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# Potential of Mesenchymal Stem Cells for Liver Regeneration

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## 1. Introduction

A wide variety of diseases, including cirrhosis, unresectable hepatic malignancy, ischemia, metabolic and auto-immune disorders, and hepatitis, whether caused by viral agents or drugs/toxins, can trigger hepatic insufficiency and failure, a life-threatening situation for which liver transplantation is the only definitive therapy [1-4]. Over 16,000 patients are currently awaiting the availability of a liver from a compatible donor [5], and many of these patients will die without ever receiving a transplant, due to the current shortage of available donor organs [6]. Furthermore, even when a patient is fortunate enough to find a compatible donor and receive a liver transplant, several factors can still thwart the ultimate success of this procedure. Operative damage, immune rejection towards the new organ, relapse of the pre-existing liver disease, and life-long side effects due to immunosuppression are among the most common complications [7, 8]. Furthermore, after liver transplantation, several long-term morbidities can arise, such as cardiovascular and retinal complications, lymphoproliferative disorders, and chronic renal failure [8-10]. Additionally, it is anticipated that the number of patients in need of liver transplantation will increase in the next decade, due to the obesity epidemic and the higher incidence of Hepatitis C infection. Therefore, new therapeutic approaches that can eliminate the need for partial or complete liver transplantation are urgently needed.

A valuable alternative to entire or partial liver transplantation is the delivery of cells capable of restoring normal organ physiology [11-19]. The use of cell therapy possesses several inherent advantages over organ transplantation: the procedure could be performed in a much less invasive way, the purified cell populations may be less immunogenic [20], and the use of autologous cells could be implemented [21].

Hepatocyte transplantation has been considered one of the most promising alternatives to liver transplantation, as these cells offer the benefit of being fully functional and are therefore able to quickly replace damaged hepatocytes upon delivery [22]. Also, the ability to cryopreserve and store hepatocytes gives the advantage of having a source of cells available when required. However, accessibility of hepatocytes at the required numbers for clinical intervention is still problematic, as human livers are required for their isolation, and

the harvesting and storing procedures are difficult and inefficient [23]. Additionally, differentiated hepatocytes have limited proliferation capabilities, and *in vitro* culture may alter their physiological and functional characteristics [24], making it difficult to obtain an adequate number of hepatocytes of sufficient quality for transplantation [25]. Further compounding these difficulties, it has been shown that, following transplantation, only a small percentage of the infused hepatocytes actually survive and durably engraft within the liver [26-30].

A better alternative to the use of adult hepatocytes is exploiting the presence, within the liver, of hepatic stem/progenitor cells (HpSCs), and the intrinsic ability of these cells to extensively expand, differentiate into all mature liver cells, and reconstitute the liver when transplanted, with minimal immunogenicity [31, 32]. HpSCs are found in the Canals of Hering in adult livers, and in ductal plates of the fetal liver. They can be easily isolated by immunoselection using an antibody against the epithelial cell adhesion molecule (EpCAM) and, they comprise approximately 0.5-5% of the liver parenchyma depending on age [33]. Although there is controversy regarding the cell surface markers that define this cell population, positivity for EpCAM, NCAM, or CD133, and negativity for AFP are currently considered to be the most accepted markers. The potential of these cells has been clearly demonstrated in numerous murine studies, and a few studies in humans have confirmed the presence and regenerative properties of HpSCs in the presence of viral hepatitis, cirrhosis or inborn metabolic disorders [34-37].

In the last decades, alternative sources of stem cells have raised great hope for improving the treatment of liver diseases. In particular, the demonstration that cells within the bone marrow contributed, at different levels, to liver parenchymal cells opened the possibility of using autologous cells to treat liver disorders/diseases [38-52]. Amongst these, mesenchymal stem cells (MSC) have been considered an ideal cell source because of their ease of isolation and expansion, their immunomodulatory properties, and their broad differentiation potential [53, 54].

## 2. Mesenchymal stem cells

Mesenchymal stem cells (MSC), also referred to as marrow stromal cells or stromal precursor cells, were first described in the 1960s by Friedenstein, and shown to belong to the bone marrow stromal microenvironment that supports hematopoietic stem cells and controls the process of hematopoiesis [55]. These cells were also shown to be able to differentiate into multiple lineages of mesenchymal tissues, including bone, cartilage, fat, tendon, and muscle [56-58]. Numerous culture methods and purification procedures such as plastic adherence, Ficoll gradient centrifugation, or cell-sorting using surface markers have all been used to enrich for bone marrow-derived MSC, with each laboratory preferring its own method of isolation. This makes the comparison of results obtained by various laboratories very difficult, since each lab is likely studying somewhat different cell populations, despite the fact that all of these cells have collectively been referred to as MSC. According to the International Society for Cellular Therapy, MSC should have several characteristics in addition to adherence to plastic. They must express CD105, CD73, CD90, but not express CD45, CD34, CD14, CD11b, CD79 or CD19 and HLA-DR surface markers. Furthermore, they must be able to differentiate into osteocytes, chondrocytes, and adipocytes [59]. Although MSC constitute a very small percentage of the nucleated cells

present in the BM, between 0.001 and 0.01%, these cells can be expanded exponentially while maintaining their original phenotype and differentiation potential, making it possible to easily obtain adequate numbers for cell-based therapies. These characteristics make MSC ideal agents for cell replacement therapies

## 2.1 Properties of Mesenchymal stem cells

In keeping with the original findings of Friedenstein, MSC are still most often isolated from the bone marrow. In humans, these “BM-MSCs” are usually collected from the superior iliac crest of the pelvis; however, they can also be obtained from the tibial and femoral marrow compartments [60], and the thoracic and lumbar spine [61]. In larger animals, BM-MSCs are isolated from the same areas. In contrast, in small animals such as mice, a bone marrow aspiration is not possible, so BM-MSCs are harvested by flushing the mid-diaphysis of the tibia or femur [61].

In stark contrast to hematopoietic stem cells, MSC can easily be expanded in culture for many passages without losing their phenotype or pluripotency capability [60]. Indeed, Bruder et al. demonstrated that human BM-MSCs can readily be propagated *in vitro* until passage 38±4; after that passage the cells turn flat and very broad, indicating they have reached senescence [62]. Moreover, by plating these cells at a low density and consistently passaging them before they have reached confluence, it is possible to accelerate their growth rate and increase their expansion capacity [63].

Over the past several years, studies have provided compelling evidence that MSC's differentiation capacity far exceeds that originally reported by Friedenstein. Indeed, *in vitro* and *in vivo* transplantation studies have now shown that MSC have the capacity to differentiate not only into mesodermally-derived cell types such as bone [64], cartilage [65], tendon [66], muscle [67], cardiomyocytes [68] and adipose tissue [69], but, even more remarkably, can also give rise to cells that developmental biology classifies as being derived from ectoderm (neurons and astrocytes [70, 71]) and endoderm (pancreatic beta cells [72] and hepatocytes [73]). This extraordinary multipotentiality has generated a great deal of interest in applying MSC to tissue repair/regeneration as well as cell therapy approaches for a variety of diseases/injuries.

