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Characterization and evaluation of hepatic differentiation potential of human umbilical cord mesenchymal stem cells

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1. INTRODUCTION

1.1 Anatomical features of human liver

The liver, the largest gland in the body, is situated in the upper and right parts of the abdominal cavity, occupying almost the whole of the right hypochondrium, the greater part of the epigastrium, and not uncommonly extending into the left hypochondrium. In the male it weighs from 1.4 to 1.6 kg, in the female from 1.2 to 1.4 kg. Its shape must be compared to a wedge, the base of which is directed to the right and the thin edge toward the left. The liver possesses three surfaces: superior, inferior and posterior. The superior surface is attached to the diaphragm and anterior abdominal wall by a triangular or falciform fold of peritoneum, the falciform ligament; the line of attachment of this ligament divides the liver into two parts, termed the right and left lobes, of different size. The inferior and posterior surfaces are divided into four lobes (quadrate, caudate, right and left lobe) by five fossae, which are arranged in the form of the letter H.

Each lobe is subdivide into lobules, liver morphofunctional units. The lobules (*lobuli hepatis*) form the chief mass of the hepatic substance; they are tiny hexagonal or pentagonal cylinders, measuring from 1 to 2.5 mm in diameter. The bases of the lobules are clustered around the smallest radicles (*sublobular*) of the hepatic veins, to which each is connected by means of a small branch which issues from the center of the lobule (*intralobular*). The remaining part of the surface of each lobule is imperfectly isolated from the surrounding lobules by a thin stratum of areolar tissue, in which is contained a plexus of vessels, the interlobular plexus, and ducts.

Each lobule consists of hepatic cells arranged in irregular radiating columns between which are the blood channels (*sinusoids*). These convey the blood from the circumference to the center of the lobule, and end in the intralobular vein**,** which runs through its center, to open at its base into one of the sublobular veins**.** Between the cells are also the minute bile capillaries. The *hepatic cells* are polyhedral in form and they contain one or sometimes two distinct nuclei. The cells usually contain granules; some of which are protoplasmic, while others consist of glycogen, fat, or an iron compound [1].

Approximately 80% of the total liver mass, consists of hepatocytes, the rest is composed by endothelial cells, Kupffer cells, stellate cells, liver natural killer cells and cholangiocytes.

Hepatocytes placed at the periphery are named as periportal cells while those located centrally are named as pericentral, perivenular or centrolobular ones. On the basis of spatial distribution of these cells there is also functional compartimentalization. In fact, periportal hepatocytes are specialized in glycogenolysis and gluconeogenesis and removal of ammonia by urea, while centrolobular hepatocytes are active in glycolysis and glycogen synthesis and metabolize ammonia by glutamine synthetase [2].

In conclusion, the liver induces conversion of the extra glucose in the body into stored glycogen in liver cells, and then converting it back into glucose when the need arises; liver produces bile (a substance necessary in the of fats) and blood clotting factors and amino acids. Moreover, the liver performs processing and storage of iron necessary for red blood cell production; synthesis of cholesterol and other chemicals required for fat transport; it converts ammonia in urea which is then excreted in the urine; and it executes xenobiotic detoxification.

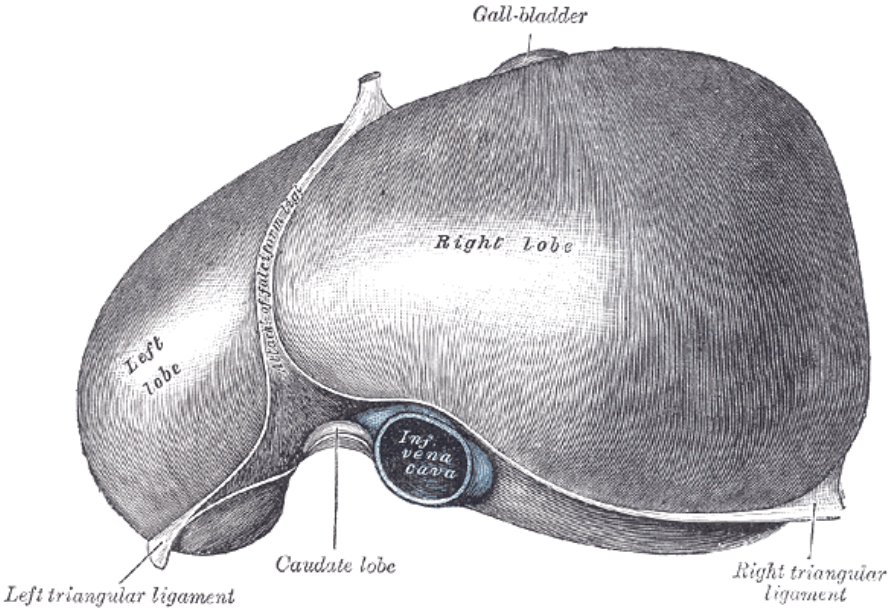


Figure 1: superior surface of liver (Grey's Anatomy, 1918)

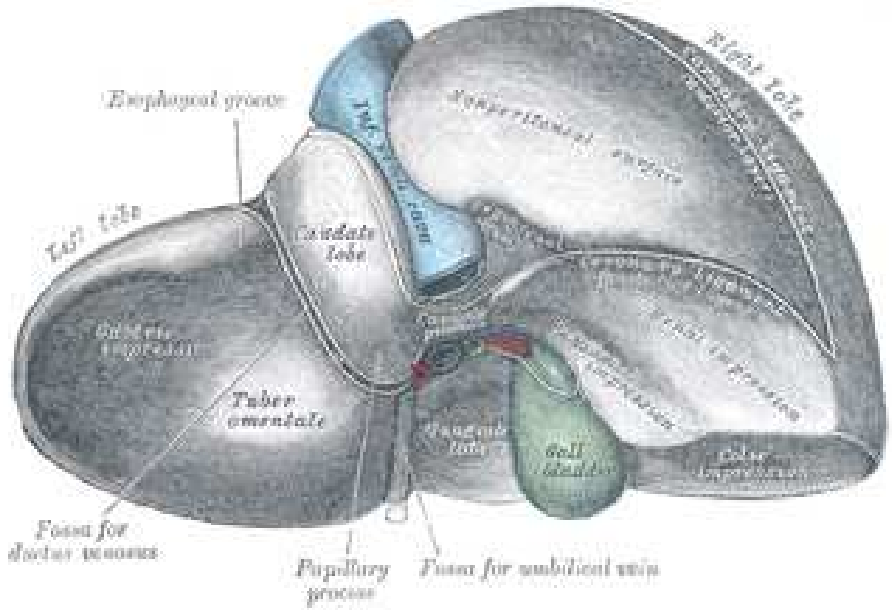


Figure 2: posterior and inferior surfaces of the liver (Grey's Anatomy 1918)

1.2 Overview of development of the liver and involved factors

The liver arises as a diverticulum from the ventral surface of the gut. This diverticulum is lined by entoderm, and grows upward and forward into the septum transversum, a mass of mesoderm between the vitelline duct and the pericardial cavity, and there gives off two solid buds of cells which represent the right and the left lobes of the liver. The solid buds of cells grow into columns or cylinders, termed the hepatic cylinders, which branch and anastomose to form a close meshwork. This network invades the vitelline and umbilical veins, and breaks up these vessels into a series of capillary-like vessels termed sinusoids, which ramify in the meshes of the cellular network and ultimately form the venous capillaries of the liver. By the continued growth and ramification of the hepatic cylinders the mass of the liver is gradually formed [1].

Many investigations were carried out on different animal species to understand which mechanisms act in early development of the liver. Advanced evidence reported that development requires a series of inductive signals from three cellular types: mesodermal cardiogenic cells, cells of the septum trasversum and endothelial cells.

In embryo mouse, around stage of 7-8 somites, the ventral wall is adjacent to cardiac mesoderm that releases fibroblast growth factors (FGFs) required to initiate differentiation toward hepatic fate [3].

Serl et al demonstrated that ventral endoderm explants in absence of FGF express pancreatic mRNA: when FGF was added at different concentrations, ventral endoderm expressed hepatic mRNA. Therefore, these data highlighted importance of FGF during early hepatic development [4]. The ventral endoderm responds to this induction phase, generating the primary liver bud. Outgrowth of this structure is induced by bone morphogenetic protein-4 (BMP-4) released from septum trasversum cells. The septum transversum mesenchyme cells are tightly associated with the cardiac mesoderm and, therefore, could contribute to the initial stage of hepatic induction as well [5].

The liver bud, after the induction mediated by FGF and BMP, expresses several liver mRNAs, including *Albumin*. By E9.5, the basement membrane surrounding the liver bud is broken, and cells delaminate from the bud and invade the

surrounding septum transversum mesenchyme, which is the source of stellate cells as well as sinusoidal endothelial cells that begin to form vessels.

Around hepatoblast stage, the cells have bipotential capacity since can differentiate to both cholangiocytes and hepatocytes [6].

Liver development is peculiar since between 11.5 and 16.5 embryonic days, the liver is also a hematopietic site. Studies demonstrated that oncostatin M, secreted by hematopoietic stem cells, controls late hepatic differentiation probably by increasing HNF 4α expression [7].

HNF 4α is involved in transforming the fetal liver into epithelial parenchima regulates hepatocyte differentiation, and is essential for maintaining sinusoidal architecture. During mouse embryonic development, aroud 14.5 embryonic day, HNF- 4α is necessary for expression of junction proteins and adhesion molecules which, within the developing hepatoblasts, facilitate the formation of bile canaliculi and convert the immature cells into a polarized hepatocyte [8].

1.3 Liver regeneration and therapeutic applications for liver diseases

When liver is subjected to the partial hepatectomy (HP) , the hepatocytes undergo one or two replicative cycles to restore loss hepatic mass, but if HP is higher than 70%, hepatocytes do not proliferate more and reach a state known as “quiescent senescence”.

The term “regeneration” imply re-growth of excised structure.Actually, in the liver, damaged cellular mass doesn’t reconstitute, rather unharmed cells expands to compensate the lost tissue. Therefore, it is more corrected to speaks about compensatory process.

Several studies tried to explain what mechanisms underlie this compensatory process. It is clear that cytokines, growth factors and metabolic networks interact together to support liver regeneration [9].

When replicative ability of hepatocytes is blocked or delayed, some cells known as oval cells, replicate and differentiate in hepatocytes.

Many studies were carried out to understand origin, characteristics and differentiative potential of these cells.

The oval cells originate together with intrahepatic biliary cells from hepatoblasts located near portal spaces. Analysis of maker expression suggested that these cells have a bipotential capacity because express hepatic and biliary markers. In addition, studies demonstrated that oval cells express typical markers of hematopietic stem cell such as Cd34, CD45, Sca-1 [10].

Unfortunately, in case of liver disease, where there is loss of liver function, the regeneration mediated by proliferative capacity of mature hepatocytes or in some cases of oval cells is no more sufficient.

In the world exists hundreds of millions of people affected by liver pathologies.

The most common causes of hepatopathy are chronic hepatitis C and B, alcoholism, nonalcoholic fatty liver disease, autoimmune, and drug-induced hepatic disorders. Many of these conditions, if not prevented and treated , can lead to progressive liver injury, liver fibrosis and ultimately cirrhosis, portal hypertension, liver failure and sometimes cancer [11].

Acute liver failure consists in a rapid decrease in liver function. Commons causes are viral hepatitis, acetaminophen and mushroom ingestion, idiosyncratic drug reactions.

In case of *autoimmune-associated disease* the body produces an inappropriate immune response against itself; and sometimes against liver tissue. PBC (Primary biliary cirrhosis), PSC (Primary sclerosing cholangitis) and autoimmune hepatitis are some possible causes.

Budd-Chiari syndrome is characterized by presence of blood clots that impede correct blood flow. The major causes are hypercoagulable disorders, liver injury, cancer and parasitic infection

Hemochromatosis, Alpha-1 antitrypsin deficiency, Wilson's disease are some genetic diseases which result in alterations of some liver functions.

Cirrhosis, consists in the loss hepatic function because liver architecture is compromised. Possible causes are chronic hepatitis, alcoholism, or chronic bile duct obstruction.

To date, organ transplantation remains an excellent therapeutic treatment for liver pathologies. More than 5000 liver transplants, each year, are performed in USA. About 20,000 people wait for liver transplantation, but only 7,000 transplants are

performed annually, therefore 1,500 patients die yearly while on the waiting list. Adverse factors such as donor scarcity, high costs and consequences from long-term immunosuppressant therapies limit the potential of organ transplantation. An alternative treatment could be hepatocytes transplant: it is defined such as “the use of living cells to restore, maintain, or enhance tissue and organ function” [12].

Cell transplantation is a less invasive and easier procedure, hepatocytes may be cryopreserved for future applications, risks associated with transplant rejection are be limited and subsequent orthotopic liver transplantation or liver directed gene therapy would remain feasible [13].

If liver architecture is not damaged, infusion of hepatocytes may be performed into the portal vein or into the splenic pulp, from where the cells migrate to the liver [14]. Certainly, the presence of physiological matrix helps engrafted hepatocytes to replicate. When, instead the liver architecture is damaged it is preferable to infuse the hepatocytes into ectopic sites such as the spleen offering so a site for long term survival [15].

To date hepatocytes therapy was performed only on three categories of liver diseases such as acute liver failure , inherited metabolic diseases and cirrhosis. Acute liver failure causes rapid deterioration of liver functions, for these reasons cell therapy could provide to restore the main functions such as metabolism of toxins, secretion of proteins and stabilisation of haemodynamic parameters. According to first studies hepatocytes transplantation in patients with acute liver failure were performed into splenic artery or the portal vein [16-17-18].

Successively, accessibility of the peritoneal cavity and intraperitoneal transplantation of hepatocytes was considered a promising strategy to create a bridge to spontaneous regeneration of the liver. Indeed, infused hepatocytes in peritoneal zone had a short life therefore cell transplantation with alginate embeding or microcarriers may offer a reasonable alternative. A report, in fact, demonstrated that use of microcarrier-attached hepatocytes into rats with total hepatectomy improved long-term survival rates [19]. Inherited metabolic liver diseases are other diseases where hepatocytes transplantation had encouraging results. In a girl with Crigler-Najjar syndromeType I with hyperbilirubinaemia, infusion of hepatocytes into portal vein, resulted in partial correction plasma

bilirubin levels for more than 11 months [20]. Another success was obtained in a woman with glycogen storage disease [21] and in a 4-year-old girl with infantile Refsum disease [22].

Cell therapy in end stage liver diseases such as cirhhosis, is certainly more problematic, because the loss of functional hepatocytes contribute to the decrease of liver function. Above all considering that liver architecture is damaged, cell transplantation into hepatic sites may be questionable. Therefore infusion of hepatocytes in the spleen is considered a valid alternative. Studies on animals with stable liver cirrhosis induced after 4 weeks of administration of carbon tetrachloride, demonstrated that rat or porcine hepatocytes [23] or syngeneic rat hepatocytes [15] or immortalized rat hepatocytes [24] infused into splenic sites improved liver function and increased survival rate. A few clinical applications were performed in cases of decompensated chronic liver disease. Certainly, use of microcarrier or supports for hepatocytes transplantation in advanced cirrhosis could prolong survival of engrafted hepatocytes [25].

Despite hepatocyte transplantation has given encouraging results, cadaveric livers scarcity, limited replicative potential of these cells, damages induced from cryopreservation and elevated number of cells necessary for transplantation, limit its potential. Therefore several groups investigated on therapeutic potential of hepatocyte-like cells derived from precursor cells or stem cells.

2. Main features of stem cells

According to general consensus pluripotent cells are capable of long-term self-renewal, have an undifferentiated state, can generate various cellular types derived from all three germ layers. Another main characteristic of stem cells is asymmetric division: one daughter cell possesses typical features of a stem cell, the other daughter cell can differentiate into well defined cellular type thanks to signals derived from the surrounding microenvironment known as its “niche”.

In living organism exist two different types of stem cells: “active stem cells” generate cells in case of regeneration such as hematopoietic, epidermis and the intestine stem cells, and “passive stem cells” give rise cells only when the adult organs have been damaged, such as stem cells of the nervous system, liver, eye.

Schofield, in 1978, was the first to enunciate the existence of niches, environments composed of cells that help stem cells to maintain homeostasis of the tissues and isolate some stem cells from proliferative or apoptotic stimuli [26]. Excessive production of stem cells, could induce the onset of cancer [27]. According to differentiative capability, stem cells can be classified in: **totipotent stem cells** give rise to all cells included extra-embryonic tissues such as zygote and cells of the first divisions of the embryo;

pluripotent stem cells generate all types of cells derived from three germ layers, but not extra-embryonic tissues, such as cells of the inner mass of the blastocyst or umbilical cord stem cells;

multipotent stem cells: give rise only some cellular types, for example the elements of the blood platelets red and white cells and adult nervous system stem cells;

unipotent stem cells generate only one type of specialized cell.

