility and advantages with respect to current therapeutical options.

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