In addition to their broad differentiation potential, MSC also appear to possess the intrinsic ability to migrate, or home, to sites of injury following systemic infusion. Importantly, from the standpoint of developing a clinically viable and safe cell-based therapeutic, MSC appear to selectively engraft and differentiate into tissue-specific cells that are missing or defective due to the disease in question, while contributing very little, if at all, to normal/healthy tissue [74-77]. For the past several years, scientists have attempted to elucidate the mechanism by which MSC are selectively attracted to sites of injury. During pathological conditions, several cytokines/chemokines are produced, which will stimulate MSC to express: 1) integrins, by which MSC will bind to endothelial cells, and 2) cytokine/chemokine receptors, by which MSC will migrate towards the inflammatory site. This complex network of signaling allows MSC to establish cell-cell contact and mediate rolling with endothelial cells. Additionally, they also transmigrate into the extracellular matrix by interacting with integrins and fibronectin stimulated by MSC-secreted ligands. Despite these insights, however, more information is required for a complete understanding

of this process. This understanding could then be used to develop means of enhancing MSC engraftment after transplantation [78].

The ability of MSC to reprogram to cells specific to other organs/tissues suggested that MSC would have to replace a significant percentage of the damaged cells within a diseased/injured organ to exert a beneficial effect. However, controversy arose in the MSC field when a series of studies were published demonstrating a reproducible therapeutic improvement in the absence of detectable MSC engraftment. These findings sparked additional studies that have now shown that MSC can also mediate tissue repair by acting as “trophic factories”, releasing specific cytokines and growth factors that modulate the activity of tissue-specific cells, suppress local inflammation, and inhibit fibrosis and apoptosis, thereby facilitating endogenous tissue regeneration [79]. Adding to the complexity of the functions/effects of MSC, it was recently discovered that MSC can transfer mitochondria or mitochondrial DNA to cells that have been damaged by ischemia and reperfusion. By transferring mitochondria or mitochondrial DNA, MSC can rescue the cells that have non-functional mitochondria, rescuing these cells and enabling regeneration of the tissue [80]. In recent years, it was also shown that MSC express an array of miRNA's, small non-coding RNA's that are involved in gene regulation [81]. It is believed that a single miRNA can regulate several different target genes and a single gene can be regulated by multiple miRNA's. Studies to date have provided evidence that miRNA's are involved with stem cell differentiation, hematopoiesis, immune response, neurogenesis, stress responses, and the development of skeletal and cardiac tissue [82-84]. These regulatory miRNA's have now been shown to be present inside microvesicles that are secreted by MSC, which are then transferred to neighboring cells to regulate their activities. This pathway provides yet another means by which MSC can communicate with injured cells. Following secretion of the microvesicles, the miRNA's contained therein can then enter the injured cell and induce differentiation and/or production of soluble mediators, and stimulate cell-cycle re-entry; the net result of these myriad actions being tissue regeneration [85].

Upon arrival at the site of injury, MSC also fulfill another vital function, which is to modulate the inflammatory microenvironment present within the damaged/diseased tissue. MSC possess an extraordinary ability to modulate immune cells, exerting these effects by releasing soluble factors and by cell-cell contact. MSC are known to inhibit proliferation and maturation of cytotoxic T cells, helper T cells, B cells, dendritic cells, and NK cells, as well as to inhibit NK-mediated cytotoxicity. These broad-ranging actions enable MSC to interfere with each component of the adaptive immune system. MSC can also stimulate the differentiation of Tregs, which can further dampen the immune response. MSC are known to release a host of soluble factors, which have been associated with their immunomodulatory properties including transforming growth factor- $\beta$ , prostaglandin-E2, inducible NOS, nitric oxide, IL-10, HLA-G, hepatocyte growth factor, and indoleamine 2,3-dioxygenase. By dampening the ongoing inflammation and/or aberrant immune reaction present within the damaged/diseased tissue, MSC facilitate the process of repair/recovery, further adding to the promise of using these cells for regenerative medicine [78].

In addition to the inherent properties of MSC that make them well suited for cellular therapy, it is important to realize that MSC can easily be genetically manipulated *in vitro*, with both viral and non-viral vectors, to enhance their immunosuppressive properties [86, 87], to deliver a protein that is missing/defective in the patient, to induce apoptosis of tumor

cells, to promote cell proliferation, to guide their migration to a specific site within the body, and even to direct their differentiation towards a specific cell lineage [88-90], making the range of clinical applications for which MSC could be used almost limitless.

## 2.2 Sources of Mesenchymal stem cells

In addition to their presence in the bone marrow, these MSC have also been identified in, and isolated from, several other tissues including cord blood (CB-MS), cord matrix (hWJSCs), amniotic fluid (AF-MS), placenta, adipose tissue (AT-MS), brain, liver, lung, and kidney [91-93]. The presence of these cells in several organs/tissues raises the possibility that they could have a crucial function in organ homeostasis, and/or repairing the tissue, and suggests that MSC isolated from these tissues may have a unique transcriptional or proteomic signature that renders these cells biased in terms of homing or differentiation towards the organ of origin. Differences also exist in the cytokine/chemokine molecules produced by MSC from various sources and in their differentiation capabilities [94, 95]. Using fetal liver MSC (FL-MS) as an example to illustrate these differences, FL-MS exhibit much more rapid growth kinetics than BM-MS, due, at least in part, to a greater abundance of transcripts involved in cell cycle regulation, DNA repair and chromatin regulation. In addition, analysis of telomerase activity and telomere length revealed that fetal liver MSC telomeres are longer and these cells possess greater telomerase activity than adult sources of MSC. As a result, these cells are more expandable and they become senescent later in culture [96]. Fetal liver MSC also express more primitive genes, such as Oct-4, Nanog, and SSEA-3 than their adult counterparts, but transcripts involved in differentiation towards more mature cells are reduced relative to MSC from other sources. More importantly from the standpoint of clinical utility, fetal liver MSC also exhibit reduced immunogenicity compared to adult BM-MS, perhaps due to expression of higher levels of HLA-G1 [97]. In addition to reduced immunogenicity, fetal liver MSC also demonstrated an enhanced immunomodulatory function than BM-MS when tested for their ability to inhibit T cell proliferation [98]. Despite all the promising characteristics of these cells, very few studies have examined their utility/potential *in vivo*. In one of these studies, rabbit fetal liver MSC were tested for their engraftment, proliferation and differentiation capabilities following *in utero* transplantation. Two routes of administration were analyzed, intrahepatic and intra-amniotic. Both approaches were safe for both the mother and the fetal recipient, but only the intrahepatic route resulted in the formation of donor-derived hepatocytes in the liver. While the levels of hepatocyte production were low, the engraftment persisted for at least 16 weeks after transplantation [99].