According to the tissue of origin, the stem cells are classified in embryonic and somatic or adult stem cells.

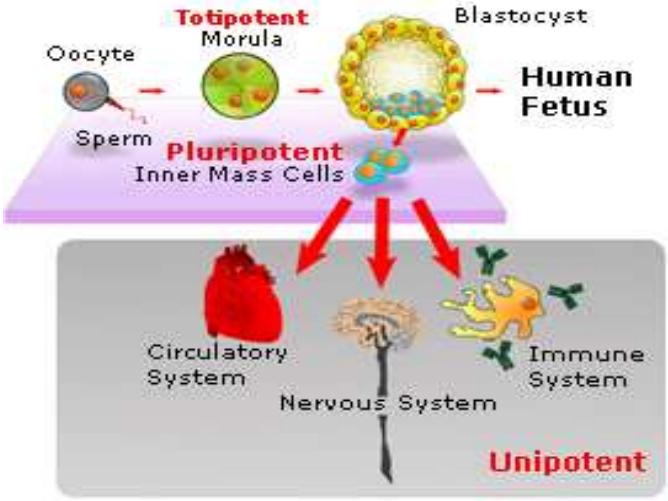


Figure. 3: Schematic rapresentation of the differentiative potential of stem cells isolated from tissues at different development phases.

2.1 Embryonic stem cells

Embryonic stem cells derive from a limited cellular population of the embryo at the stage of blastula or blastocyst. The blastocyst consists of 50-150 cells and three structures: the trophoblast, a layer of cells surrounding the blastocyst; the blastocoel, a cavity inside the blastocyst, and the inner cell mass (ICM) formed by a group of about 30 cells, defined as embryonic stem cells, located at one end of the blastocoel. According to general consensus, embryonic stem cells maintain indefinitely an undifferentiated state, are permanently diploid, maintain normal karyotype, are immortal, can propagate indefinitely and differentiate into cellular types derived from three embryonic germ layers [28].

For these reasons, embryonic stem cells are considered a potential source for the treatment of several diseases [11].

However research on embryonic stem cells had some limitations: their use in fact requires the removal of the embryo within 14 days from fertilization and in some cases the graft of these cells can induce teratoma formation and immune reaction into recipient host. For these ethical and practical issues the researchers carried out studies on adult stem cells [29].

2.2 Adult stem cells

Somatic or adult stem cells are responsible of the maintenance of structural and functional tissue integrity, supporting the replacement of damaged or destroyed cells following to diseases of different origin and nature. Adult stem cells can be isolated from tissue samples obtained either by children or adults, can self-renewal, proliferate for elevated number of passages,do not induce teratoma formation. Until a few years ago, it was commonly believed that stem cells found in adult tissues had a limited ability to transform into other cellular types present in organs to which they belong. Only recently it was shown that some adult stem cells can also generate cells pertinent to other organs, different by the one from which they derive. As an example, hematopoietic stem cells of bone marrow, after transplant can differentiate into muscle [30]; stem cells derived from nervous tissue and muscle can reconstitute the hematopoietic system in a mouse model [31] [32]; bone marrow cells can repopulate the liver after transplantation or can be differentiated into cells that express neuronal markers [33-34]. According to some authors this capacity, known with term 'plasticity' may be attributed in whole or in part to the phenomena of cell fusion [35]. Classifically, the main source of adult stem cells is bone marrow.

2.3 Bone marrow mesenchymal stem cells (BM-MSC)

The stromal fraction of bone marrow is essentially composed by adult mesenchymal stromal cells, which are further capable of self-renewal and able to differentiate into several cell types of the connective tissue (chondrocytes, adipocytes, and osteoblasts) as well as other cell types of ectodermal and endodermal origin. Friedenstein et al. were the first to demonstrate that bone marrow stromal cells, were able to generate clonal colonies of fibroblasts, therefore named colony forming units fibroblasts (CFU-F) [36].

Bone Marrow derived Mesenchymal Stem Cells (BM-MSC) are able to undergo ex vivo expansion, grow on plastic surfaces, and express a number of markers that are also shared by several differentiated phenotypes. In fact, it was demonstrated that BM-MSC express transcripts typical of osteoblasts, chondrocytes, endothelial cells (EC), epithelial cells, and neurons [37] .

BM-MSC reproducibly express CD44, CD73, CD90, CD105, CD166, other markers are: CD49e, CD51, CD54, CD59, CD71. According to general consensus is widely accepted that CD117 is not expressed in human BM-MSC, albeit being reported in other MSC populations [38]. Indeed, BM-MSC do not express markers typical of endothelial/hematopoietic cells such as CD31,CD14, CD34, CD45, CD79, CD86, and glycophorin A (CD235a) [39-40]. Several studies support the idea that use of MSC in vivo should be safer than that of embryonic stem cells (ESC), because they have higher chromosomal stability and do not induce neoplasms formation in the recipient host [41-42].

2.3.1 Differentiation potential of BM-MSC

The bone marrow derived MSCs were defined pluripotent cells since capable to differentiate into various connective cellular types especially osteoblasts, chondrocytes, adipocytes.More recently some researchers have demonstrated that these cell can also differentiate into nervous cells.

First works on the osteogenic differentiation by BM-MSC demonstrated that BM-MSC can grow in standard media and a colonial fraction of these cells express alkaline phosphatase [43]. Osteogenic differentiation by stem cells is accomplished in presence of factors that induce formation of mineralized bone such as dexamethasone, ascorbic acid and β-glycerophosphate [44]. In particular, dexamethasone induces the early stages of osteogenesis and the differentiation of MSCs into osteoblasts, associated to the increase in expression of alkaline phosphatase [45-46].

Ascorbic acid and β-glycerophosphate, are then essential for late stages of osteogenesis: formation and mineralization of the extracellular matrix [47].

BM-MSC differentiated into osteoblasts-like cells express markers such as osteocalcin [48] osteopontin and osteonectin.

Similarly to osteogenic differentiation, adipogenic differentiation requires the presence of specific inducers in the medium. Dexamethasone is one of the main inducers, its addition alone to the culture medium is sufficient to induce adipogenic differentiation in stromal mesenchymal cells [49]. Janderova and collaborators demonstrated that the simultaneous presence of dexamethasone,

insulin, indomethacin and isobutylmethylxanthine in the medium, is certainly a necessary condition to occur adipogenic differentiation [50].

Friedenstein et al. were the first to demonstrate the formation of cartilage tissue from bone marrow stromal cells. Several studies showed that BM-MSC differentiate into chondrocytes in presence of some members of the family of transforming growth factors such as β-IGF but also TGF-β1 and TGF-β3 [51-52].

Other inducers of chondrogenic differentiation are: BMP-2 [53], BMP-6 [51], IGF-1 [Insulin Growth Factor] [52], bFGF [54] and dexamethasone, although the latter is less efficient than TGF-β [55].

The MSCs can also differentiate to cardiomyocytes [56], and recently some studies demonstrated their capacity to generate hepatocyte-like cells [57-58].

Despite adult human bone marrow is the most common MSC source, the number of cells useful for regenerative medicine applications is extremely low (0.001% to0.01%) [59]. Moreover, number of MSC from bone marrow significantly decreases with donor age [60]. Many researchers have therefore searched alternative sources of MSC in another tissue, with easier accessibility, such as extraembryonic tissues: placenta, amniotic membrane, and umbilical cord.

3. Immunological properties: interactions between MSC and immune system

In the last years, the interest for MSC in regenerative medicine is increased, due also to their immunomodulatory and hypo- immunogenicity properties.

Many reports evidenced the capacity of MSC to interact with adaptive and innate immune system in a cell-contact mediated fashion but also by secretion of soluble factors [61]. Main immunological features of MSC consist in the inhibition of T-cell proliferation and dendritic cell (DC) differentiation [62]. Some studies suggest that MSC can modulate T-cell proliferation by their low expression of costimulatory molecules and the absence of class II HLA [61-63]. In addition, the immunosuppressive capacity of MSC may also be mediated by the secretion of soluble factors, and by the induction of T-cell anergy and regulatory T-cells (Tregs), with important consequences for post-infusion therapies. [64-65].

Recent studies demonstrated that the secretion of key soluble factors is often a consequence of cross-talk between MSC and T-lymphocytes, therefore it cannot be considered a constitutive process [66].

Several reports indicate that MSC express non classical type I HLAs such as HLA-G and its soluble form HLA-G5 [63-67-68], HLA-F and HLA-E. To date, HLA-E expression has been observed only in BM-MSC. These HLA 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 [69-70].

Di Nicola and colleagues suggested that transforming growth factor-β (TGF-β) and hepatocyte growth factor (HGF) are two possible mediators for suppression of T-cell proliferation in mixed lymphocyte reaction. The same researchers showed that T-lymphocytes inhibited by BM-MSC, are not in apoptotic, in fact they can be re-stimulated with cellular and humoral activators and therefore actively proliferate [61].

Recently, Ren and colleagues observed that the adhesion molecules ICAM-1 (inflammatory cytokine-induced intercellular adhesion molecule-1) and VCAM-1 (vascular cell adhesion molecule-1) are critical for the MSC immunosuppression on T cells, and are inducible by the parallel presence of IFN-γ and inflammatory cytokines (as IL-1 and TNF-α) [71].

Anergy is another mechanism underlying BM-MSC mediated T-cell suppression. It was supposed that BM-MSC determine T-cell arrest in G0-G1 phase by inhibition of cyclin D2 expression [72]. MSC can induce immune suppression, stimulating the production of CD8+ regulatory T-cells that inhibit allogeneic lymphocyte proliferation [73] and interfering with dendritic cell (DC) differentiation maturation and activation [74].

In addition, MSC inhibit B-cell proliferation and activation in a dose-dependent manner and modulate their differentiation, chemotactic abilities and antibody production [75].

Recently, Németh and co-workers demonstrated that BM-MSC, in a murine sepsis model, actively reprogram macrophages through prostaglandin E2 (PGE2)

stimulation of the EP2 and EP4 receptors, Consequently, reprogrammed macrophages produced more IL-10, reducing neutrophil infiltration in tissues (which is a component of multi-organ dysfunction) and increasing neutrophil numbers in the circulation leading to bacterial clearance [76].

These data were confirmed from recent work of Mei and collaborators. These researchers demonstrated that MSC improve survival in sepsis by reducing inflammation, while enhancing bacterial clearance [77]. MSC express also IDO (indoleamine deoxygenase) and NO (nitric oxide), molecules involved into regulation of immune responses by MSC [78-79].

3.1 MSC and Tolerance induction

Tolerance to self antigens is a very important process for the correct development of the human immune system. A novel class of lymphocytes known as Treg (regulatory T) cells, takes part in the peripheral and central tolerance mechanisms. These lymphocytes exert active suppression by cytokine expression or by promoting the so-called infectious tolerance [80].

HLA-G is a non-classical type Ib HLA molecule, characterized in trophoblast cells. It mediates tolerance towards the semi-allogeneic embryo together with other factors such as EPF (early pregnancy factor), HLA-E, etc [81-82].

HLA-G is expressed in different MSC populations, such as BM-MSC [83] and WJ-MSC [63].

This molecule induces the expansion of CD4+CD25+FoxP3+ Tregs which would contribute to the suppression of effector responses to alloantigens [80-83].

HLA-G has two isoforms, the first one is a membrane-bound isoform, implicated in direct cell-cell contact and the second one is shed from the cellular surface (HLA-G5). Furthermore recent reports suggested a possible cooperative interplay between different immunomodulatory molecules co-expressed by MSC.

Recently, Diaz-Lagares and co-workers demonstrated the existence of a functional interplay between NO and HLA-G establishing an immune tolerance. HLA-G is a target of protein nitration, a reaction which is favored by increased NO in the extracellular space. Nitration of HLA-G renders it sensitive to metalloprotease-

dependent shedding. Therefore, HLA-G should exert a tolerogenic action in a paracrine fashion [84].

Gonzalez et al demonstrated on experimental rheumatoid arthritis that adipose tissue (AT)-derived MSC were involved to the generation and activation of Treg cells [85]. Moreover, Madec and co-workers showed in an experimental model of diabetes, that MSC exerted a protective function on NOD mice by inducing regulatory T cells.

In fact, MSC reduced the ability of diabetogenic T cells to infiltrate islets. Moreover, MSC suppressed both allogeneic and insulin-specific proliferative responses in vitro, an effect linked to IL-10 production by Treg cells [86].

In a report, Zhao and co-workers highlighted as in NOD mice autologous Tregs (which are functionally impaired by the underlying disease) can be modulated by co-culture with cord blood stem cells, reverting the autoimmunity via systemic and local immune modulation and promoting beta cells regeneration [87]. In conclusion all these data supported idea that MSC, could be a good candidates for engraft, because they could be evade an immune response and induce peripheral host tolerance.

3.2 Contrasting evidence in vivo for use MSC

Several data in the literature highlighted the ability of MSC to modulate immune cells proliferation and activation in vitro settings, instead few reports showed the potential generation of immune and memory responses by MSC when administered in vivo. This constitutes a serious issue in cellular therapy, since xenogenic and allogeneic MSC should be eliminated by the host immune system previous to exert any beneficial action.

Data in the literature showed that they are being used in almost every disease setting where autoimmunity or tissue regeneration have to be targeted. Therefore, in reality one should not be surprised that in some model organisms (above all for xenogenic approaches) MSC fail to deliver the expected outcome when applied in vivo. According to the general opinion, negative results in MSC administration in vivo ought to provide instead key information on the molecular mechanisms of MSC-mediated immune modulation, above all in allogeneic settings, for which

most hopes are placed on MSC as a possible “off the shelf” therapy for a number of diseases.

One key question which remains still to be solved is to define actual levels of immune privileges of MSC when transplanted in vivo. WJ-MSC should be recovered in high numbers and should auspicably be banked in parallel to cord blood units. This should lead to the increase of cell numbers available for both autologous and HLA-matched heterologous administration. On the other hand, once the immune mechanisms of the different MSC populations are fully characterized and validated in most in vivo settings, then the path to use of HLA-mismatched heterologous cells will be opened. Griffin and co-workers reviewed the multiple aspects of allogeneic mesenchymal stem cell therapy. While examining the evidence for and against the use of MSC as immunoprivileged cells in vivo, the authors stressed the concept that a better understanding of the mechanisms of MSC-immune cell interactions in vivo is tantamount for the success of allogeneic therapy [80].

In the literature contrasting reports exist on the maintenance of the immune privileges of MSC in vivo settings. Sundin and co-workers demonstrated that allogeneic MSC, transplanted in human patients undergoing HSCT (hematopoietic stem cells transplantation), didn’t induce production of alloantibodies in the host, while anti FCS (fetal calf serum)antibodies were detectable. These antibodies seemed however clinically insignificant, while the important datum is that in vivo, in human subjects, no humoral response was detected against MSC [88].

In a parallel report, Rasmusson and colleagues showed that human BM-MSC were resistant to CTL (cytotoxic T lymphocytes), failing to induce IFN-γ or TNF-α. Therefore the authors stated that only an “abortive” activation program should be induced in fully differentiated CTL [89]. Subsequently Morandi and co-workers highlighted that human MSC can process and present viral or tumor antigens to specific CTL with only limited efficiency. This is due to defects in the antigenprocessing machinery, some of whose components are not expressed in MSC [90].

In the literature exist a study that speaks about on the use of allogeneic and xenogenic MSC as effective in muscular regeneration. In particular the researcher Shabbir and his collaborators demonstrated that dystrophic hamsters treated with porcine or human MSC exhibited 1 month after infusion both muscle regeneration and attenuated oxidative stress [91].

Moreover, in a recent report, Quevedo and co-workers highlighted the restoration of cardiac function in chronic ischemic cardiomiopathy by long-term engraftment and the differentiative ability of allogeneic porcine MSC [92].

Recently, Chen and co-workers demonstrated by in vivo experiments of excisional wound healing, that allogeneic and syngeneic BM-MSC had similar engraftment ability and resulted in enhanced wound healing, without effects on the numbers of CD45+ leukocytes and lymphocytes [93]. In contrast to these other reports have raised doubts about the general validity of this theory. Eliopoulos and co-workers claimed that allogeneic BM stromal cells were rejected by MHC class I and class II mismatched recipient mice [94].

In another work, Nauta and co-workers further extended this concept suggesting that allogeneic MSC are immunogenic and stimulate donor graft rejection [95].

For both papers, MSC characterization was not optimal, the cells used by Eliopoulos and colleagues were negative for CD90 expression and positive for both CD80 and class II MHC [94] therefore bearing key differences to standard bone marrow stromal cells immunophenotype. In experiments carried out from Nauta and co-workers, MSC can differentiate in three cellular lineage (although proved exclusively by histochemical methods), but their characterization was limited to CD106, CD45, CD14 and CD31 [95].