Despite their many unique characteristics and promise of offering MSC primed for repair of specific tissues, the inherent difficulty in obtaining organ-specific MSC such as those derived from liver, will likely preclude their widespread use in a clinical setting. Ideally, for cellular therapies, one would like a readily available source of cells that could be used as off-the-shelf therapeutics. MSC are present in significant numbers in discardable tissues such as cord blood, placenta and amniotic fluid, and these MSC have the ability to be expanded and frozen without loss of viability or differentiative potential, making MSC from these tissues an attractive option. Indeed, both AF-MS [100] and CB-MS [101] were shown, upon transplantation *in vivo*, to give rise to hepatocytes, suggesting they have definite potential as cellular therapeutic for treating liver diseases. Having considered these two very different

MSC examples, a summary of the properties of the main sources of MSC that are currently being tested for therapeutic purposes appears in **Table I**, to provide a better overall picture of the similarities and differences inherent to MSC isolated from various tissues.

MSC Cells	Purification	Special Characteristics	Advantages	Disadvantages
<b>BM- MSC</b>	Aspiration from the iliac crest, concentration by Ficoll gradient and adhesion to plastic. Number of cells obtained: 1-10 CFU/10 <sup>6</sup> MNC (0.01-0.001% of MNC)	- Positive for cell surface markers: CD105, CD73, CD90, CD29, CD44, CD166, STRO-I. - Negative for cell surface markers: CD34, CD45.	- Easy to expand in vitro up to 15 passages with minimal spontaneous differentiation. - Easy to cryopreserve. - Higher capacity for osteogenic differentiation compared to AD-MSC - Cells can be isolated from the patient, modified and injected back into the same patient.	- Invasive and painful extraction. - The number of cells obtained is small. The amount decreases with the patient's age, the presence of osteoporosis, and exposure to chemotherapy or radiation, and varies with gender.
<b>CB- MSC</b>	Collection of umbilical cord blood, purification of MNC by Ficoll gradient and concentration by adhesion to plastic. Number of cells obtained: 0-2.3 clones/10 <sup>8</sup> MNC (1.10 <sup>3</sup> -5.10 <sup>3</sup> cells/sample)	- Express lower levels of CD90 and CD105 compared to BM-MSC and AT-MSC. - Express hematopoietic genes at higher levels, and consequently higher levels of growth factors than BM-MSC.	- Umbilical cord is discarded at birth; therefore, it represents an untapped source of MSC that can be obtained non-invasively. - Longer survival and expansion in vitro compared to BM-MSC and AT-MSC.	- Low frequency, difficulties and inconsistency in isolation. Only MSC can only be successfully isolated from 10-30% of the collected cord blood samples. - Difficult to differentiate into adipocytes, compared to BM-MSC and AT-MSC. - Slow to differentiate into chondrocytes in vitro, compared to BM-MSC and AT-MSC.
<b>AT- MSC</b>	Lipoaspiration of the fat tissue, collagenase treatment to separate the stromal cell populations and concentration by adhesion to plastic. Number of obtained cells: 1% of adipose cells are MSC.	- Positive cell surface markers: CD49. - Negative cell surface markers: CD106, STRO-I.	- More abundant than BM-MSC or CB-MSC. - Faster proliferation in culture compared to BM-MSC. - More easily differentiable to adipocytes. - Large amounts of fat are discarded during liposuction; therefore, large availability of cells for cell bank. - Similar immunosuppressive ability to BM-MSC. - Cells can be isolated from the patient, modified and injected back into the same patient.	- Do not differentiate well into chondrocytes. - More heterogeneous population than BM-MSC after purification; however, the cells can be sorted to increase homogeneity. - Differentiate less actively to osteoblasts compared to BM-MSC and CB-MSC. - Differentiate more easily to adipocytes compared to CB-MSC.
<b>AFS</b>	Amniocentesis, concentration by adhesion to plastic and selection by c-kit. Number of obtained cells: 0.9-1.5% of cells in the amniotic fluid are MSC.	- Similar surface markers to BM-MSC but lower expression of CD44 and CD105. - Express primitive cell marker: SSEA4. - Express more primitive genes: Oct-4 and Nanog.	- Higher proliferation rate than BM-MSC due to long telomeres. - Even though they express more primitive markers, expansion in vitro and in vivo is not associated with chromosomal instability.	- Less ability to differentiate into chondrocytes than BM-MSC. - Sorting for c-kit should be done to eliminate abundant skin cells derived from fetus.
<b>hWJSCs</b>	Extraction of Wharton's Jelly from umbilical cord, collagenase and hyaluronidase treatment, and final disruption of the tissue by a needle. Number of obtained cells: 4.10 <sup>5</sup> cells per sample; 10-15.10 <sup>3</sup> cells per centimeter of cord (usually 15cm/sample).	Express primitive cell marker: SSEA4. - Express more primitive genes: Oct-4 and Nanog.	- Umbilical cord is discarded at birth; therefore, it represents an untapped source of MSC that can be obtained non-invasively. - Higher frequency of MSC than BM or cord blood. - MSC can be isolated from all samples, in contrast to CB-MSC. - Extensive and faster proliferation compared to BM-MSC. - Similar immunosuppressive ability to BM-MSC. - Higher potential to differentiate into chondrocytes than BM-MSC.	- Sorting strategies are encouraged as the population of cell obtained from the Wharton's Jelly is heterogeneous. - Less potential to differentiate into adipocytes than BM-MSC.

Table 1.

## 2.3 Mesenchymal stem cells for treating liver disease

### 2.3.1 *In vitro* models to study Mesenchymal stem cell differentiation

One could convincingly argue that the best way to study the differentiation potential of MSC is to perform studies *in vitro*, as studies of this nature allow for creation of a carefully controlled microenvironment which greatly facilitates the delineation of the pathways/mechanisms by which MSC commit to specific lineages and undergo reprogramming. In contrast to *in vivo* studies, where the researcher has little or no control over the myriad local and systemic cues and factors present within the recipient, performing *in vitro* studies enables the researcher to definitively establish the true multipotential capability of MSC at the single cell level, or at the level of a clonally-derived population. Indeed, *in vitro* studies have now revealed that MSC are able to transdifferentiate into cells of the three germ layers, including neuronal and glial cells [102-105], cardiomyocytes [106-110], endothelial cells [111-113], and insulin-producing beta cells [114, 115]. The discovery of this tremendous potential has prompted researchers to perform microarrays studies to understand the molecular mechanisms responsible for the commitment and differentiation of MSC along each of these lineages [116-118]. It is hoped that understanding these pathways will pave the way for the development of methods for efficiently driving MSC differentiation down specific lineage pathways to create the cell type required for therapy. These studies also provided vital information regarding key genes and signaling pathways that are directly involved in maintaining MSC in an undifferentiated state, helping to characterize this cell population and providing clues as to methods for expanding these cells for longer periods of time while maintaining their multipotency.

While these findings were exciting and highlighted the vast potential of MSC for cellular therapy, the most important ability, from the standpoint of therapies for the liver, would be the ability to differentiate into hepatocytes. Accordingly, several protocols have now been developed for the *in vitro* differentiation of both murine and human BM-MSCs into hepatocytes [1, 119-125]. These MSC-derived hepatocytes exhibit the same morphology and antigenic profile as native hepatocytes, and they appear to be functional, based upon uptake of low-density lipoprotein, urea production and storage of glycogen. These initial findings with BM-MSCs have now been extended to include MSC derived from adipose tissue, amniotic fluid, CB, and Wharton's Jelly, with adipose-derived MSC showing the greatest propensity to differentiate *in vitro* to putative functional hepatocytes. It was initially hypothesized that CB-MSCs might harbor an innate capacity to differentiate into hepatocytes, since they constitutively express early as well as more mature hepatic markers and functions [126]. However, this initial assumption was not realized. After several studies, it became clear that CB-MSCs differentiate only partially, displaying early and some mature hepatic markers/functions but lacking the expression of other proteins that are critical for liver development [123, 126]. While this discovery initially reduced the enthusiasm for the use of CB-MSCs as therapeutics for liver disease, it is important to realize that the immature nature of the hepatocytes they form could still enable them to treat disorders such as metabolic liver disease, in which generation of fully functional mature hepatocytes is not required, as long as the transplanted cells produce adequate levels of the missing/defective enzyme for correction. This limited differentiation capacity does, however, likely preclude their use for treating conditions such as acute hepatic failure.



Only a few groups have analyzed *in vitro* differentiation of hWJSCs. Zhang et al applied a one-step protocol with HGF and FGF-4 and found that, after 21 days, cells expressed hepatocyte markers such as Albumin, AFP, and CK-18 [127]. In other studies, Lin et al. induced differentiation of the cells by co-culturing them with mice liver tissue previously treated with thioacetamide, a chemical used to induce chronic fibrosis of the liver. Two days after induction, hWJSCs expressed hepatic markers, providing evidence that, with the appropriate stimuli, hWJSCs can very rapidly reprogram to adopt a hepatocytic fate. AF-MSCs were also tested for their ability to differentiate *in vitro* into hepatocytes. The differentiation process employed by these investigators consisted of two steps: first, the MSC were treated for 1 week with EGF and FGF to commence induction along the hepatic lineage; and second, a maturation step, during which the cells were treated with dexamethasone and oncostatin-M for 2 weeks. The MSC-derived cells obtained at the end of this 2-stage induction protocol expressed several hepatic markers/functions, including albumin production, uptake of low density lipoproteins, glycogen storage, and urea production, promoting the investigators to cautiously refer to them as hepatocyte-like cells [100, 128, 129].

Collectively, the results of these *in vitro* studies provide compelling evidence that MSC derived from a variety of sources all possess the ability to give rise to what appear to be functional hepatocytes, albeit at varying levels. This suggests that MSC could represent viable cellular therapeutics for treating liver disease, and thus provide a much-needed alternative to whole or partial liver transplantation.

### 2.3.2 *In vivo* models to study Mesenchymal stem cell differentiation

Despite all the knowledge that can be gained from performing *in vitro* studies, they are inherently limited by the need to supply all of the requisite factors to observe the desired differentiation or reprogramming. This becomes problematic when one wishes to discover/investigate novel properties of MSC, since, in most cases, these factors are not yet known. Adding to this problem is the lack of suitable assays to rigorously establish that the "hepatocyte-like cells" generated *in vitro* are, in fact, bona fide hepatocytes that perform all of their required physiologic functions. For this reason, scientists are forced to resort to *in vivo* transplantation studies in the hopes that the required mediators/factors conditions are present within the microenvironment of the target organ, and can coax the transplanted MSC to reprogram towards the desired cellular fate. Performing studies *in vivo* also has the advantage of ensuring that all of the appropriate cues are present to influence migration and homing of MSC to the tissue/organ in question; an essential issue to consider if the ultimate goal is to develop therapies using MSC. Transplantation *in vivo* also provides the opportunity to examine the ability of the MSC-derived cells to seamlessly integrate into the existing cytoarchitecture and adopt appropriate behavioral characteristics. Ideally, studies of this nature would be performed with human MSC and their derivatives, to ensure the clinical translation of the results obtained. Due to ethical and practical issues, however, studies of this nature can clearly not be performed in human subjects. Thus, at the present time, investigators can only test the ability of human stem cells to engraft/differentiate within a xenogeneic setting, using suitable small or large animals as recipients.

#### 2.3.2.1 Mesenchymal stem cells differentiate *in vivo* into hepatocytes

The exciting *in vitro* findings discussed above suggested that MSC could serve as cells for repairing the injured or failing liver. Importantly, MSC can be grown quite readily in culture

for extended periods of time without any seeming loss of differentiation capacity. This has two important implications for their use in cellular-based liver therapies. The first of these is that a very small marrow aspirate could be taken from the patient and adequate cells obtained for transplantation, through extensive expansion *in vitro* following isolation. Secondly, by virtue of their ability to be expanded in culture without loss of *in vivo* potential, MSC could be harvested from the patient's own marrow even if the liver disease present was the result of an underlying genetic defect, since MSC are quite amenable to genetic modification/correction using a wide range of viral and non-viral vector systems. Following genetic manipulation, a pure population of genetically corrected autologous MSC could thus be propagated to generate sufficient numbers of cells to achieve meaningful levels of engraftment following transplantation. Based on these promising characteristics, MSC have now been tested in a wide variety of injury/disease model systems for their ability to generate hepatocytes and correct these liver defects. Using MSC isolated from a variety of mouse, rat, and human tissues, investigators have now provided evidence that MSC can mediate varying degrees of correction/repair of the liver following injury due to partial hepatectomy [126, 130-133], treatment with the toxin CCl<sub>4</sub> [134-145], injury induced by allyl-alcohol [146, 147], and treatment with 2-acetylaminofluorene [139].

Unfortunately, these studies are confounded by the problem of each group of investigators using MSC defined in different ways, ranging from specific antigen profile to simple plastic adherence. The use of differing definitions for "MSC" can likely explain, at least in part, the differing outcomes, even when using a similar injury model system. One thing that is quite clear from these studies looked at as a whole, however, is that MSC appear to be able to exert beneficial effects in a wide range of injuries and disease states within the liver. Another issue that needs clarification is whether fusion plays a major role in the beneficial effects, since the fusion of donor MSC with host hepatocytes has not yet been addressed in detail in any of these injury/disease models.