In a more recent report, Prigozhina and colleagues further suggested that allo-transplantation of MSC (again from mice) leads to loss of their in vitro immunosuppressive potential, failing to reduce GVHD (graft versus host disease) [96]. However, again some remarks should be made on the characterization process (based on positivity to four MSC markers alone) and on the isolation protocol from BM, placenta and WJ. In fact, initial passages in culture showed massive contamination with CD45+ cells (up to 93% in BM preparations, and 64% in placenta preparations. Data in the literature speak about on the possible

application of human MSC in an animal model of disease. Chiavegato and co-workers recently showed that human amniotic fluid-derived stem cells were rejected after transplantation in the myocardium of normal, ischemic, immuno-suppressed or immuno-deficient rat. A more accurate analysis of the results reported allowed to establish that AF-derived cells were positive for both CD80 and CD86 B7 co-stimulators, leading the authors to suppose that these cells should act as donor APC (antigen presenting cells) [97]. Again, the lack of an extended characterization in vitro of cells prior to their use in vivo led to disappointing in vivo results. The data obtained so far claim the need for caution in the administration of MSC for pre-clinical or clinical trials. The characterization of cells before implantation is a sine qua non which must be observed in all cases, since otherwise it would lead to contrasting data on the therapeutic efficacy of these cells. Moreover, we must consider that animal models (and in particular mouse) are not perfectly equal to the human counterpart. As we stated previously, there are differences between the immunomodulatory molecules expressed in vitro by human and mouse MSC. Indeed, Ren and co-workers have recently highlighted that while immunosuppression in murine MSC is driven by iNOS-derived NO secretion, human and primate MSC use IDO as an immunosuppressant molecule [98].

This is a first proof that animal models do not always mirror exactly the conditions of the human disease or the behaviour of human cells. When examining the contrasting reports on the beneficial effects of MSC in GVHD, some reports clearly evidenced that in BM-transplant receiving mice, allogeneic BM-MSC failed to prevent GVHD in mice [99-100]. On the contrary, Ringden and colleagues demonstrated that allogeneic human BM-MSC contributed to alleviate GVHD in BM-transplant recipient patients [101]. Again the species-specific differences between intrinsic immunomodulatory potency of MSC may explain the striking differences between animal models and actual clinical effects in patients [100]. Moreover, it is expected that when human cells are being used, the extended characterization of markers expressed should be viewed as an additional “safety” feature to prevent immune reactions in the recipient host.

3.3 Allograft Rejection: MSC and Immunosuppressants

Immunosuppressive therapy associated with organ or islet transplant is accompained and limited by a number of heavy side effects. Therefore MSC are ideally viewed as cellular therapy devices which should completely abrogate the need for immunosuppressants [102].

Recently some reports evidenced that MSC immune modulation should exert a beneficial effect on allogeneic islets transplanted in diabetic subjects. In fact, Ito and co-workers demonstrated that co-transplantation of islets and BM-MSC improved islets graft morphology and function. The authors hypothesized that this improvement was due to the promotion of graft revascularization by MSC [103]. In a parallel report, Li and co-workers co-transplanted allogeneic BM-MSC and allogeneic islets under the kidney capsule of diabetic mice. They found a reduction of rejection by MSC, which exerted suppressive effects acting on T lymphocyte subsets (both naïve and memory cells) and dendritic cells [104].

In addition, Longoni and colleagues employed allogeneic and syngeneic rat MSC to determine the effects on rejection of islets administered through the portal vein. The authors showed normalization of glucose levels and low-grade rejections for up to 15 days, together with reduction of proinflammatory cytokines. Moreover, the authors demonstrated that the same effect was obtained with both syngeneic and allogeneic MSC, and at levels comparable to those achieved with standard immunosuppressive therapy [105]. Indeed, as MSC properties are being determined by researchers, it is clear that in some cases MSC and immunosuppressant drugs may have the same target (i.e. lymphocyte proliferation) [106]. Recently, Popp and co-workers reported that MSC should act synergistically with mycophenolate mofetil (MMF) to induce long term acceptance of solid organ allograft in a rat heart transplantation model [107]. In another report, Ge and colleagues demonstrated that MSC also synergize with rapamycin to attenuate autoimmune responses and promote cardiac allograft tolerance [108].

3.4 MSC-Derived Differentiated Cells: immune properties In Vitro and In Vivo

The main feature of undifferentiated MSC consists in the hypoimmunogenicity, this could be a key factor in the application of these cells as cellular therapy vectors. When the host reparative processes are limited, it provide to the use of cells capable to differentiate to mature cytotypes and replacing existing malfunctioned cells. Therefore the question whether differentiated MSC should lose their immunomodulatory features is of striking importance to decide whether to infuse differentiated cells alone or mixed with undifferentiated cells (which should protect them from immune system attacks). Le Blanc and colleagues, were first to define the HLA expression and the immunological properties of differentiated MSC compared to their undifferentiated counterpart. The researchers performed differentiation of MSC towards bone, cartilage or adipose, and the differentiated cells upregulated expression of HLA class I, but not class II. Moreover, with respect to control cells, IFN-γ-driven expression of HLA class II at the surface of cells was clearly diminished. In vitro alloreactivity was not seen for all of the differentiated cells, even after IFN-γ pre-treatment (besides, IFN-γ increased MSC suppression of mixed lymphocyte cultures) [109].

Recently, Liu and co-workers, using a novel in vivo model of osteogenesis, demonstrated that differentiated allogeneic MSC maintained their immunomodulatory potential and were detectable 4 weeks post-implant in the regenerated tissue, where they secreted bone matrix proteins, without evidence of cell clearance. No evidence of the induction of a frank memory response was obtained by the authors. Indeed, after in vivo implantation, MHC II expression was detectable even if IFN-γ was not the critical factor for this expression [110]. In a recent report, Zheng and coworkers highlighted, in rheumatoid arthritis (RA), the capacity both allogeneic MSC and MSC-derived chondrocytes to blocked the response of type II collagen-reactive T cells isolated from RA patients, and the suppressive effects mediated by TGFβ1 [111]. Another in vitro set of experiments published by Chen and co-workers, examined the xenogenic immunogenicity of rat MSC, differentiated into bone, adipose and cartilage. In particular these researchers demonstrated that xenogenic MSC-derived chondrocytes were

chemotactic to human dendritic cells (DC), and upon differentiation upregulated the two costimulatory B7 molecules (CD80 and CD86, which were absent in untreated cells), leading to DC maturation [112].

Therefore, rat MSC differentiated towards a chondrogenic fate didn't show the same immunomodulatory features as their human counterpart, while osteogenic and adipogenic cells maintained their immune privilege. A higher number of studies, which still lack for the most complex differentiation protocols (e.g. hepatocytes or beta cells), are required to definitively shed light on the immune properties of differentiated MSC. This valuable fruitfully used to design better transplantation strategies which should take into account the expected reactions that the recipient could manifest against donor cells.

3.5 MSC and inflammation targeting

Several reports showed that MSC after systemic infusion have the tendency to migrate to sites of tissue injury and inflammation, and initially accumulate there [113].

Therefore it was supposed that inflammatory sites should also be the sites where MSC are scavenged more easily by resident and migrating phagocytes. There is growing evidence that MSC plays a role in reducing inflammation in vitro and in vivo. Neurological disorders seem to benefit from MSC treatments based on the anti-inflammatory and oxidative stress-dampening properties of MSC. In particular, Kemp and co-workers recently described the secretion of SOD3 (superoxide dismutase 3) by MSC. SOD3 exerts a direct antioxidant activity, attenuating tissue damage and reducing inflammation, and thus could have a neuroprotective role [114].

Interestingly, the authors showed that SOD3 secretion by BM-MSC was upregulated synergistically by inflammatory stimuli (e.g. TNFα and IFNγ), rather than by the exposure of cells to elevated ROS (reactive oxygen species) concentrations. Moreover, Liao and colleagues demonstrated that human umbilical cord-derived MSC exerted anti-inflammatory and pro-angiogenic effects in a rat model of intracerebral haemorrhage [115].

Further reports of MSC action in inflammatory in vivo settings indicated that MSC may reduce inflammation in obstructive sleep apnea [116], ischemia/reperfusion induced acute renal failure [117], liver fibrosis [118], experimental colitis [119], asthma [120], acute myocarditis [121], and myocardial infarction [122]. In addition, one of the first reports of use of umbilical cord blood-derived mononuclear cells in diabetes pointed out that NOD mice treated with UCB mononuclear cells normalized blood glucose levels and increased their lifespan. In treated mice a reduction of insulitis was also detected [123]. Another key question is the role of inflammatory cytokines in the regulation of the differentiation potential of MSC, and the in vivo effects of such interactions. Wehling and colleagues, recently, demonstrated that chondrogenesis in human MSC was inhibited by both IL-1β and TNF-α [124]. This study pointed out that in inflamed joints the cellular reparative mechanisms may fail if not supported by the contemporary administration of specific antagonists of these inflammatory cytokines. These issues arose from immature cells implantation in inflamed cartilage, and would be avoided if the differentiation process was carried out pre-implantation. The accumulated evidence strongly suggests not only that MSC preferentially home in inflamed tissues, but that they also can attenuate inflammation by the secretion of a number of mediators. Their usefulness has been demonstrated in several in vivo models of acute and chronic inflammatory diseases.

4. Features of Human umbilical cord

The umbilical cord is the only organ that dies when life begins. It is an extraembryonic formation that form by fifth week of development and its function consists to protect the vessels that transport oxygen and nutrients between mother and fetus. By the end of the third week of development the embryo is attached to placenta via a connecting stalk (fig.3). At approximately 25 days the yolk sac forms and by 28 days at the level of the anterior wall of the embryo, the yolk sac is pinched down to a vitelline duct, which is surrounded by a primitive umbilical ring. By the end of the 5th week the primitive umbilical ring contains 1) a connecting stalk within which passes the allantois (primitive excretory duct), two umbilical arteries and one vein; 2) the vitelline duct (yolk sac stalk); and 3) a canal which connects the intra- and extraembryonic coelomic cavities. By the 10th week the gastrointestinal tract has developed and protrudes through the umbilical ring to form a physiologically normal herniation into the umbilical cord . Normally these loops of bowel retract by the end of the third month. *[from encyclopedia reproduction Harvey J. Kliman, M.D., Ph.D.Yale University School of Medicin]*.

At term gestation, the umbilical cord weighs around 40g and its length is approximately 60-65cm. It is covered by layers of squamous-cubic epithelial cells that constitute the umbilical cord epithelium (UCE). It is supposed that UCE derives from amniotic epithelium. Mizoguchi et al. demonstrated that the cells of epithelium express not only mucous epithelial keratins , as found in the amniotic epithelium, but also stratified epithelial keratins and cornified cell envelope (CCE) associated proteins [125]. The inner tissue architecture of the umbilical cord normally consists two arteries and one vein embedded within a matrix of mucous connective tissue composed by fibroblastic-like cells, miofibroblasts and occasional mast cells and by loose substance rich in proteoglycans. In the umbilical cord there are neither capillaries nor lymphatics.

4.1 Wharton's Jelly : structure and function

The most abundant component of extracellular matrix of the umbilical cord is Wharton's Jelly, a connective mucous tissue composed mainly by the amorphous component rich in glicosaminoglycans (GAGs) and mainly in hyaluronic acid (HA) and proteoglycans, with a few collagen fibres. The main role of Wharton's jelly is to prevent compression, torsion and bending of the vessels, which provide two-way flow of blood between the maternal and fetal circulation. [126].

In the Wharton's Jelly exist two cellular types: myofibroblasts and fibroblast-like cells. The first ones are not defined as either as fibroblasts or smooth muscle cells despite have muscle-specific cytoskeletal filaments. The term of "myofibroblast" was assigned because these stromal cells are positive for vimentin [127], a marker typical of fibroblasts, and desmin [126], a marker of muscle cells. The fibroblast-like cells instead have similar features to the fibroblasts, they produce collagen and other extracellular matrix components.

Several studies demonstrated that Wharton jelly cells (WJC) support ex vivo hematopoietic expansion [128] and in vivo engraftment of hematopoietic stem cells [129].

Weiss and collaborators showed that WJC express osteopontin protein [130], a major component of the hematopoietic stem cell niche and a regulator of hematopoietic progenitor cells [131].

Raio et al. highlighted that WJC are source of hyaluronic acid (HA), another main element of the hematopoietic stem cell niche [132].

Therefore, both the expression of the osteopontin gene and the presence of HA confirm that WJC are facilitators of hematopoietic expansion.

WJC secrete cytokines similar to those produced by BM-MSC, moreover synthesize granulocyte-macrophage colony stimulating factor (GM-CSF) and granulocyte colony stimulating factor (G-CSF).

WJC, conversely to BM-MSC, are slower in the differentiation towards adipocytes, have a higher frequency of CFU-F, feature a shorter doubling time [133], and can be isolated with 100% success.

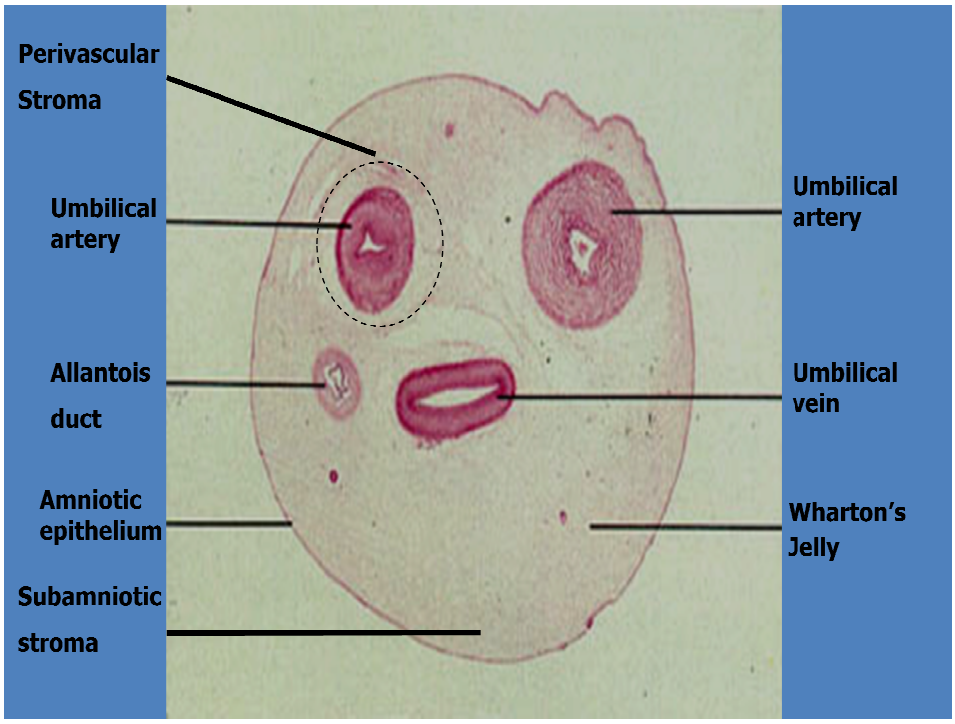


Figura 4: sezione trasversale del cordone ombelicale

4.2 Phenotypical characterization of Wharton's Jelly mesenchymal stem cells

WJ-derived mesenchymal stem cells (WJ-MSC) 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 [63- 134].

WJ-MSC usually show a phenotype which resembles that of BM-MSC.

Immunocytochemistry experiments highlighted that WJ-MSC lack expression of CD14, CD31, CD33, CD34, CD45, but not CD56 conversely to BM-MSC [135]. Moreover, both BM-derived and WJ-derived MSC do not express HLA-DR [40-130-136-137]. On the other hand, WJ-MSC express at protein level: CD73, CD90, CD105, HLA class I [138], as well as CD10, CD13, CD29, CD44, CD49e, and CD166 [38-63-130-139-140]; all of them were also characterized as BM-MSC markers [130-141-142]. WJ-MSC express mesodermal markers such as vimentin and α-smooth muscle actin. Also CD117, the receptor for the stem cell factor (SCF), a typical marker of the hematopoietic stem cell lineage, has been demonstrated in WJ-MSC. In addition , a subset of WJ-MSC expresses nestin [63], an intermediate filament of the neuroectodermal cellular lineage expressed as a precursor of neurofilaments, also observed in pancreatic progenitors capable of differentiating toward β cells, as well as in human and rat BM-MSC populations [143-144].

Several recent reports were carried out to value the similarities (and differences) of WJ-MSC with respect to other MSC populations. Recently it was showed that WJ-MSC express, at mRNA and protein levels, GATA-4, GATA-5, GATA-6 [63], transcription factors involved in different developmental pathways of mesoderm- and endoderm-derived organs. Previously, only GATA-4 expression had been reported in BM-MSC [145]. La Rocca et al demonstrated that WJ-MSC express connexin-43 [63], a molecule expressed typically in embryonic and myocardial cells and 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 [146]. Moreover, WJ-MSC are capable to undergo self-renewal, a key feature of all MSC, maintaining their replicative potential together with their undifferentiated state. In this respect, it was reported the expression of Nanog and Oct3/4A, factors responsible for maintaining long-term self-renewal and the undifferentiated state also in ESC. WJ-MSC isolated by nonenzymatic methods express a subset of epithelial cytokeratins (CK) [147] such as CK-8, CK-18, CK-19, while CK-7 was not detected [63]. On the other hand, only CK-18 and CK-19 expression have been demonstrated in BM-MSC [148].