Another issue that has complicated interpretation of the data generated from these studies in liver, as well as those conducted looking at the potential of MSC to mediate repair in other organ systems, is the observation that a therapeutic benefit is often observed in the absence of any evidence of engraftment of the transplanted MSC within the damaged organ. Instead, it appears that the transplantation of MSC somehow stimulates the host's liver to repair itself without the donor cells actually having to persist long-term within the recipient. These findings led to a great deal of debate as to whether MSC can actually generate hepatocytes or if, perhaps, all the effects they produce are simply mediated through release of soluble factors. Meticulously executed *in vitro* studies have now provided definitive evidence that MSC can under appropriate conditions be reprogrammed into cells with all of the characteristics of functional hepatocytes that can currently be assessed in culture [37, 135, 148-151]. Thus, it is now presumed that if these hepatocyte induction protocols work well in cultured MSC, *in vivo* organ-specific microenvironment of the recipient liver is likely to be even better suited for inducing the transplanted MSC to differentiate into hepatocytes. Therefore, it seems safe to presume that the beneficial effects of MSC thus far observed in animal injury models have been mediated, at least in part, by MSC differentiating to hepatocytes.

However, the other capabilities of MSC cannot be ignored and may be equally important in the observed therapeutic effects. A variety of evidence from animal studies has now indicated that both MSC's direct differentiation and their indirect effects through secretion

of factors which stimulate the regeneration of endogenous cells are likely to play important roles in promoting tissue recovery [79, 152-156]. In support of this conclusion, MSC were shown to provide significant therapeutic benefit during acute hepatic failure by releasing chemotactic cytokines that reduce leukocyte infiltrates and hepatocytes death and increase hepatocyte proliferation [156, 157]. For example, recent studies by Tsai et al. showed that the direct injection of MSC into rats with CCl<sub>4</sub>-induced liver fibrosis resulted in a significant reduction in the liver fibrosis. However, although MSC engrafted, they did not differentiate into albumin-producing cells, but secreted cytokines that promoted liver regeneration and thereby restored liver function [144].

In addition, other studies have now revealed an additional property of MSC that may indicate that they are ideally suited for treatment of liver diseases involving fibrosis: the ability to enhance fibrous matrix degradation, likely through the induction of metalloproteinases [136, 158-164]. Moreover, other researchers have found that MSC are able to prevent liver fibrosis by suppressing the function of activated hepatic stellate cells, inducing their apoptotic death and diminishing collagen synthesis [155]. Studies like those by Lin et al. have shown that MSC may utilize multiple mechanisms to exert their effects, both engrafting and differentiating into albumin-producing cells, and producing metalloproteinases that significantly reduced the collagen deposits in a rat model of chronic liver fibrosis [165]. However, these promising results must be interpreted carefully and with tempered enthusiasm, because other studies have suggested that under different conditions, transplanted MSC may actually contribute to the myofibroblast pool and thus enhance the fibrotic process within the liver [159, 166-169]. This has led to the current feeling within the field that the effect of MSC will probably vary with the nature of the liver injury/disease that is being treated, the specific experimental model in which the therapy is being tested, and perhaps even the time frame of MSC application, such that MSC could be beneficial if administered at certain stages of disease progression and harmful if administered at other stages. Thus, it appears that the therapeutic potential of MSC may have to be investigated for each specific disease/injury to be treated to delineate the optimal time frame and population to be administered to achieve the desired effect, ensuring they provide benefit rather than harm.

### 2.3.2.2 The fetal sheep model

All of the afore-mentioned studies exploring the therapeutic properties of MSC in model systems generated by inducing an external stress, such as chemical- or radiation-induced injury or by depleting a specific cell type in the recipient, e.g., partial hepatectomy, have provided compelling evidence that MSC represent valuable cellular therapeutics for liver disease. However, they have also revealed that whether MSC will exert their beneficial or harmful effects is dictated largely by the presence of activated cells and the microenvironment within the injured or diseased organ at the time of transplantation. What is clear is that the microenvironmental conditions that surround MSC play a crucial role in determining the fate adopted by MSC *in vitro* and *in vivo*. The cloning of Dolly the sheep certainly represents the most dramatic example of the power that the microenvironment can exert on cell fate [170]. The microenvironment, in this case the cytoplasm of an enucleated egg, induced the nucleus taken from an adult somatic cell to completely reset its developmental gene expression clock and reveal its true potential. To fully exploit the vast therapeutic potential of MSC, a deep understanding of the mechanisms that control the cell

fate and their efficient application to drive differentiation towards the hepatocytic lineage are urgently needed; such understanding will require an adequate model system.

The ideal experimental model would allow transplantation of human MSC, which could then engraft and differentiate/reprogram under normal physiological conditions, in the absence of injury/insult. Additionally, such a model should allow the generation of a broad spectrum of differentiation states of the donor-derived cells in the desired tissue at adequate levels to enable delineation of the mechanisms that participated in their generation. Irrespective of the source of donor cells and mechanisms involved in reprogramming, however, the first key step for proper function is for the cells to reach the target organ. The circulatory system provides an efficient stem cell distribution system throughout life. During fetal life, a series of well-established migratory processes, likely employing the circulatory system, ensure that adequate numbers of appropriate stem/progenitor cells reach the target tissues/organs when needed. This carefully regulated migration is accomplished by the dynamic expression of an array of adhesion molecules and release, by the tissue, of specific chemokines/chemoattractants that alert the circulating stem cells when and where they are most needed. Once the stem cell reaches the target tissue, the permissive milieu induces the entering stem cells to proliferate and differentiate to produce the required type(s) of cells. The existence of this highly permissive milieu is very likely associated with the continuous need for new cells during fetal development.

With these permissive aspects of the developing early gestational-age fetus in mind, we reasoned that it might represent a perfect platform in which to study the properties of human MSC. The transplanted MSC could piggy-back on the naturally occurring migratory pathways, and thus be efficiently disseminated throughout the fetus to the various developing tissues. Once there, they would then be naturally influenced by the host proliferation/differentiation environment to adopt a specific cellular fate, assuming that the transplanted cells harbor the potential in question. By performing the transplant at a point in development when all the organs had begun to differentiate but there was still a need for exponential growth and differentiation, we hypothesized that the fetal milieu would support the possibility of reprogramming of cellular fate through a bombardment of proliferation/differentiation stimuli without forcing the transplanted cells to adopt a specific fate by damaging/inducing regeneration within a particular organ. If the supposition that the appropriate microenvironmental influence can induce a cell with a mature phenotype to regress into an undifferentiated state, directly reprogram a cell to an alternate fate, and/or induce a primitive stem cell to start differentiating into a new lineage, then the fetus should represent an ideal model system in which to examine the full potential of MSC, and other adult stem cells.

In addition to providing a unique signaling environment that can drive migration and differentiation of the transplanted MSC, the fetus also represents a unique recipient from an immunological perspective. In contrast to other model systems routinely used to study stem cell transplantation and the therapeutic potential of MSC, the fetal sheep recipient has an immature, but functioning immune system. In early immunologic development, before thymic processing of mature lymphocytes, the fetus appears to be largely tolerant of foreign antigens [171, 172]. Therefore, if the transplant is performed at the appropriate stage of development, the fetus is able to support the engraftment/differentiation of MSC (and other adult stem cells) in the absence of irradiation or other myeloablative therapies. Furthermore, exposure to foreign antigens during this period often results in sustained tolerance, which

can become permanent if the presence of antigen is maintained [100, 173]. By taking advantage of this so-called “window of opportunity” and performing the transplant during the “pre-immune” stage of development, it is possible to reach significant levels of allogeneic sheep cells and xenogeneic human cells within the fetal sheep, in the absence of irradiation or other myeloablative therapies [174-180], to create a lifelong chimera [181], and induce stable, donor-specific immune tolerance.

In addition to the unique characteristics of the fetus itself as a recipient, there are several additional advantages of selecting sheep as an animal model: 1) sheep are fairly close in size to humans during development and throughout life, which should greatly facilitate, or even eliminate the need for, scale-up of the protocol for clinical human therapies once promising results have been obtained in the fetal sheep model, 2) the physiology and developmental processes are similar and therefore, sheep have been for decades the model to study normal fetal growth and fetal abnormalities [182-185], 3) in contrast to mice and rats, sheep are outbred, and thus present a diverse genetic background, just like humans, 4) the development of sheep immune system has been extensively studied and it closely parallels that of humans [186-194], 5) the long lifespan and large size allows the study of cellular fate in the same animal for several years after transplantation, which provides critical answers about long-term efficacy and safety of the therapy in question. Collectively, these properties make the fetal sheep an ideal model in which to test the therapeutic potential of MSC and obtain results that could readily be translated into clinical studies.

#### 2.3.2.2.1 Results obtained in fetal sheep model

In order to investigate the *in vivo* differentiation potential of human MSC in the absence of injury/selective pressure, we isolated several clonal MSC populations from adult BM by magnetic sorting, using an antibody against Stro-I [195]. Although the antigen recognized by this antibody has not yet been identified, we found that by triple-labeling BM cells with antibodies against Stro-I, CD45 and GlyA and selecting for Stro-1+CD45-GlyA- cells, we can reliably obtain a homogenous population that is highly enriched, both phenotypically and functionally, for MSC. This selected population has therefore been used for all of our studies to examine human MSC differentiative potential.

To rigorously test whether MSC could generate significant numbers of hepatocytes *in vivo*, we examined the ability of clonally-derived human MSC from adult BM to generate functional albumin-producing hepatocytes *in vivo* following transplantation into fetal sheep recipients, comparing two routes of administration, intraperitoneal (IP) and intrahepatic (IH) [40]. Human hepatocytes formed after transplantation of BM-MSC into fetal sheep were then identified by HEP-1 staining, coupled with human-specific fluorescence *in situ* hybridization. Our results showed that, although MSC efficiently generated significant numbers of hepatocytes by both routes of administration, the IH injection resulted in a 5-fold increase in the number of hepatocytes generated, when compared to the IP route ( $12.5\% \pm 3.5\%$  versus  $2.6\% \pm 0.4\%$ ) [196]. In addition to higher levels of hepatocytes, the route of cell administration also exerted a marked effect on the pattern of distribution of the generated hepatocytes. Sheep that received an IP injection exhibited a preferential periportal distribution (in acinar zone 1) of donor-derived hepatocytes that produced high levels of albumin [40], while IH-transplanted animals contained donor-derived (human) hepatocytes dispersed throughout the parenchyma (acinar zone 2) that expressed minimal amounts of albumin. Previous results have demonstrated that localization of the hepatocyte within the

liver is strictly associated with the levels of synthesis of certain plasma proteins, such as albumin. Hepatocytes localized in the periportal area of the liver produce higher levels of albumin, compared to hepatocytes situated in other lobular zones [197-199]. These studies thus provided compelling evidence that MSC represent a valuable source of cells for liver repair and regeneration and demonstrate that, by altering the site of injection, generation of hepatocytes occurs in different hepatic zones, and the resultant hepatocytes exhibit differing functionality, just like their naturally-occurring counterparts. These results are highly relevant for designing a potential cellular therapy for liver regeneration, as depending on whether the overall goal of the therapy is to provide hepatocytes to restore the liver architecture or to achieve normal levels of a secreted therapeutic protein into the circulation, different routes of injection would likely be needed. However, if one wishes to achieve functional repopulation of the liver, it is possible that a transplantation approach combining both routes of administration would be the most effective.

In other studies, we evaluated the ability of MSC derived from the fetal kidney to form hepatic cells *in vivo* and *in vitro* [200]. Like their BM counterparts, these cells gave rise to significant numbers of human albumin-producing hepatocyte-like cells upon *in utero* transplantation into fetal sheep. Furthermore, after culture in specific inducing media, cells with hepatocyte-like morphology and phenotype were obtained, suggesting that metanephric-derived MSC could also serve as a source of cells with hepatic repopulating ability. Similar results were also obtained in the fetal sheep model, using a novel, adherent MSC-like cell population isolated from umbilical cord blood, which the authors termed unrestricted somatic stem cells, or "USSC" [201]. This cord blood-derived MSC population was capable of giving rise to albumin-producing human parenchymal hepatic cells at levels of >20% in the recipient liver, in the absence of any injury or genetic defect. Importantly, cell fusion was not required for hepatocyte formation in any of these studies, demonstrating that, at least in this model, human MSC isolated from several different sources all had the ability to directly reprogram to functional hepatocytes.

Another key aspect to assessing the utility of stem cell therapy for regenerative medicine for the liver, and for other organs as well, is the mechanism whereby the transplanted cells replace/repopulate the recipient liver [40]. Indeed, there has been a great deal of controversy about the mechanism by which MSC reprogram and differentiate into other cell lineages, such as hepatocytes. Several researchers have shown that cell fusion could be one of the mechanisms by which MSC appear to give rise to hepatocytes, rather than true reprogramming/transdifferentiation [202]. Furthermore, evidence suggests that the means by which the transplanted MSC contribute to the recipient liver is strictly dependent on the model system employed. For example, an animal model in which proliferation of endogenous hepatocytes has been arrested, such as those using chemical-induced injury, will require replication of the transplanted cells and therefore, favoring transdifferentiation of the transplanted MSC. On the other hand, in an animal model that promotes proliferation of endogenous and MSC-derived hepatocytes, both mechanisms are possible, but fusion seems to be favored.