In addition, the expression of neuroectodermal markers such as glial fibrillar acidic protein (GFAP) and neuron-specific enolase (NSE) was described in earlier reports for both WJ-MSC and BM-MSC [144-149-150-151].

Umbilical cord derived cells can differentiate toward endoderm-derived organs, hepatocyte nuclear factor 4α (HNF-4α) expression by WJ-MSC, in fact might suggest a possible role in regeneration of key cell types such as hepatocytes and pancreatic endocrine cells [63]. In particular, some studies demonstrated that HNF-4α is dispensable for early endodermal specification, but essential for maintaining the differentiated hepatocyte expression pattern [152].

4.3 Differentiation capacity of WJ-MSC

WJ-MSC are multipotent cells, capable of giving rise to different mature cytotypes . Most studies agree in 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 was considered part of the minimal criteria stated in 2006 to uniformly define MSC properties [153]. The effectiveness of the differentiation of MSC toward the mature connective cytotypes is defined by phenotypical and morphological criteria.

The standardized protocols to obtain osteogenic differentiation of MSC [47-154] resulted 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 [63-155]. Moreover, differentiated MSC should express specific proteins, such as osteonectin, osteocalcin, periostin, runx2 [156.]. After adipogenic differentiation protocols [49-50], differentiated adipocytes should be demonstrated by lipid-specific histological stains such as Oil Red O [50-63]. 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 [52-157], the differentiated cells can be specifically stained by Alcian blue or Safranin O-Fast Green [158]. 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 [159].

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 [150]. 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 specifi c 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 [130].

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 [136]. 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 applications 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). The authors showed that differentiated cells expressed in vitro cardiac myosin injected into the viable myocardium bordering an experimental infarcted area, were incorporated in the vasculature and occasionally were positive for cTnT [160]. Other reports claimed a supportive role for several MSC populations in terms of suppression of infl ammation in acute myocardial infarction models, microenvironment-driven direct differentiation, as well as paracrine effects on the repairing myocardium [161-162].

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) [163].

Endothelial differentiation. As demonstrated previously for human ESC [164], human WJC can be differentiated into EC after culturing in low serum medium supplemented with vascular endothelial growth factor (VEGF) and bFGF [50].

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 [164-165]. 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 [50]. More recently, Chen and colleagues [166] 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.

4.4 Hepatogenic differentiation potential of WJ-MSC

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 [167].

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 [168-169-170]. 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 fi rst inductive phase, and oncostatin M (OSM), involved in the final differentiation phase [171]. Differentiation protocols should be based on the parallel administration of these factors [172], or follow a stepwise process [173]. 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 [172], in 3D scaffolds [174], or in co-culture systems with fetal or adult hepatocytes [175]. Most differentiation experiments have been performed using low (1%) serum culture media. 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 [172], most published data refer to multiple markers, whose expression is assessed at both the protein and mRNA levels [176]. 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 [147-168-177-178].

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 [168]. In addition, in a very recent study, Zemel and collaborators [179] 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 [63]. In fact, we demonstrated that WJ-MSC 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 [152-180-181]. 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) [172-182]; ammonia metabolization and urea production (determined by colorimetric or fluorometric assays) [176-183-184]; selective uptake of vital stains (eg, indocyanine green, which is uptaken exclusively by hepatocytes) [185]; secretion of plasma proteins (eg, albumin, determined by ELISA) [186-187].

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 [176] showed that WJ-derived MSC, differentiated with a multistep protocol, express functional inducible CYP3A4.

4.5 Hepatogenic differentiation ability of WJ-MSC with respect to other MSC populations

Among the different extrahepatic sources of differentiated cells to be used for regenerative medicine applications (reviewed in ref. [168], MSC are emerging as a useful cytotype, as different reports published in the last few years have indicated..

Bone marrow mesenchymal stem cells. Lee and colleagues, for the fi rst 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 [57] (see table 1).

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 [58].

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 [188]. Lysy and colleagues further investigated, with in vitro and in vivo experiments, the hepatocyte differentiation ability of BM-MSC, evaluating the expression of hepatospecific 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 [189]. 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 nanofi brous 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 [187].

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 HSCderived from bone marrow differentiated into hepatocytes when cultured in the presence of sera from mice with damaged liver [190]. 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 [191]. 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 fortransplant, 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 [178-192] .

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 [184]. More recently, Taléns-Visconti and collaborators carried out a study comparing BM-MSC and hADSC [173]. 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 [178]. In a more recent work, Aurich and coworkers performed experiments in which adipose tissue-derived MSC (AT-MSC) were transplanted into liver of immunodefi cient 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 [193].

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 [194].

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 [195]. 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 [172].

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 [196]. 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 other 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 [176]. 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 [197]. 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 [176]. Interestingly, more recent data suggest a supportive role of undifferentiated human umbilical mesenchymal stem cells (WJ-MSC) in rescuing injured liver functions and reducing fibrosis in vivo. Tsai and coworkers infused undifferentiated WJ-MSC 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, WJ-MSC 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 [118]. 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 [198]. 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 [199]. More recently, Zhang and coworkers [200] 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 [201] 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 fi broblastoid 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 [202].

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 [203]. 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 [182].

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 [204]. Further studies should be carried out to better evaluate the functional features of differentiated cells as well as their engraftment success rate.

4.6 Formation of hepatocyte-like cells in vivo by extrahepatic stem cells

To date, several reports demonstrated that extrahepatic stem cells, after transplantation in animal models, express hepatic markers, but this does not imply that these cells show transdifferentiation into true hepatocytes. Certainly, these studies are an important basis for future clinical applications.

Beeheirde and collaborators, infused human umbilical cord blood non-hematopoietic somatic stem cells into severe combined immunodeficiency mouse (SCID mouse). After 7 days they found that these cells did not alter the architecture of liver, in fact they integrated very well in the tissue, expressed human albumin only after transplantation, while continuing to expresse human α-1 antitripsin gene also after transplantation. Another very important aspect of this work consisted in the downregulation of human β-2 microglobulin gene by infused stem cells. β-2 microglobulin is an integral part of the major histocompatibility complex and his absence renders the residual heavy chain inactive as an antigen. Therefore it should be assumed that these transplanted stem cells should by-pass killer-T-cells through absence of this molecule [205].

In addition another study carried out by Ishikawa et al., highlighted that two cellular types, CD34+ and CD45+ populations isolated from human umbilical cord blood, after transplantation in newborn NOD/SCID (non-obese/ severe combined immunodeficiency) β-2 microglobulin ^{null} mice, after 4-5 months, can express human albumin. Moreover, this study demonstrated that generation of hepatocytes from engrafted stem cells is not the result of cell fusion between mouse and human cells, because it was demonstrated by FISH that generated hepatocytes had a human centromeric DNA [206].

Kakinuma et al. carried out studies that demonstrated that human umbilical cord blood cells(hUBC), both in vitro and in vivo can differentiate in hepatic progenitor cells. After 21 days h-UBC, cultured in a medium supplemented by factors promoting hepatic differentiation, expressed some hepatic molecules such as cytokeratin-18 (CK18), alpha-fetoprotein (AFP), glutamine synthetase (GS) and albumin. After transplantation in liver-injured SCID mice, transplanted cells released albumin into the sera of recipient host, suggesting that these cells develop

into functional hepatocytes even if they appear at frequencies of 0.1%-1.0% in recipient livers. Probably this low frequency is due to the xenogenic nature of transplantation, therefore other studies could been necessary to evaluate the behaviour, after transplantation, of syngeneic cells [194].

Newsome and collaborators confirmed observation of other groups described previously. These researchers highlighted that umbilical cord blood cells, transplanted into irradiated NOD/SCID mice generated hepatocytes. After characterization by immunocytochemistry, immunofluorescence and immunohistochemistry, the cells were engrafted into recipient mice. The presence of HepPar1, antigen specific for human hepatocytes, and the presence of human DNA in hepatocytes derived from transplanted stem cells, allowed demonstrating that these cells were not generated by fusion of human and mouse cells [207]. In addition, Nonome and colleagues studied the behaviour of umbilical cord blood cells ($CD34^+$ and $CD34^-$ cells) after induction of hepatogenic differentiation in vitro and after transplantation in irradiated mice and in mice with injury liver. In vitro experiments highlighted that already by 7 days the HUBC changed their shape and expressed hepatic molecules such as albumin, AFP, CK-19, GS and transferrin.

In vivo, the HUBC, $CD34^+$ and $CD34^-$ cells were infused into two groups of NOD/SCID mice with Fas-mediated liver injury or simply irradiated. After 4 weeks, immunohistochemical analyses and RT-PCR demonstrated the expression of albumin and Hep-Par 1, while fluorescent in situ hybridization analysis showed the presence of Y chromosome in mouse of the Fas ligand/transplantation group but not in irradiated mice group [208].

Another category of adult stem cells that can generate hepatocytes are mesenchymal stem cells. In the last years the interest for these cell is increased, below in table are reported all reports carried out both in vitro and vivo on mesesenchymal stem cells differentiate into hepatocyte-like cells (see table n.1).

Table N°1: list of in vitro and vivo experiments on hepatogenic differentiation capacity by MSC derived from several sources [209]

MSC population	**Hepatic Differentiation Protocol**	**Analysis of markers expression**	**Functional assays**	**REF.**
Human bone marrow mesenchymal stem cell	Two steps – seven weeks protocol: Pre-conditioning: DMEM plus EGF and bFGF for 2 days. Serum-free IMDM, supplemented with HGF, bFGF, nicotinamide for 7 days. Serum-free IMDM plus OSM, dexamethasone, ITS, thereafter.	I. AFP, albumin, G6Pase, TO, CK18, TAT,CYP 2B6, 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	[57]
Umbilical cord blood-mesenchymal stem cell		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 cell (BM-MSC)	Co-colture of rat BM-MSC with fetal rat liver cells. Fibronectin matrix coated plates. One step – three weeks protocol Stem Span SFEM medium, plus DMX, SCF, HGF, EGF, FGF-4.	Albumin, AFP and CK18. by RT-PCR	None.	[58]
Rat Bone marrow-derived mesenchymal stem cells	Co-colture of MSC with liver stellate cell (LSC). One step – three 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	[188]

Human bone marrow–derived mesenchymal stem cell	Collagen type I-coated dishes. Two steps - four weeks protocol: IMDM plus HGF, FGF-4, ITS, nicotinamide for 10 days IMDM plus OSM, DMX, 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.	[189]
Human BM-MSC	2D or 3D culture conditions (biomimetic scaffold) Two-steps - three weeks protocol: One week plus DMEM-LG, 15% FBS, HGF, DEX. 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	[187]
Adult hematopoietic stem cell (HSC)	Laminin, gelatin, and hyaluronic acid coated plates One step – one week protocol IMDM plus10% 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.	[190]
Human adipose tissue stem cell (hADSC)	Fibronectin-coated dishes One step – four 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.	[184]

Adipose tissue derived stem cell (ADSC) Bone-marrow-derived mesenchymal stem cells	Two steps – Three weeks protocol Pre-conditioning: DMEM plus EGF and bFGF for 2 days. DMEM plus HGF, bFGF, nicotinamide for 7 days. DMEM plus OSM, DMX, ITS up to 21 days.	I. Albumin, Thy-1, AFP, CYP 3A4, CYP2E1, CK-18, CK-19, HNF- 4α, C /EBPβ by Real Time-PCR. II. Albumin and AFP by IHC.	None	[173]
Adipose–derived stem cell (ADSC)	Collagen type I-coated dishes. Three steps – three weeks protocol DMEM plus Activin A and FGF-4 for 3 days. HCM plus HGF, FGF-1, FGF-4, OSM, ITS, DMX, DMSO and nicotinamide for 10 days. HCM plus nicotinamide and DMX 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.	[178]
Human umbilical cord blood cells (UCB-cells)	One step - three 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 immunoflorescent staining analysis III. Expression of albumin by immunohistological analysis	*In vivo*: UCB cells transplanted in liver-injured SCID mice. Albumin expression.	[194]
Human umbilical cord blood-derived mesenchymal stem cells	Two steps – four weeks protocol IMDM plus 10% FBS, DMX, ITS, HGF for 2 weeks. IMDM plus 10% FBS, DMX, 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	[195]
Human umbilical cord blood-derived mesenchymal	One step – four weeks protocol IMDM supplemented with 10% FBS, HGF,	I. AFP and albumin expression by radioimmunoassay. II. CK-18 by immunocytochemistry	*In vitro:* urea production assay; PAS staining for glycogen	[172]

stem cell	FGF-4,.	III. AFP, albumin, CK-18 expression by RT-PCR.		
Umbilical cord matrix stem cell (UCMSC)	Rat tail collagen type I coated plates. Three steps – three weeks protocol IMDM plus bFGF, EGF. for 2 days; IMDM plus HGF, bFGF nicotinamide, ITS for 10 days. IMDM plus OSM, DMX, 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; Cytocrome P450 3A4 assay; *In vivo:* UCMSC transplantated in SCID mice after partial hepatectomy. Expression of albumin, AFP, fibronectin.	[176]
Umbilical cord mesenchymal stromal cells (UC MSC)	One step - three weeks protocol IMDM supplemented with 1% FBS, 40 ng/mL HGF and 10 ng/mL 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	[201]
Umbilical cord mesenchymal stromal cells (UC-MSC)	Two steps – four weeks protocol DMEM/F-12 supplemented with 50ng/ml HGF, 10ng/ml bFGF, 50mg/ml ITS+ premix for 16 days; DMEM/F-12 supplemented with 20ng/ml OSM, 10^{-6} M DMX, 50mg/ml ITS+ premix for two weeks	I. Albumin, AFP, CK-19 by IF. II. Albumin, AFP, CK-19, G-6P, 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.	[202]

Human placenta-derived multipotent cells	Culture dishes untreated or coated with fibronectin or poly-L-lysine. Two step – three week protocol I step: 60% DMEM-LG, 40% MCDB201 plus ITS, linoleic acid, BSA, DMX, ascorbic acid, EGF, PDGF-BB for 16 hours. II step: 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;	[203]
Human amniotic membrane-derived mesenchymal stem cell	Collagen type I-coated dishes. One step – three weeks protocol α-MEM plus 10% FBS, hHGF, hFGF-2, OSM, DMX	I. Albumin, AFP, CK18, α1-AT, HNF-4α.by RT-PCR. II. Albumin, CK18, AFP by ICC. III. Albumin, AFP by IF.	*In vitro*. PAS stain for glycogen	[204]
Human amniotic fluid derived mesenchymal stem cell Human bone marrow-derived mesenchymal stem cell	Collagen gel type I coated plates. Three steps – three 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, DMX, trichostatin A. Phenobarbital was added 18 days after differentiation	I. albumin, AFP, CK-18, HNF_1 α, C/EBP, CYP 1A1 by real-time PCR. II. AFP, albumin, CK-18, HNF_1 α, C/EBP, CYP1A1 by IF	*In vitro*: PAS stain for glycogen; urea assay	[182]
Mesenchymal stem cells derived from human fetal lung	One step – three 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	[205]

5. OBJECTIVES

The liver is a parenchymatous organ assigned to a variety of important functions : metabolizing dietary molecules, xenobiotic detoxifying, storing glycogen and urea production.

Hepatocytes represent 80% of total liver mass, instead the remaining part constituted by cholangiocytes, kupffer cells, stellate cells, hepatic specific natural killer and endothelial cell. The hepatocytes are polarized epithelial cells, and they serve endocrine and exocrine functions. Through basal surface, they secrete of serum factors into venous blood flow, and through apical surface, the hepatocytes secrete bile into canaliculi that join the bile ducts [210].

Today, a wide variety of liver diseases lead to the impairment of liver functions such as acute and chronic liver failure, cirrhosis, metabolic liver disease. The liver has an elevated replicative potential, even if 70% of hepatectomy, replicative activity of hepatocytes decreases, therefore requiring medical interventions.

Liver transplantation is the main treatment for some end-stage hepatic diseases, but adverse factors such as rejection, complications associated to long term immunosuppressants administration, and scarcity of donors limit its therapeutic potential.

Alternative medical interventions could be provided by hepatocytes transplantation, a simpler and less invasive procedure. However, this therapeutic approach is limited by several factors: only 20-30% of transplanted hepatocytes survive upon transplantation, multiple transplantation attempts are required to achieve significant liver repopulation, and finally paucity of cadaveric livers and limited replicative potential of the hepatocytes. [211].

For this reason many researchers searched alternative sources of cells for transplant such as adult stem cells. Two different stem cells types are capable to differentiate into hepatocytes: hepatic stem cells (oval cells and hepatic progenitor cell populations) and extra-hepatic stem cells (bone marrow derived mesenchymal stem cells and Wharton’s jelly mesenchymal stem cells, WJ-MSC). Certainly ,the phenotypic characterization of adult stem cells is an important objective for defining the characteristics of different cell populations, as well as for determining

the safety of their use for regenerative medicine applications. In this research project, one of the objectives was to characterize (by the application of molecular biology, and immunological techniques), the expression pattern of WJ-MSC, focusing on transcription factors typical of embryonic stem cells, responsible for undifferentiated state and pluripotency, of endodermal markers suggesting a probable differentiation capacity toward endodermal cellular lineages, and on the expression of immuno-modulatory molecules potentially involved in the establishment of a immune tolerance in the host suggesting a possible critical role in clinical applications.

After a comprehensive characterization of the WJ-MSC, the other main objective will consist to differentiate these cells into hepatocyte-like cells applying a two step protocol: an induction phase and maturation phase, miming in vitro the liver devolopmental processes.

To confirm hepatic differentiation capacity by WJ-MSC, we will continue our analysis by molecular biology, flow cytometry and immunostaining, the expression of early and late hepatic molecules and the expression of enzymes involved in some typical hepatic functions such as detoxyfication of xenobiotics, glycogenolisys, glycogensynthesis and gluconeogenesis.

Another objective needed to establish if WJ-MSC could be considered as probable candidates for future clinical applications is the analysis of immuno-modulatory molecules involved in the establishment of an immune tolerance (or the existence of an immune privilege) in the host. In particular, we will highlight the expression of immuno-modulatory molecules both in undifferentiated cells and in hepatocyte-like differentiated cells to evaluate if the differentiation process will modify immune properties of WJ-MSC.

6. MATERIALS AND METHODS

6.1 Cellular isolation protocol of Wharton's Jelly Mesenchymal Stem Cells

Isolation protocol was adopted from our previously published data [63]. All umbilical cords were obtained after mother's agreement according to tenets of the Declaration of Helsinki and local ethical regulation. After normal vaginal or caesarean delivery, after full-term birth, umbilical cords were stored aseptically in cold saline and cellular isolation was started within six hours from partum. The cords were washed in warm HBSS (Gibco), and then were cut in small pieces about 1.5 cm length, sectioned longitudinally so that to exhibit the Wharton Jelly under amniotic membrane. Different incisions, without vessels removal, were made within matrix with sterile scalpel to increase area exposed to the contact with medium composed by DMEM low glucose (Sigma), supplemented with 10% FCS (fetal cow serum, PAA), 1x NEAA (non-essential aminoacids, Sigma), 1x antibiotics-antimycotics (GIBCO), and 2mM L-glutamine (Sigma).This isolation protocol does not use enzymatic activity to dissociate cells from the embedding matrix but is based on cells migratory ability. Cord pieces were left for 15 days with medium changed every second day. Therefore, the slow degradation of the matrix allowed growth factors and signalling molecules to exit from the cord and continuing provide a positive stimulation to the cultured cells.

After 15 days of culture, cells widely adhered to the plastic surface, and were cultured until confluence.

6.2 Cell culturing and passaging

After reaching confluence, cells were removed from flasks with Tryple Select (Invitrogen) and were cultured for up to 15 passages corresponding to about 60 population doublings. For immunocytochemical analysis, cells were plated in 8-well chamber slides (BD Bioscience) and were used after reaching 90% confluence. For RNA extraction, cells were cultured either in 6-well tissue culture plates or in 25cm^2 tissue culture flasks (Corning).

6.3 Immunocytochemical analysis

Immunocytochemistry detects the expression of specific antigens recognized by primary antibody which are bound by secondary antibody.

Cells, after removed the medium, were washed with PBS, fixed and dehydrated with methanol for 20 minutes at -20C°. Rinsed with PBS, cells were treated for 3 minutes with Triton X-100 0,1% in PBS 1X. Removed Triton-X, two washes were performed with PBS followed by addition of 0,3% hydrogen peroxide to inactivate endogen peroxidases. After 20 minutes in a blocking solution (complete medium with 10% of serum in PBS 1X, in a ratio of 1:10), cells were incubated with specific primary antibodies for 1hour and 30 minutes at room temperature. After another wash with PBS, cells were incubated with species-specific secondary antibodies for 10 minutes. Subsequently, streptavidin- peroxidase (DAKO-Cytomation) was added followed by 3.3'-diaminobenzidine (DAB chromogenic substrate solution, DAKO). At the end, Hematoxylin (DAKO) was used to counter stain the nuclei of the cells.

Immunopositivity was scored using a semiquantitative approach. Three independent observers (FC, LM,GLR) evaluated the immunocytochemical results and semi-quantified the percentage of positive cells for each specimen. Ten high-power Fields were examined in each culture slide and counting of the cells was performed at 40X magnification. The antibodies used in the present study, with indications of the working conditions used, are listed in table n.2

Antigen	Host	Manufacturer	Diluition
Albumin	Mouse	Sigma	1.100
B7H3	Rabbit	Santa Cruz	1:100
CYP 3A4	Mouse	Santa Cruz	1:50
Cytokeratin 8	Mouse	Sigma	1:200
Cytokeratin 18	Mouse	Sigma	1:800
Connexin 32	Mouse	Santa Cruz	1:100
Connexin-43	Mouse	BD Laboratories	1:50
HNF 4α	Rabbit	Santa Cruz	1:100

Table N°2: List of antibodies used in the present study

6.4 Total RNA extraction

Total RNA extraction from Wharton's jelly cells was made by RNAspin mini RNA isolation Kit (GE Healthcare).

The cells were lysed by adding 350µl of Buffer RA1 and 3.5 µl of β-mercaptoethanol, and were mixed vigorously. The obtained lysate was filtered through RNAspin Mini Column and was centrifuged for 1 minute to 10000 rpm. After centrifugation, the mini filter was discarded and the filtrate was transferred to a new 1.5 ml tube where were added 350µl of ethanol 70%. After mixing, the lysate was pipetted 2-3 times and was transferred to a RNAspin Mini Column, placed in a 2ml tube and centrifuged at 8500 rpm for 30 seconds. After centrifugation , the column was placed in a new tube, were added 350µl of MDB (Membrane Desalting Buffer), and centrifugation was performed for 1 minute at 10000 rpm to dry the membrane. Each sample was incubated for 15 minutes with 95µl of a mixture containing DNase to avoid a possible DNA contamination. Following washes were carried out with specific buffers supplied by the kit (RA2 and RA3), the column was transferred into a 1.5 ml tube (nuclease-free). The RNA was eluted from the filter in 100µl of RNase-free H2O (by centrifugation at 10000 rpm for 1 minute). The RNA extract was stored at -20 ° C until use. The concentration of RNA extracted was determined by spectrophotometer with a wavelength of 260nm. Only samples with A260/A280 ratio over 1-6 were considered usable for the following analyses.

6.5 RT-PCR (Reverse Transcription Polymerase Chain Reaction)

Qualitative RT-PCR was performed using Phusion High-Fidelity RT-PCR kit (Finnzymes). RT-PCR consists of two phases: retro-transcription where RNA is converted in DNA complementary (cDNA) and amplification of cDNA.

After treatment with DNase, 2 μg of RNA were added oligo dT and oligo N to select only mRNA from total RNA. Subsequently were added 5μl of Buffer Phusion 10x, 1μl dNTP mix, 1μl of RNase inhibitor, 1μl of AMV reverse transcriptase and RNase/DNase free water until to reach a final volume of 50μl. The reaction comprised a reverse transcription step of 50 minutes at 42 C° and an inactivation phase of 5 minute at 92 C°.

Subsequently, at 2 μl cDNA were added 10pM of specific primers, 4μl of 5x Phusion Buffer, 0,4μl 10mM dNTP, 0,6 μl DMSO, 0,2 μl of Phusion DNA Polymerase and water until to reach a final volume of 20μl. The amplification reaction was performed according to five steps. The initial denaturation of 30 second at 98C°, followed by another denaturation step of 10 second at 98 C°, the annealing phases of 30 second at specific-primers temperatures, the extension step of 30 second at 72C°, and finale extension for 10 minutes at 72 C°.

The specific primers pairs used in this study are listed in table 3.

Table 3: list of PCR primers used for the present study

Name	Product size	Primers
ABCG2	255	Forward 5-ATGGTGTATAGACGCCTGA-3 Reverse 5-GGGACAGGTATGTGAAAAGC-3
Actin, beta	350	Forward 5-AAACTGGAACGGTGAAGGTG-3 Reverse 5-TCAAGTTGGGGGACAAAAAG-3
α -A1AT	271	Forward 5-GGGAAACTACAGCACCTGGA-3 Reverse 5-CAGCTTCAGTCCCTTTCTCG-3
AFP	250	Forward 5-AGCTTGGTGGTGGATGAAAC-3 Reverse 5-GTCCCTCTTCAGCAAAGCAG-3
Albumin	180	Forward 5-ACATTCACCTTCCATGCAGA-3 Reverse 5-CTCCTTATCGTCAGCCTTGC-3
BAAT	346	Forward 5-CCAAACTGGACATGGTGAAT-3 Reverse 5-TCCTCCATTCCTTCTTTCCT-3
B7H3	170	Forward 5-CCCTCCCTACAGCTCCTACC-3 Reverse 5-CAGCAGGATGACTTGGGAAT-3
BSEP	258	Forward 5-TCCTACATCGGAATCCAAGC-3 Reverse 5-CCGAGGGTTCAAAAATGAAA-3
CD66F	228	Forward 5-TCTACCTGACTGCCCCAGA-3 Reverse 5-AGCCAAACCAAGGCTGACT-3
CD80	259	Forward 5-AGGGCCTCCTTAGATCCCTA-3 Reverse 5-TTAGCTGCCATGAGATGTGC-3
CD86	250	Forward5-TCCTGGCTGAGAGAGGAAGA-3 Reverse 5-AGACTGCCCCATCCCTTAGT-3
CK-8	216	Forward5-TCTGGGATGCAGAACATGAG-3 Reverse 5-AGACACCAGCTTCCCATCAC-3
CK-18	263	Forward5-CTGCTGCACCTTGAGTCAGA-3 Reverse 5-GTCCAAGGCATCACCAAGAT-3
CK-19	295	Forward5-ATGAAAGCTGCCTTGGAAGA-3 Reverse 5-CCTCCAAAGGACAGCAGAAG-3
Connexin 26	215	Forward 5-ACTGTGGTAGCCAGCATCG-3 Reverse 5-AGGCTGAAGGGGTAAGCAA-3
Connexin 32	218	Forward 5-TCAGTGAGGAGGGATGTGG-3 Reverse 5-TGGGGACTAGAGGCAGAGG-3
Connexin 40	203	Forward 5-GTGTGTGTGTGGGTGCTGA-3 Reverse 5-GATGGGCAGGTGAGTCAGA-3
Connexin 43	240	Forward 5-CTTCAAGCAGAGCCAGCAG-3 Reverse 5-TACCCCATACACCCCCAGT-3
Connexin 45	234	Forward 5-GCCAACATGGCAAAACTGT-3 Reverse 5-CCTGGTTCAACAAGCCAAC-3
FXR	365	Forward 5-CCAGCCTGAAAATCCTCAAC-3 Reverse 5-GGATTCCCTGGAGCCTTTTA-3
FGFR2	263	Forward 5-AGACTCTTTGGCGTTGGAGA.3 Reverse 5-TTCATCTTGCACGGCTATTG-3
FGFR4	221	Forward 5-GACACAGTGCTCGACCTTGA-3 Reverse 5- GTATTGGGAGGCAGGAGGTT-3
GATA-4	270	Forward 5- CCAGAGATTCTGCAACACGA-3 Reverse5-ATTTTGGAGTGAGGGGTCTG-3
GATA-5	259	Forward 5-GAATGGCCGGTGATGTATGT-3 Reverse 5-TGAAGCTGATGCCAGACAAC-3
GATA-6	259	Forward5-ACTAACCCACAGGCAGGTTG-3 Reverse 5-GGTACAAAACGGCTCCAAAA-3
G-6-Pase	323	Forward 5-GTACAGGGAGAGCTGCAAGG-3 Reverse 5-ATACCAGTGCCCATTGCTTC-3

GYS2L	348	Forward 5-AGCTGAATGCACTGTGATGG-3 Reverse5-TCTGTCGTTGGTGGTGATGT-3
HCC-4	336	Forward 5-ATCTTTGCCCTCTGGGTCTT-3 Reverse 5-TTTCTTTCCCTGTCCTGCAA-3
HLA-A	262	Forward5-TGGGACTGAGAGGCAAGAGT-3 Reverse5-ACAGCTCAGTGCACCATGAA-3
HLA-DR	349	Forward5-GCACAGAGCAAGATGCTGAG-3 Reverse 5-AGTTGAAGATGAGGCGCTGT-3
HLA-E	245	Forward 5-CAAGGGCCTCTGAATCTGTC-3 Reverse 5-CGTGTTAGCCAGGATGGTTT-3
HLA-F1	202	Forward 5- TGGAGTTGCTCCGCAGATA-3 Reverse 5-TCCACAAGCTCTGTGTCCTG-3
HLA-F3	230	Forward 5-TGGAGTTGCTCCGCAGATA-3 Reverse 5-GTCCCACACAAGGAAGCTGT-3
HLA-G-C	287	Forward 5-GCTCCCACTCCATGAGGTATT-3 Reverse 5- CTGGAGGGTGTGAGAACTGG-3
HNF-1 α	164	Forward 5-GCCCAGGAGAAGAAAGAGGT-3 Reverse 5-GGTTCACAGGCTCCTTTGTC-3
HNF-1 β	192	Forward 5-TCCCCTCATGGAGAAACATC-3 Reverse 5-CACAGGAAGCTGGCATGTTA-3
HNF-3 α	228	Forward 5-TCCACAGTTGGACATGGTGT-3 Reverse 5-TTCCACGGCTTAAAATCTGG-3
HNF-3 β	322	Forward 5-AGGAGGAAAACGGGAAAGAA-3 Reverse 5-TGGATTTCACCGTGTCAAGA-3
HNF-3γ	266	Forward 5-TTGGCCACCATTCTGTGTAA-3 Reverse 5-AAATTCCCCACACCCTAACC-3
HNF-4α	238	Forward5-CGAGCAGATCCAGTTCATCA-3 Reverse 5-TTCCCATTTTTCTGGTGAGG-3
HNF-6	318	Forward 5-TAAAACCTCGGTGGAAAAGC-3 Reverse 5-AAAAGATGTCCGCTCAATGG-3
ISL-1	360	Forward 5-TCAAGAAGTCTGAAGCGACT-3 Reverse 5- AAGACCACCGTACAACCTTT-3
MDR-1	425	Forward 5-ACAAAGCGCCAGTGAACTCT-3 Reverse 5-TCACAGGCAGTTTGGACAAG-3
MDR-3	255	Forward 5-GGGAGATAAGGGGACTCAGC-3 Reverse 5-TGCTCCTTGACTCTCCCATT-3
Nanog	209	Forward5-CTCCATGAACATGCAACCTG-3 Reverse 5-CTCGCTGATTAGGCTCCAAC-3
Nestin	275	Forward 5-TATAACCTCCCACCCTGCAA-3 Reverse 5-AGTGCCGTCACCTCCATTAG-3
NKX 2.5	316	Forward 5-CATGGTATCCGAGCCTGGTA-3 Reverse 5-GAGCTCAGTCCCAGTTCCAA-3
Oct 3/4A	273	Forward 5-AGTGAGAGGCAACCTGGAGA-3 Reverse 5-GTGAAGTGAGGGCTCCCATA-3
Oct 3/4 B	194	Forward 5-TATGGGAGCCCTCACTTCAC-3 Reverse 5-CAAAAACCCTGGCACAAACT-3
Sox-2	323	Forward 5-GCGAACCATCTCTGTGGTCT-3 Reverse 5-ACATGGATTCTCGGCAGACT-3
TDO2	292	Forward 5-CCTGCGATCAACTGTGAGTG-3 Reverse 5-AGAGCATCGTGGTGCTGAAC-3
Transferrin	338	Forward 5-GGTACCAGGAAACCTGTGGA-3 Reverse 5-CTACGGAAAGTGCAGGCTTC-3

6.6 Agarose gel electrophoresis

After amplification of cDNA sequences through specific primers, the amplified were loaded in 2% agarose gel and were run in a Borax-EDTA 1x buffer. Sybr Safe DNA Gel Stain (Invitrogen) was used to stain DNA. All samples were loaded with Loading Buffer 10X (TBE 1X, bromophenol blue, glycerol and SDS 10%). Following staining gels were evaluated by the Safe Imager ^{TM} 2.0 Blue-Light Transilluminator. The size of DNA bands was estimated by parallel run of molecular weight markers.