Using the fetal sheep model made it possible for us to show that MSC could give rise directly to cells within the liver without the need for first forming hematopoietic elements [41]. In more recent studies, we have now shown that the ability to directly contribute to liver repopulation without the need for a hematopoietic intermediate enables the transplanted MSC to rapidly begin contributing to the growing liver, producing cells with

hepatic markers within as little as 24 or 48 hours post-transplantation [41]. The findings of these more recent studies confirmed our prior findings regarding the lack of a need for fusion, and furthered our understanding of the mechanism of hepatic repopulation by demonstrating that the generation of hepatocytes occurs independently of the transfer of either mitochondria or membrane-derived vesicles between the transplanted donor cells and the cells of the recipient liver [41]. These findings thus provide strong evidence to support genetic reprogramming and differentiation of the transplanted stem cells. The lack of fusion as a requirement for liver repopulation was in contrast to the results of numerous other studies employing injury models, raising the possibility that the efficacy and mechanism of stem cell repair will likely depend upon not only the stem cell population being transplanted, but also the nature of the injury/defect within the liver, and therefore the conditions within the hepatic microenvironment at the time of stem cell transplantation.

Therefore, we performed studies to begin delineating the mechanism(s) of hepatocyte formation following transplantation of human MSC in the fetal sheep model, which we felt would be ideal for this analysis given the robust generation of human-derived hepatocytes. We labeled human BM-MSC with CFSE, which irreversibly stains the plasma membrane [203, 204], or DiD, which labels all cell membranes, membrane-derived vesicles, and intracellular organelles such as mitochondria [205-207]. Consequently, pre-immune fetal sheep were IP injected with either CFSE-positive MSC alone or CFSE-positive MSC in combination with DiD-positive MSC. After transplantation, peripheral blood and peritoneal lavage were assayed for the presence of the cells. At 20h post-transplant, cells were already present in the peripheral blood, and all transplanted cells had exited the peritoneum by 96h. Confocal microscopic analysis for the presence of CFSE+ or DiD+ cells revealed that the transplanted cells initially appeared in the liver at 25h post-transplant, and their numbers then increased, reaching a maximum at 40h post-transplant. The next step was to evaluate if the cells, once in the liver, commenced proliferation before or after initiating differentiation towards tissue-specific cells. At all time points after transplantation, 95% of the CFSE+ or DiD+ cells were also positive for Ki67, indicating that the cells had already begun, or simply continued, to proliferate upon arrival to the liver. These results confirmed that the higher levels of the cells observed at later time points was likely due to the proliferation of the initial MSC that engrafted in the liver and not a result of more cells engrafting in the organ. These studies have important clinical implications, since they suggest that, independently of the low initial percentage of MSC engraftment into a certain tissue, the real contribution of the cell to that tissue does not only depend on the initial engraftment levels but also on the tissue's intrinsic proliferative capacity. Following engraftment of transplanted cells into the liver, hepatoblasts were generated that, due to their intrinsic proliferative capacity [208], continued proliferating and further contributing to the chimeric tissue [196]. In contrast, if one were developing a therapy for which the transplanted cell needed to differentiate into a quiescent cell, such as a terminally differentiated neuron, the contribution of that cell to the tissue would be limited to the initial levels of engraftment.

We next examined the timeline of MSC differentiation into organ-specific cell types in the liver, identifying differentiation of the transplanted cells by their simultaneous positivity for CFSE or DiD and  $\alpha$ -Fetoprotein, since during normal fetal liver development, hepatocytes acquire the expression of this protein [209, 210]. At 25h post-transplant, cells that were positive for CFSE or DiD, were already expressing  $\alpha$ -fetoprotein, indicating that the transplanted MSC were not only present in the tissue at this first time point of analysis, but

they were already differentiated into a hepatocyte-like phenotype. These results thus showed, for the first time, that transplanted MSC engraft within the recipient liver, proliferate, and rapidly commence hepatocytic differentiation. To begin unraveling the mechanism by which MSC seemingly gave rise to hepatocytes in the fetal liver, we performed fluorescence in situ hybridization (FISH) using a human- and a sheep-specific probe, coupled with confocal microscopy for the CFSE or DiD labels. The complete lack of hybridization of the nuclei of CFSE+ or DiD+ cells to the sheep probe conclusively demonstrated that the transplanted human MSC gave rise to hepatocytes independent of fusion or membrane vesicle/organelle transfer, and by true reprogramming/transdifferentiation [211]. In fact, we observed a sequential differentiation program, in which cells gradually expressed markers of differentiation, from the most undifferentiated cell to the mature fully differentiated cell type in the organ in question. Understanding the complete pathway of differentiation could ultimately make it possible to provide a cell driven to a precise point in differentiation to correct of a disease by providing exactly the cell type most needed.

Despite the significance of our findings in the fetal sheep model, it is important to note that, even when using an optimal route of injection, the overall levels of liver engraftment may still be too low to achieve cure in many clinical situations. While the fetus has long been presumed to be immune-naïve, recent studies in mice have suggested that this may not be the case, since syngeneic hematopoietic stem cells engraft at higher levels than allogeneic cells of the same phenotype following *in utero* transplantation. Thus, it is possible that some rudimentary immune surveillance exists within the fetus and limited the levels of engraftment within the liver. MSC are well known for their immune-evading and immunomodulatory properties, but studies in murine and swine models have provided evidence that MSC are not completely invisible to the recipient's immune system, nor immune-inert. Indeed, upon *in vivo* administration, MSC are able to trigger immune responses, resulting in rejection of the transplanted cells [212-216]. Based on these prior studies, we hypothesized that further reducing the immunogenicity of the MSC prior to transplant might enable us to achieve even higher levels of engraftment and hepatocyte generation, both in this "pre-immune" fetal model and, perhaps, even in recipients with a more developed/mature immune system. To test this hypothesis, we genetically modified human MSC to stably express proteins known to exert potent immunomodulatory/immune-evading properties. The proteins we selected were derived from the ubiquitously prevalent human cytomegalovirus (HCMV). This virus is well known to possess multiple immune evasive strategies, which enable it to enter a state of latency in which it is invisible to immune surveillance, only to re-emerge when conditions are favorable, such as during the period of immuno-suppression following bone marrow or solid organ transplant, and wreak havoc on the immuno-compromised patient. HCMV accomplishes its immuno-evasion due largely to its unique short region (US) proteins. We therefore used a retroviral vector to genetically modify MSC to stably express members of the HCMV US protein family that are known to specifically reduce cytotoxic T cell recognition by different mechanisms, and compared the immunogenicity and immunomodulatory properties of these "US-MSC" to unmodified MSC and to MSC transduced with an empty control vector. Our results revealed that MSC expressing US6 (MSC-US6) and US11 (MSC-US11) exhibited the most pronounced reduction in HLA-I expression and accordingly, induced the lowest level of human or sheep PBMNC proliferation in mixed lymphocyte reactions. Moreover, as there are controversial reports



regarding whether reduction in HLA-I expression by HCMV US proteins renders infected cells more susceptible to NK killing [217, 218], we next examined whether forced expression of US6 or US11 predisposed MSC to NK lysis. To our surprise, expression of US6 or US11 protein did not increase the ability of NK cells to target MSC. Moreover, expression of US11 actually protected MSC from NK cytotoxic effects [219]. Based on these promising *in vitro* results, we transplanted MSC-US6, MSC-US11 and MSC-E (control cell line transduced with the empty vector) into fetal sheep recipients by IP injection. Tissues were collected at 60 days post-transplant and analyzed for engraftment and hepatocytic differentiation of the transplanted cells. Using both quantitative PCR and immunofluorescence, we determined that expression of either the US6 or the US11 HCMV protein on the transplanted MSC led to significantly enhanced levels of liver engraftment compared to those seen with MSC-E. However, although the increased levels of engrafted cells translated into increased levels of cells expressing HEPAR-I, many of these did not express albumin or Ov-6 [220]. This suggests that the hepatocytes generated by transplantation of these genetically modified cells were of a broad range of differentiation, not immature and not completely mature at the time of tissue collection. These results clearly show that by enhancing the immuno-evasive MSC properties, the levels of engraftment and hepatocyte generation can be significantly increased to provide a more successful regenerative therapy, even in the context of a fetal recipient whose immune system is presumed to be largely immature.