6.7 Induction of hepatogenic differentiation

Isolated WJ-MSC through non enzymatic procedure were cultured in 25 cm^2 tissue flasks (Corning) and then expanded in 75 cm^2 and 175 cm^2 until to reach necessary number for experimental. WJ-MSC, around fifth and/or sixth passage, were cultured with a proprietary protocol (Anzalone et al, patent in preparation) in a medium supplemented with hepatic inducers. For three weeks, hepatic differentiation medium contained 1% FCS (fetal cow serum, PAA), 1x NEAA (non-essential aminoacids, Sigma), 1x antibiotics-antimycotics (GIBCO), and 2mM L-glutamine (Sigma), 10 ng/ml of human Fibroblast growth factor-4 (Miltenyi Biotech), 20 ng/ml of human Hepatic growth factor (Miltenyi Biotech), 1x of Insulin-trasferrin-selenite (Sigma) and 0,1 µM of Dexamethasone (Sigma). After 3 weeks, the cells were cultured for another two weeks with the same medium supplemented with all inducers previously described, and with addition of 10ng/ml of oncostatin M (PROSPEC). In differentiation experiments, control cells were represented by WJ-MSC themselves but cultured in a classical growth medium with 10% of FCS (fetal cow serum, PAA), 1x NEAA (non-essential aminoacids, Sigma), 1x antibiotics-antimycotics (GIBCO), and 2mM L-glutamine (Sigma). Both treated and control WJ-MSC were analyzed at the end of third, fourth and fifth week of hepatic differentiation process.

For immunocytochemical analysis and PAS Staining, cells were plated in 8-well chamber slides(BD Bioscience). For RNA extraction, cells were cultured either in 25cm^2 tissue culture flasks (Corning), and for flow cytometry analysis in 75 cm^2 tissue culture flasks (Corning). In addition, WJ-MSC were cultured in 96 well

plates (Braun) for CYP3A4 assay and in 12 well plates for Glucose 6-Phosphatase assay.

6.8 Periodic Acid Schiff staining

Periodic Acid Schiff staining (PAS-staining) is a method that permit to detect glycogen and mucopolysaccharides presence in several tissues. The periodic acid oxides the diol functional groups in glucose and other sugars, creating aldehydes. In presence of Schiff reagent these aldehydes react generating a purple-magenta colour.

WJ–MSC, plated into 8-well chamber slides, were fixed with a solution composed by 37% phormaldeyde and 95%.ethanol, for 1 minute. After washing with water for 1 minute, on the samples was added periodic acid for 15 minutes at room temperature. Subsequently to several washes with distilled water, was added Schiff reagent for 15 minutes at room temperature.

At the end, to remove Schiff reagent, was performed another wash with running water and the samples were stained with Gill Hematoxylin for 90 seconds. Slides were air-dried and observed with inverted microscope.

6.9 Flow cytometry

Briefly, treated and control WJ-MSC, detached with Tryple (Gibco) from 75 cm^2 flasks, were divided in several centrifuge tubes at concentration of $1x10^6$ cells/ml of PBS. Then the cells were conjugated with fluorescent antibodies for 15 minutes at room temperature and subsequently centrifuged at 1200 rpm for 5 minutes. Samples were read at the flow cytometer (see table 4).

Table 4: list of antibodies for flow cytometry analysis

Antigen	Clone	Conjugated	Dilution	Manufacturer
IgG1	G18-145	FITC	undiluited	Becton Dickinson Biosciences, San Jose, CA
IgG2	PC10	PE	undiluited	Becton Dickinson
albumin	188835	unconjugated	01:20	R&D Systems
αFP	189506	unconjugated	01:40	R&D Systems, Minneapolis, MN
CD29	mar-04	APC	undiluited	Becton Dickinson
CD31	WM59	FITC	undiluited	Becton Dickinson
CD34	581	FITC	undiluited	Becton Dickinson
CD44	G44-26 (also known as C26)	APC	undiluited	Becton Dickinson
CD45	2D1	PerCP	undiluited	Becton Dickinson
CD73	AD2	APC	01:11	Miltenyi Biotec GmbH, Bergisch Gladbach, DE
CD90 (Thy-1)	5 E10	FITC	01:20	Becton Dickinson
CD105	SN6	FITC	undiluited	Abcam, Cambridge, MA
CD117	YB5.B8	PE	undiluited	Becton Dickinson
CD276	FM276	APC	01:11	Miltenyi Biotec GmbH
CK18	C-04	FITC	01:20	Abcam, Cambridge, MA
CK19	RCK108	PE	01:20	Santa Cruz Biotechnology, Santa Cruz, CA
E-Cadherin	36	FITC	1/100	Becton Dickinson
EpCAM	EBA-1	FITC	undiluited	Becton Dickinson
HLA ABC	W6/32	FITC	1/300	Abcam Cambridge, MA
HLA DR	L243 (G46-6)	PerCP	undiluited	Becton Dickinson
HLA-G	87G	PerCP	undiluited	eBioscience Inc., San Diego, CA
HLA-E	3D12HLA-E	APC	undiluited	eBioscience
Oval cell	OV-6	unconjugated	1/100	Santa Cruz Biotechnology

6.10 Cardiogreen staining

Cardiogreen or indocyanine green(SIGMA) is a dye that forms non covalent fluorescent complexes with some plasma proteins, principally albumin but also ribonuclease A, transferrin and cytocrome C. Indocyanine green linked with albumin is rapidly taken up by the liver and then excreted unchanged into bile. For this reason this dye is used to evaluate liver function and cardiac output.

WJC plated in chamber slides, at the concentration of $4x10^4/5x10^4$ cells for well, after hepatogenic differentiation, were marked with cardiogreen with a final concentration of 1mg/mL and incubated at 37C° for 15 minutes. After washing with PBS, the cells were observed with an inverted phase-contrast microscope. The control cells, cultured in a normal growth medium were subjected at the same protocol.

6.11 Glucose 6-Pase assay

Glucose 6- phosphatase is a key enzyme in the glucose metabolism in particular in the gluconeogenesis and glycogenolisys. Glucose 6-Pase activity assay was based on capacity of this enzymes to transforme glucose-6 phosphate in a molecule of glucose and inorganic phosphate. This compound in presence of lead nitrate generates a precipitate of lead phosphate that with addition of ammonium sulphide originates lead sulphide, a brown precipitate.

According to protocol used, provided by Professor Etienne Sokal (University of Louvain, Belgium), we added to cells a working solution pre-warmed at 37C°, composed by lead nitrate, glucose-6 phosphate and tris-acetate buffer. After 20 minutes, the working solution was removed, three washing were performed with distilled water and subsequently, ammonium sulphide was added for 30 seconds. The presence of brown precipitates was observed by optical microscope.

6.12 CYP450 3A4 metabolic activity assay

Detoxyfication of xenobiotics such as toxins and drugs, is one of the main functions of the liver.

This process is performed by cytochrome oxidases, known with term CYP 450.

In the liver exists several isoforms of these enzymes, such as CYP450 3A4 and CYP450 2B6.

P450-Glo™ CYP3A4 Assay (Luc-PFBE) evaluates the activity of the cytochrome P450 oxidase enzymes that is directly proportional to the produced luminescence.

After addition of luminogenic substrate to the culture medium, intracellular enzymes convert this substrate to D-luciferin that is released into medium (non-lytic assay), which in presence of detection reagent produces light.

Kit P450-Glo™ CYP3A4 Assay (Luc-PFBE) (Promega) was performed on hepatocyte-differentiated WJ-MSC and control cells. Briefly, the cells plated in 96-well plates, , were previously incubated for 48 hours, with an inducer of the enzyme, rifampicin (Sigma) at the final concentration of 25μM, with or without addition of ketoconazole (Sigma) at concentration of 10 μM, a known inhibitor of CYP 3A4. The medium change was performed daily.

After washing with phosphate buffered saline, PBS, (Sigma) the cells were incubated with 50 μM of luciferin-PFBE, a luminogenic substrate, for 4 hours in the dark. Subsequently, 50 μl of medium from each well were transferred to a 96-well opaque white luminometer plate and 50 μl of Luciferin detection reagent were added. After an incubation of 20 minutes at room temperature, samples were analyzed with a luminometer (Promega) using integration times of 0,5 second.

To determine background luminescence, luminogenic substrate was added in medium without cells.

6.13 Statistical analyses

During the reading of the luminometer, the light output of the luciferase reaction is proportional to CYP activity. Obtained data by were plotted using MS Excel software and statistical analyses were performed using GraphPad Prism 4 software (GraphPad Software, San Diego, USA).

The statistical methods used were nonparametric analyses.

In particular, significance of differences of luminescence levels, expressed in RLU, between different conditions, control cell with rifampicin, treated cell in presence of rifampicin and treated cell in presence of rifampicin and rifampicin together ketoconazole, was analyzed by Mann-Whitney test. Values were considered significant for $p < 0.05$

7. RESULTS

7.1 Morphological features of Wharton's Jelly Mesenchymal Stem cells WJ-MSC

To isolate WJC from the umbilical cord matrix, we used a non enzymatic method. In our experimental model, cord sections bearing all of the substructures, are placed in culture medium, and cells are left free to exit from the cord, based on their migratory ability (which is a key characteristic of all cells named as "mesenchymal"). The adoption of this method avoids the unsafe exposure of cell surface molecules to lytic enzymes. Moreover, the umbilical cord matrix (the "niche" of such stem cells) is still in contact with cells adherent to tissue culture plates. The slow degradation of the matrix allows growth factors and signalling molecules to exit from the cord and continuing provide a positive stimulation to the cultured cells. As visible in figure 4, WJ-MSC grow robustly on plastic surfaces, and maintain a fibroblastoid morphology both at low and high confluence. (see figure 5).

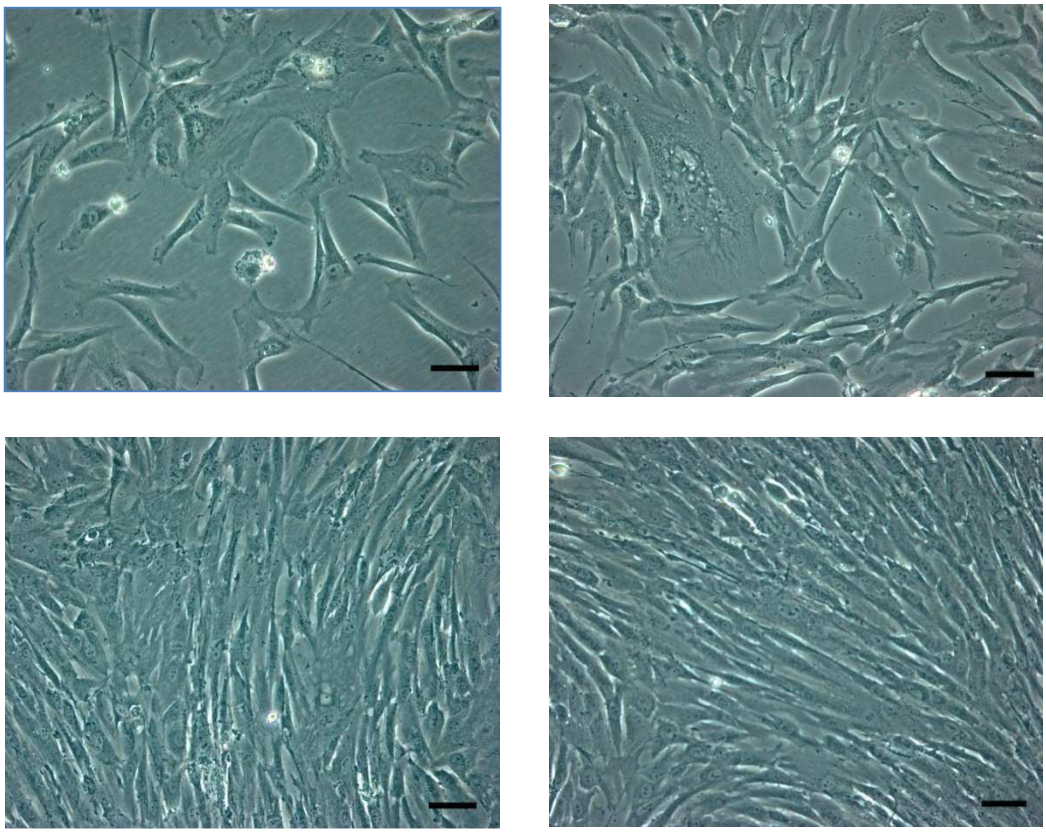


Figure 5: phase-contrast panel of micrographs of WJ-MSC at different confluences. Magnification 20x. Bar: 70µ.

7.2 Phenotypical characterization of undifferentiated WJ-MSC by RT-PCR and ICC

Studies previously performed by our research group demonstrated that these cells can proliferate for several passages in culture, maintaining a constant telomere length over time, and a stable karyotype.

In addition, molecular analyses highlighted the presence of mRNA of some endodermal molecules, such as GATA-4, -5, -6, and HNF4α (GATAs expression is shown in figure 6), assuming that these cells may differentiate into endoderm-derived cell types such as hepatocyte-like cells.

We therefore further characterized these cells by immunocytochemistry analysis and RT-PCR, evaluating expression of endodermal and mesodermal markers typical of differentiated cells and markers of embryonic and mesenchymal stem cells. Moreover, to understand if these cells can be reliable candidates for cellular therapies we also analyzed expression of immune-modulatory molecules potentially involved in the establishment of a immune tolerance in the host.

As shown in figure 7, undifferentiated WJ-MSC express some tissue–specific markers: BAAT (Bile acid-CoA:amino acid N-acyltransferase), a liver enzyme involved into bile metabolism conjugation, in particular in the step of conjugation before excretion into bile canaliculi; ISL-1 (insulin gene enhancer protein), a protein that links enhancer region of insulin gene, playing an important role in regulation of insulin gene; NKX2.5 (cardiac-specific homeobox, NK2 transcription factor related, locus 5 Drosophila) involved in hearth formation and development.

Molecular analysis also demonstrated that WJ-MSC express ABC carriers such as ABCG2 and MDR-3, responsible of multi-drug resistance phenotype. In literature, several works have recognized the presence of ABC carriers in adult and embryonic stem cells.

A key feature of WJ-MSC is the capability to undergo self-renewal, maintaining their replicative potential together with their undifferentiated state. In this respect, as shown in figure 7, these cells express Nanog, Oct3/4A, Sox2, responsible for maintaining a long-term self-renewal and the undifferentiated state in embryonic stem cells.

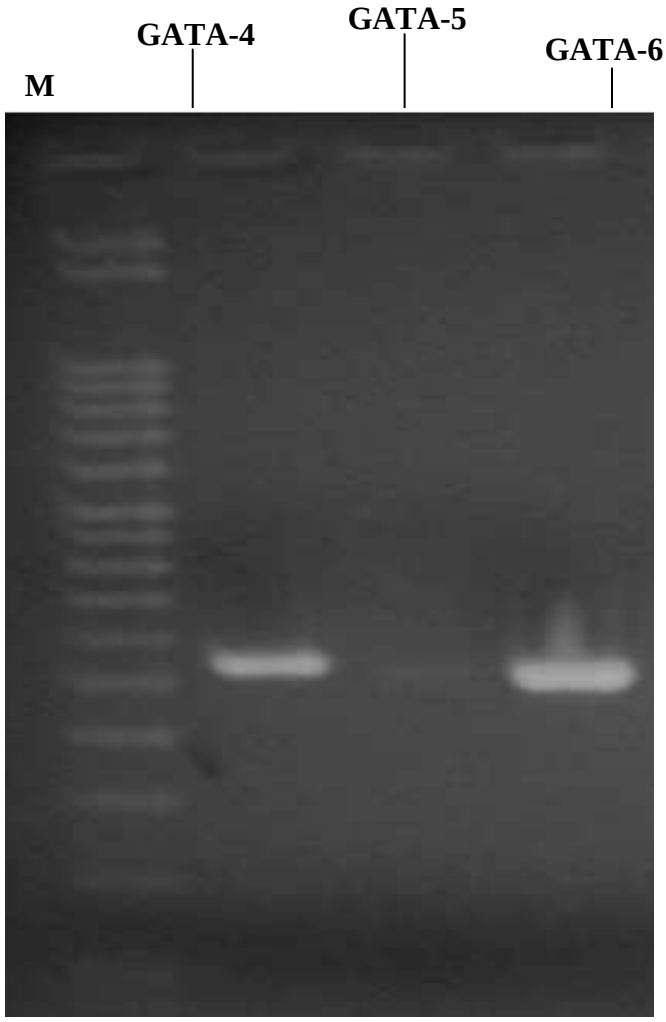


Figure 6: RT-PCR analysis of expression of endodermal markers in WJ-MSC by RT-PCR. Marker 50 bp.

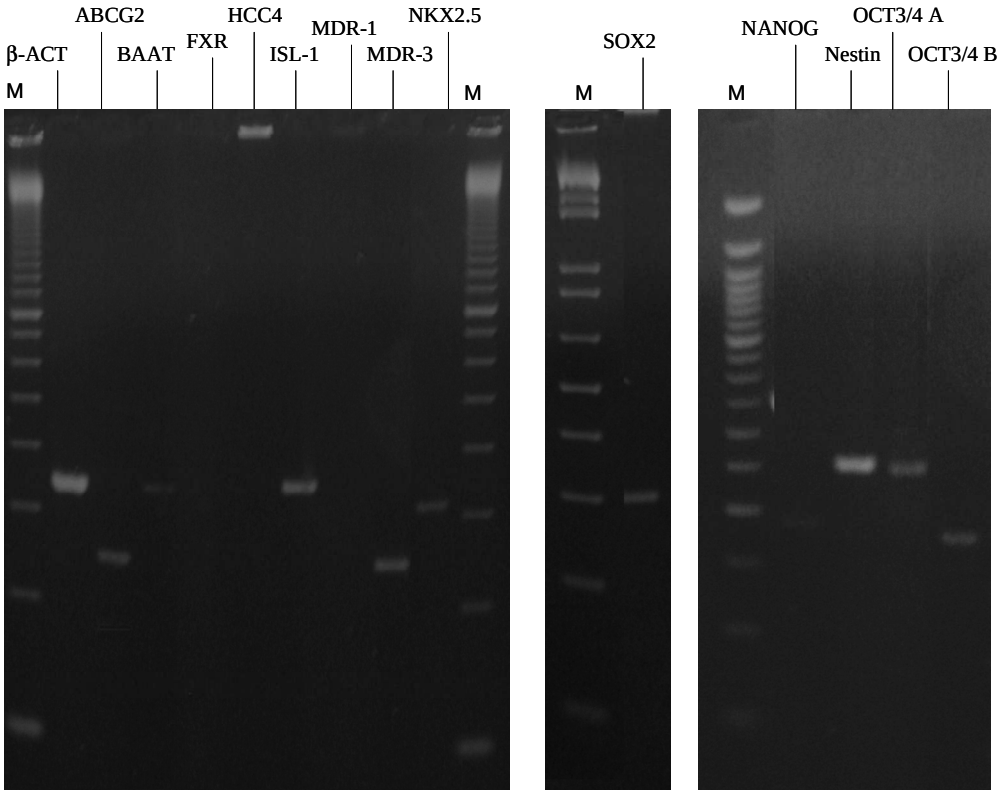


Figure 7: Analysis by RT-PCR of expression of tissue specific markers and self-renewal markers typically expressed in embryonic stem cells. Marker 50 bp.

Moreover, we evaluated the expression of hepatocyte-specific markers by WJ-MSC, demonstrating that also early and late hepatic molecules such as alphafetoprotein (AFP) and albumin are expressed by undifferentiated WJ-MSC at the mRNA level. (see figure 8)

Taken together, these data extend our previous published results aiming to the characterization of markers expressed by undifferentiated cells. This analysis is of fundamental importance for the correct definition of the actual phenotype of these cells.

To further extend our characterization effort, we investigated the expression of hepatic markers by RT-PCR on undifferentiated cells. Figure 8 shows the results of this analysis: WJ-MSC express factors belonging to the Fox A protein family, also known as Hepatocyte nuclear factors, and involved in liver development at early or late stages such as HNF-1α, HNF-1β, HNF-3α and HNF-4α. Moreover, WJ-MSC expressed at messenger level also a hepato-specific enzyme such as tryptophan 2,3 dioxygenase, involved in tryptophan metabolism. On the contrary, undifferentiated cells did not express Glucose-6-phosphatase, a key enzyme involved in glycogen metabolism in liver. Alpha-1-antitrypsin, a serine protein inhibitor, was expressed by WJ-MSC, as well as epithelial cytokeratins such as CK-8, -18, -19. It is important to note that most of these “in vitro” markers are considered useful for characterizing differentiated cells, but cannot obviously constitute a reliable evidence on their own. This initial effort will guide us through the choice of reliable markers and enzymatic activities which should be induced or clearly upregulated in WJ-MSC subjected to hepatic differentiation.

In liver there are 7 types of connexins, and connexin-32 represents the 90% of the total connexins. Studies in literature, evaluated the role of connexin-32 in liver specific processes such as xenobiotic biotransformation, ammonia detoxification, albumin secretion and glycogenolysis. RT-PCR analysis, illustrated in figure 9, showed expression by our umbilical cord cells of connexins 26, 43, data also confirmed by ICC (see in figure 10 panel D), and 45 but not of connexin-32 and connexin-40.

For some of the selected markers which were investigated in undifferentiated cells by RT-PCR analyses, we also searched for their expression at the protein level.

Figure 10 shows the results of ICC analyses on undifferentiated WJ-MSC. As shown, undifferentiated cells are widely positive for hepatocyte-specific epithelial cytokeratins, namely CK-8 and CK-18, at the cytoplasm level. Moreover, as visible in figure 10 (panel C), HNF4α expression has been demonstrated in the vast majority of cells. As visible, this transcription factor shows a clear nuclear localization, therefore suggesting that also in undifferentiated cells it is active in the induction of regulated genes. The other evidences we reported here, namely the expression of albumin and alpha-fetoprotein, should be better explained by the strong nuclear positivity of this marker, since both have inducible elements responding to HNF4α binding in their promoter region. Moreover, we demonstrated the expression of connexin-43 with a clear membrane staining, therefore suggesting that gap junctions are extensively formed between these cells, with the obvious functional consequences.

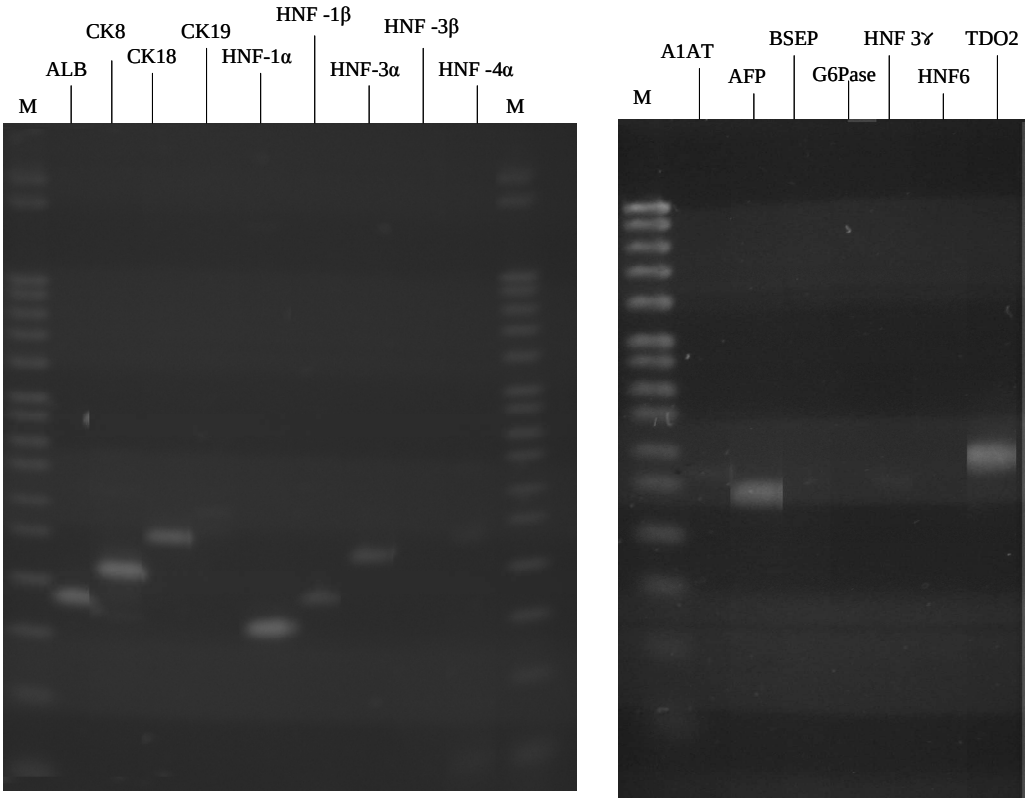


Figure 8: Analysis by RT-PCR of expression of early and late hepatic molecules in undifferentiated WJ-MSC. Marker 50 bp

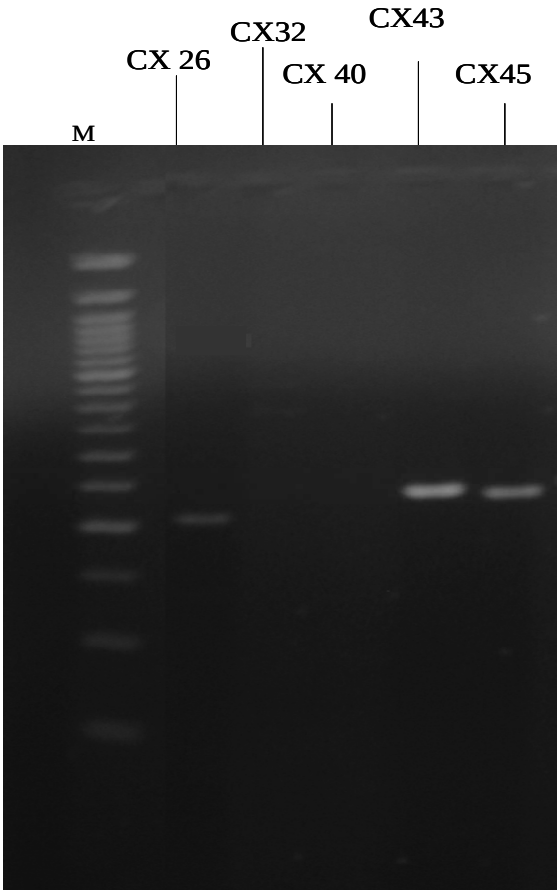


Figure 9: Analysis by RT-PCR of expression of connexins in undifferentiated WJ-MSC by RT-PCR

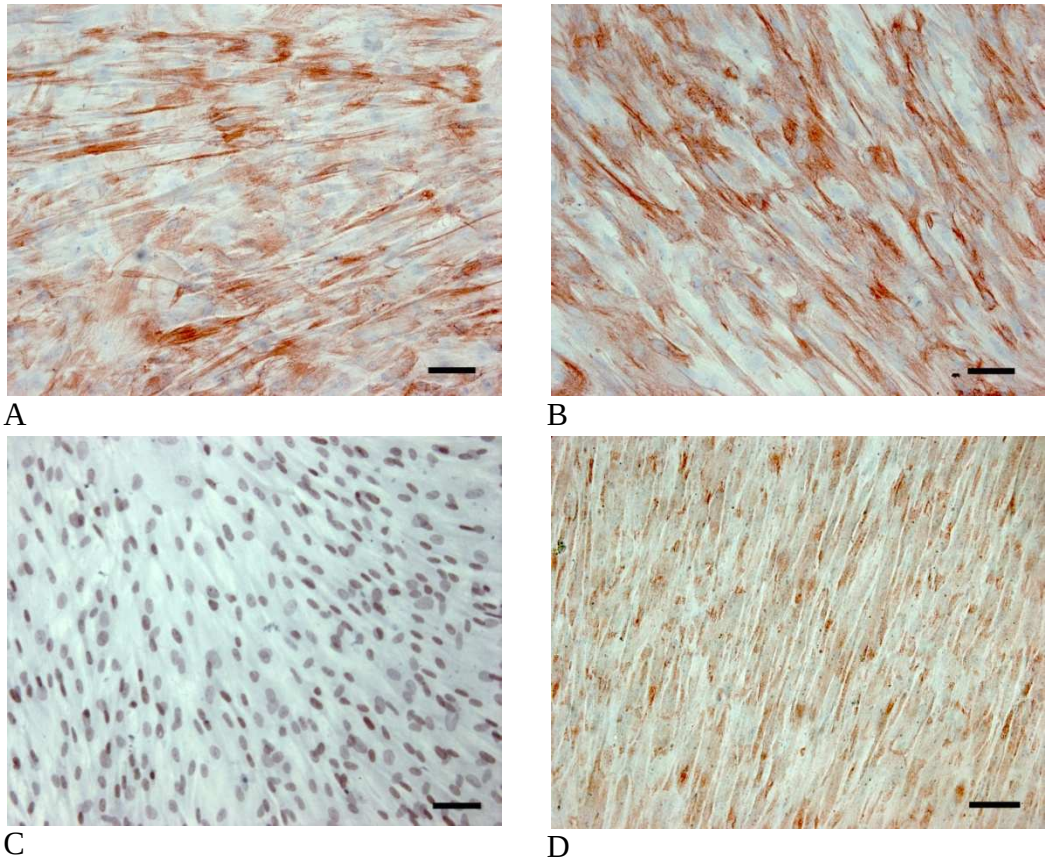


Figure 10: Immunocytochemical analysis of expression of CK8, CK18, HNF-4α and connexin 43 in undifferentiated WJ-MSC.A: CK-8, B:CK-18; C: HNF-4α, D:connexin 43. Magnification: 20X. Bar: 70µ.

7.3 Expression of immunomodulatory molecules by WJ-MSC

According to general consensus mesenchymal stem may be considered good candidates for cellular therapy also for their features of hypoimmunogenicity and immune response modulation. Therefore, we investigated the expression of immunomodulatory molecules by WJ-MSC to understand if these cells could express molecules that would be useful in vivo to evade or modulate the host immune response. As shown in figure 11, by RT-PCR analyses we evaluated the expression of first class non classical MHC (major histocompatibility complex) molecules, known to be involved in tolerance induction of natural killer cells and in the establishment of the tolerance process of mother toward fetus, such as HLA-E, HLA-G, HLA-F (isoforms 1 and 3). Moreover, we investigated also the expression of CD66f, a glycoprotein expressed by placental cells, capable to induce secretion of anti-inflammatory cytokines and abolish induced-antigen proliferation by T cells. As shown, all of these molecules are expressed by undifferentiated WJ-MSC at the messenger level. For some of these molecules, this is the first reported evidence of expression in any MSC subpopulation, and this should further increase the interest towards these cells as immunomodulators in vivo. We also confirmed our previous data showing that WJ-MSC express B7-1 (CD80) and lack B7-2 (CD86). Literature data on BM-MSC point out that both co-stimulators should not be expressed in these cells, but there is supporting evidence that the combination of the expression of CD80 and lack expression of CD86 may favour a synergistic role in the induction of tolerance, as suggested by literature data. In fact, CD86 acts on T-lymphocytes mainly by CD28 binding, while CD80 alone may act via the CTLA-4 receptor, therefore resulting in a co-inhibition mechanism, with respect to the classical co-stimulatory role.

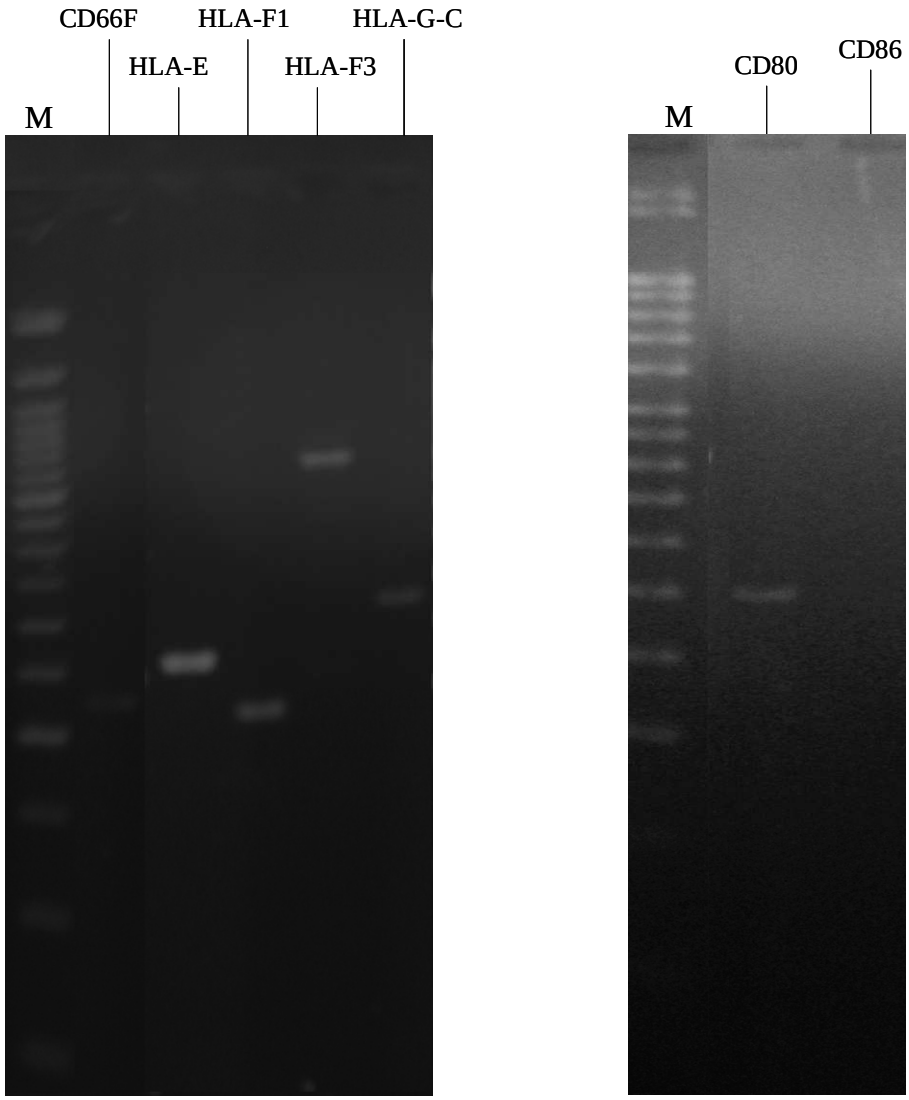


Figure 11: Analysis by RT-PCR of expression of immuno-modulatory molecules involved in tolerance processes by undifferentiated WJ-MSC.