### 3. Clinical trials

Despite the promising results obtained in animal models, the use of MSC to treat liver diseases is still in its infancy, and very few clinical trials using these cells have been performed. Several concerns still exist over this therapy regarding the best administration route, and the possibility of cellular fusion, with the inherent risks that may accompany the presence of hepatocytes that are potentially genetically unstable within the environment of a diseased liver. In 2007, Mohamadnejad *et al.* reported that infusion of BM-derived MSC via a peripheral vein was found to be well tolerated and to have a definite therapeutic effect, since the quality of life of all 4 transplanted patients was improved by 12 months post-infusion, and the model for end-stage liver disease (MELD) scores for 2 of the 4 patients improved significantly during the course of the trial.

Another 8 patients with end-stage liver disease due to different etiologies received 30-50 million BM-derived MSC injected into a peripheral vein or the portal vein. Treatment was well tolerated by all patients, and liver function improved as verified by MELD scores. However, both of these trials lacked a control arm, and the number of patients was very small. Another study examining the safety and efficacy of umbilical cord-derived MSC (UC-MSC) in 45 patients with decompensated liver cirrhosis demonstrated that both patients that received UC-MSC and those in the control arm that received saline suffered no significant side-effects or complications. However, in patients treated with UC-MSC there was a significant reduction in the volume of ascites when compared with control. Also, UC-MSC therapy significantly improved liver function, as evidenced by the increase of serum albumin levels, decrease in total serum bilirubin levels, and decrease in the sodium model for end-stage liver disease scores [151]. Forty patients with end-stage liver failure due to chronic hepatitis C were selected for a controlled study in which 10 received autologous

bone marrow-derived mesenchymal stem cells that were pre-induced to the hepatic lineage *in vitro* prior to transplant. Three groups were included in this trial: one in which 10 patients received the MSC by an intrasplenic route, another in which the 10 patients received cells by intrahepatic route, and a control group consisting of the remaining 20 patients. Patients in all groups were followed up using clinical and laboratory parameters and evaluated by MELD scores, fatigue scale, and performance status. Both transplanted groups, regardless of administration route, showed significant improvement when compared to the control [172]. Another phase 1 trial, in which four patients with decompensated liver cirrhosis were included, demonstrated that, after infusion of approximately 32 million bone marrow derived MSC through a peripheral vein, MELD scores of 2 patients improved by the end of follow-up as well as the quality of life of all four patients [173].

Collectively, these studies provide hope that BM-derived cells may prove to be a valuable resource for cell-based therapies for liver disease. However, the results of these studies must be interpreted with some trepidation, given the limited number of patients enrolled in each trial and the lack of appropriate controls in some of the studies. Furthermore, since the cells in these trials were autologously-derived, there was no way for the investigators to assess the actual engraftment, persistence, or differentiative potential of the transplanted cells, leaving the mechanism responsible for the observed clinical improvements open to speculation.

#### 4. Conclusion

Presently, chronic liver disease constitutes one of the leading worldwide causes of death. It can be triggered by a wide array of insults, including, but not limited to hepatitis infection, alcohol consumption, exposure to toxic chemicals, and congenital defects. Currently, the only definitive treatment for chronic liver disease is whole or partial liver transplantation. Due to the limited availability of donor livers and the severe morbidity and mortality associated with this treatment, there is an urgent need for new therapeutic approaches. While hepatocyte transplantation represents an option, the limited availability of donor livers and the inability to maintain and expand hepatocytes in culture precludes this option from becoming a clinically viable treatment option. MSC offer several advantages such as: extensive expansion *in vitro*, multipotent differentiative capacity, the ability to selectively and efficiently migrate to sites of injury following systemic infusion, their potent immunomodulatory and trophic properties, and the ease with which they can be genetically modified, making it possible to use autologous cells, even in the case of underlying genetic disease. MSC can be isolated from a wide range of human tissues and, despite subtle differences, they all share the same beneficial characteristics, making MSC transplantation a promising approach for liver repair/regeneration. However, in order to maximize MSC capabilities for improving/recovering the liver mass and/or function depending on the particular disease/injury, several issues must still be resolved: selection of the most therapeutic MSC source; standardization of the protocols for unequivocally isolating the desired MSC population from each tissue; more complete *in vitro* and *in vivo* characterization of the differentiative potential of the cells; and further optimization of the route, cell dose, timing, and degree of desired MSC differentiation. Once these questions have been answered, the knowledge gained during *in vitro* and *in vivo* studies in animal models could be safely and efficiently translated into humans to develop an appropriate and successful therapy for chronic liver disease.

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## 6. References

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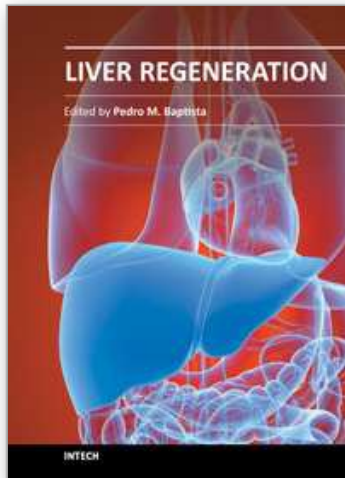
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## **Liver Regeneration**

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Doctors and scientists have been aware of the "phenomenon" of liver regeneration since the time of the ancient Greeks, illustrated by the mythic tale of Prometheus' punishment. Nevertheless, true insight into its intricate mechanisms have only become available in the 20th century. Since then, the pathways and mechanisms involved in restoring the liver to its normal function after injury have been resolutely described and characterized, from the hepatic stem/progenitor cell activation and expansion to the more systemic mechanisms involving other tissues and organs like bone-marrow progenitor cell mobilization. This book describes some of the complex mechanisms involved in liver regeneration and provides examples of the most up-to-date strategies used to induce liver regeneration, both in the clinic and in the laboratory. The information presented will hopefully benefit not only professionals in the liver field, but also people in other areas of science (pharmacology, toxicology, etc) that wish to expand their knowledge of the fundamental biology that orchestrates liver injury and regeneration.

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