7.4 Hepatogenic differentiation of WJ-MSC: methodology and morphological evidences

After the extended phenotipycal characterization of WJ-MSC, we tried to induce hepatogenic differentiation in our cells. We developed a novel protocol of induction (Anzalone et al, patent in preparation) in order to partly replicate in vitro some of the inductive processes acting in vivo during liver morphogenesis and hepatocytes specification. As a pre-inductive step, we cultured WJ-MSC, for 3 weeks, in a medium with 1% FCS, antibiotic/antimycotic, non essential aminoacids, HGF (hepatocyte growth factor), FGF-4 (fibroblast growth factor-4), sub-micromolar concentration of dexamethasone, and insulin-transferrin-selenite (ITS) supplement. After 3 weeks, the cells were cultured in a maturation medium composed by all factors previously described with the addition of oncostatin M, for another 2 weeks. Time points which were considered for the following analytical phase were: 3, 4, 5 weeks. Parallel controls were performed in which cells were cultured in the standard growth medium for same timeframe of treated cells, with medium changes at the same periodicity. Both control and differentiated cells were cultured on plastic surfaces in order to strictly determine the effects of growth factors administered, without the overlapping inductive effect of a three dimensional substrate further providing signals (e.g. matrigel).

Applying this hepatic differentiation protocol in two steps we tried to mimic in vitro the succession of inductive processes occurring in vivo during hepatogenesis During early liver development phase, the cardiac mesodermal cells release FGF-4, to induce hepatic pre-differentiation step into ventral cells. Successively, at hthe stage of hepatoblasts, septum transverse is broken and these cells contact endothelial cells which release HGF, enhancing therefore hepatic fate by these cells. Fetal liver is also an hematopoietic site, and studies demonstrated that hematopietic stem cells, residents in the organ, release OSM contributing to maintain hepatic differentiated phenotype.

The monitoring of the differentiation process by phase-contrast microscopy, alongside differentiation experiments, allowed to determine first of all a clear morphological switch of treated cells compared with undifferentiated controls. In fact, at the considered time points (after 3-4-5 weeks), the cells cultured in an

hepatic differentiation medium appeared with a quite regular polygonal morphology, resembling cultured mature hepatocytes. The control cells, indeed, maintained fibroblastic-like morphology, as shown in figure 12.

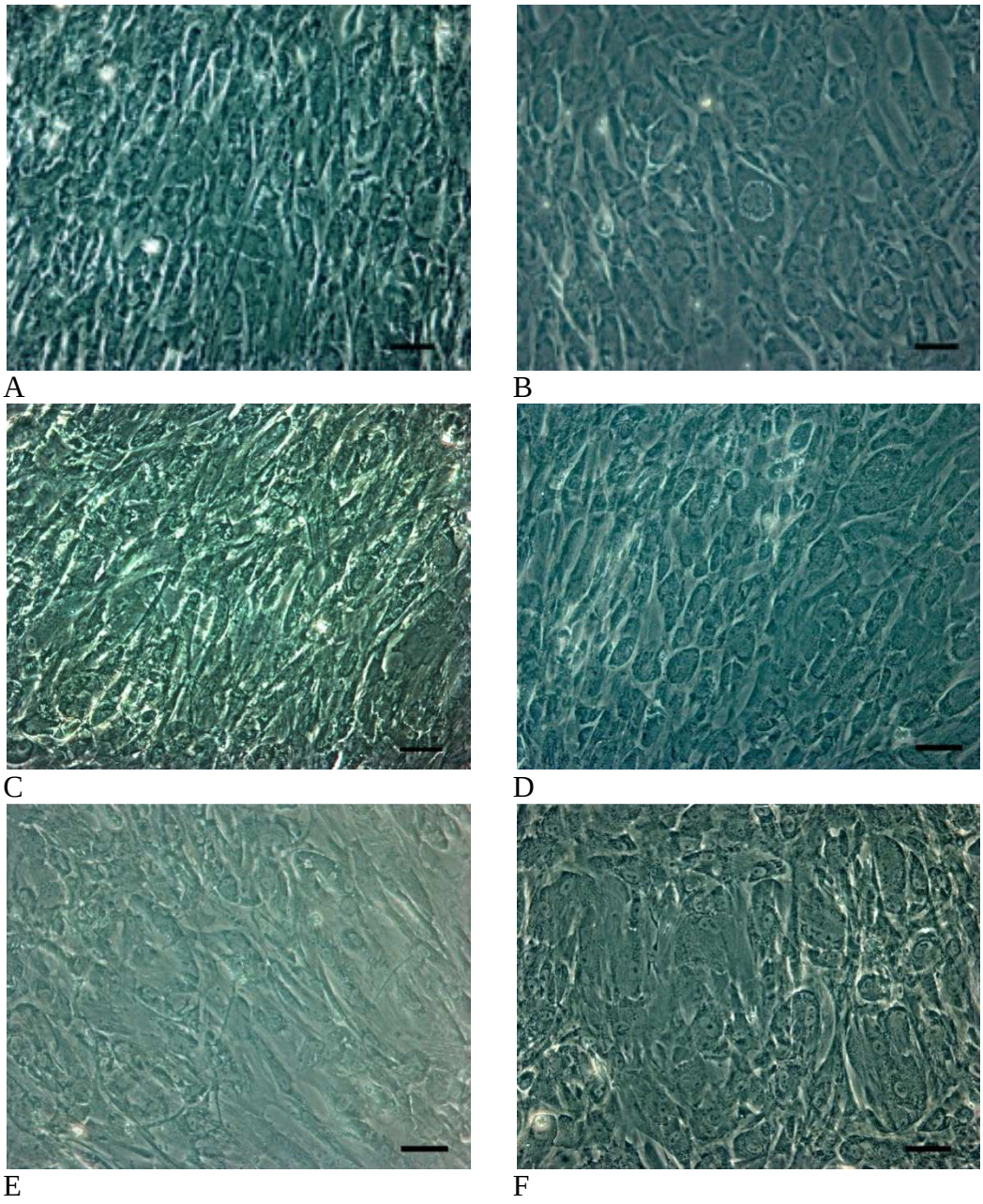


Figure 12: Phase-contrast micrographs panel of control cells (A; C; E) and hepatic induced cells (B; D; F) at different steps of hepatic differentiation. A-B: 3 weeks; C-D: 4 weeks; E-F: 5 weeks. Magnification 20 x. Bar: 70µ.

7.5 Periodic Schiff Acid staining

To confirm the acquisition of hepatocyte differentiation we performed PAS staining on treated and control cells. This stain highlights the presence of glycogen intracellularly. As shown in figure 13 A-C-E, control cells showed a pale blue staining, while treated cells, at all of the analyzed time points, showed a polygonal shape typical of hepatocytes and an intense diffused purple-magenta stain strongly suggesting the presence of glycogen deposits (Figure 13 B-D-F).

7.6 Early and late hepatic expression by RT-PCR in differentiated WJ-MSC

We performed RT-PCR analysis to determine the expression of selected genes by untreated and differentiated cells at the different time points considered. As summarized in table 5, we observed that undifferentiated cell express some hepatic differentiation molecules such as albumin, HNF4α, CK18, CK19, CK8. After 3 weeks of treatment in an hepatic differentiation medium, WJ-MSC, at a stage which should resemble hepatoblasts, started expressing molecules and receptors for factors involved during liver development process. In particular, we demonstrated that since week 3 there is a clear induction of HNF6, a nuclear factor involved in hepatocyte differentiation, as well as GYS2L (glycogen synthase-2, liver specific isoform), an enzyme involved in glycogen metabolism. This datum provides a further confirmation of the acquired ability of these cells to produce and store glycogen as already demonstrated by PAS staining.

After 4 weeks of treatment the cells maintained the expression of above mentioned molecules, and interestingly switched off the expression of CK19. In particular, this “cytokeratin switch” is known to be 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 population. Moreover, from fourth week onwards, cells started expressing the mRNA for BSEP (Bile salt export protein), a transmembran protein involved in bile transport across hepatocyte membrane towards biliar capillaries. At fifth week of hepatic differentiation, the switch of expression of CK-19 was maintained, as well as the

other de novo induced molecules mentioned above. Part of these data were further analyzed and confirmed at the protein level by ICC and FC techniques see below). Taken together, these data clearly suggest that the protocol we used for differentiating hepatocytes from our undifferentiated starting population clearly causes the de novo expression of specific mRNAs of the mature hepatocyte lineage.

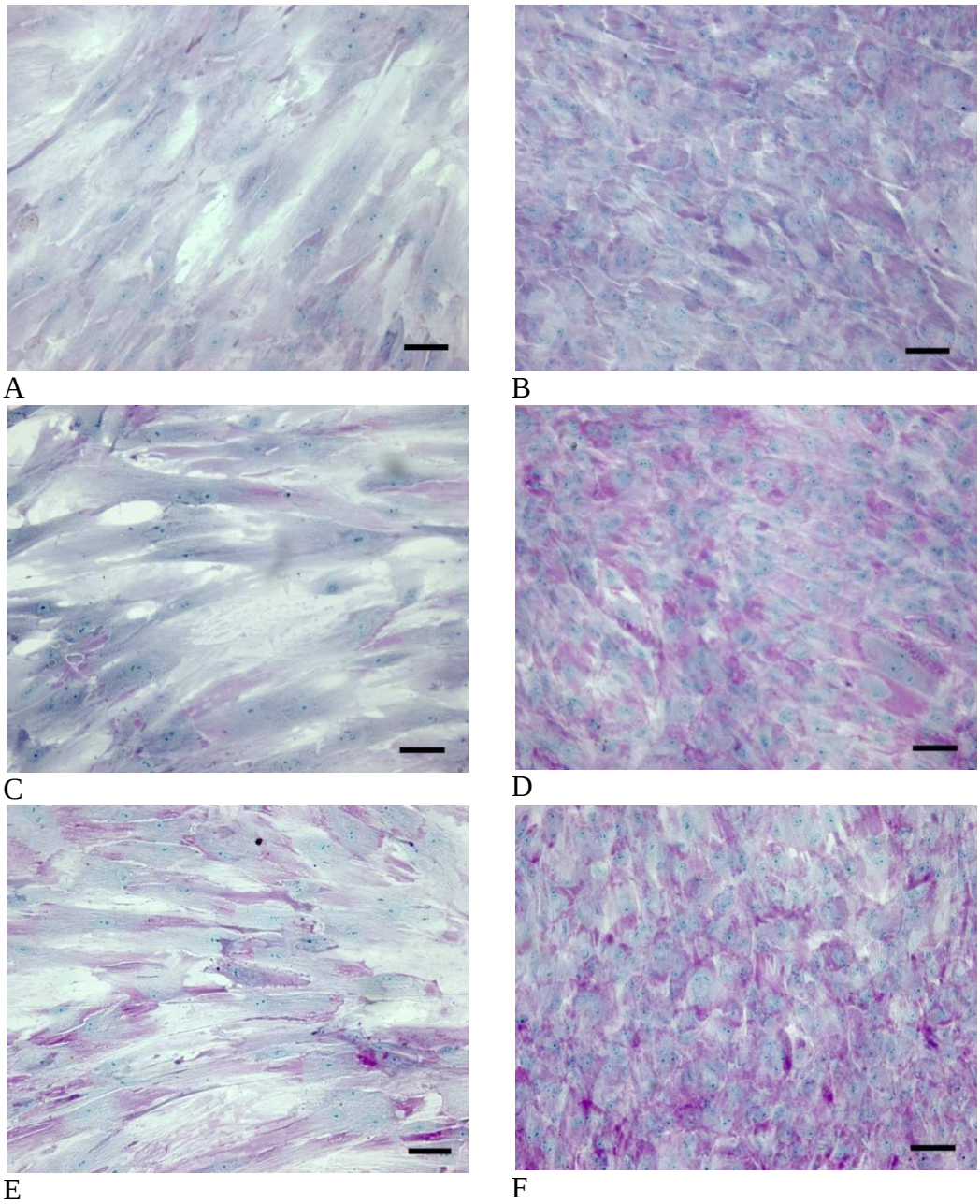


Figure 13: Microscopic demonstration of glycogen deposits. Micrographs panel of control cells (A-C-E) and treated cells (B-D-E) at 3-4-5 weeks, after PAS staining. A-B: 3 weeks; C-D: 4 weeks; E-F: 5 weeks. Magnification 20x. Bar: 70µ

Undifferentiated WJ-MSC	**WJ-MSC at III weeks hepatic differentiation**	**WJ-MSC at IV weeks hepatic differentiation**	**WJ-MSC at V weeks hepatic differentiation**
Albumin + (*)	Albumin + (*)	Albumin + (*)	Albumin + (*)
CK8 + (*)	A1AT +	A1AT +	A1AT +
CK18 + (*)	CK8 +	BSEP +	BSEP +
CK19 +	CK18 + (*)	CK8 +	CK8 +
HNF4α + (*)	CK19 +	CK18 +(*)	CK18 +
Transferrin +	GYS2L +	CK19 -	CK19 -
	HNF4α + (*)	GYS2L +	GYS2L +
	HNF6 +	HNF4α + (*)	HNF4α + (*)
	FGFR2 +	HNF6 +	HNF6 +
	FGFR4 +	FGFR2 +	FGFR2 +
	Transferrin +	FGFR4 +	FGFR4 +
		Transferrin +	Transferrin +

Table 5: expression of early and late hepatic molecules at the mRNA level in undifferentiated and hepatic-induced cells, at different weeks (*) indicates data cross-confirmed by ICC and FC at the protein level.

7.7 Characterization of differentiated WJ-MSC by flow cytometry analysis

Flow cytometry analysis of undifferentiated and hepatocyte-differentiated cells was performed to analyze the expression of both MSC core markers and epithelial or hepatic specific molecules. As shown in figure 14, undifferentiated and differentiated cells at 3-4-5 weeks expressed CD29,CD44and HLA-ABC, at comparable levels with respect to the undifferentiated counterpart. Moreover, both undifferentiate3d and hepatocyte-differentiated cells lack expression of hematopoietic markers such as CD34, CD45 and HLA-DR. In addition, we showed that the differentiation protocol resulted in a clear down-regulation of CD105 expression, while levels of CD31, CD117, OV-6, remained similar between the two conditions. In particular, the results on the expression of OV-6, a marker for oval cells, thought to be a progenitor population resident in liver, which antigen is not yet known, show that also undifferentiated cells are highly positive to this marker, whose levels appear to be unaffected by the differentiative stimuli applied. The putative identity of OV-6 antigen with one member of the cytokeratins family in mice, further points out its limited value as specific differentiation marker, also in the light of the number of CK molecules we demonstrated to be expressed in WJ-MSC.

Epithelial markers such as EP-CAM (Epithelial cell adhesion molecule, involved into interactions between lymphocytes and epithelial cells) and E-Cadherin (epithelial cadherin, a Ca2+ dependent transmembran protein) were expressed in control WJ-MSC and in all stages of hepatic differentiation. As shown in figure 15, is possible observe a net increase in the expression of both markers at most time points.

AFP characterizes early hepatocytes, and flow cytometry analysis showed that its levels of expression were maintained higher than undifferentiated cells at all the considered time points of the differentiation process (Figure 16A).

Also for albumin expression flow cytometry showed that its expression was higher in differentiated cells with respect to undifferentiated ones (see figure 16B), further confirming what observed with ICC analyses.

Figure 17 shows the results of flow cytometry analysis of Cytokeratin-18 and -19 expression. As visible, CK-18 clearly increases at fifth week of differentiation, while Cytokeratin 19 is expressed at lower levels which further decrease in differentiated cells. Therefore, flow cytometry analysis confirmed the "cytokeratins switch" in differentiated cells, as already showed by RT-PCR analyses.

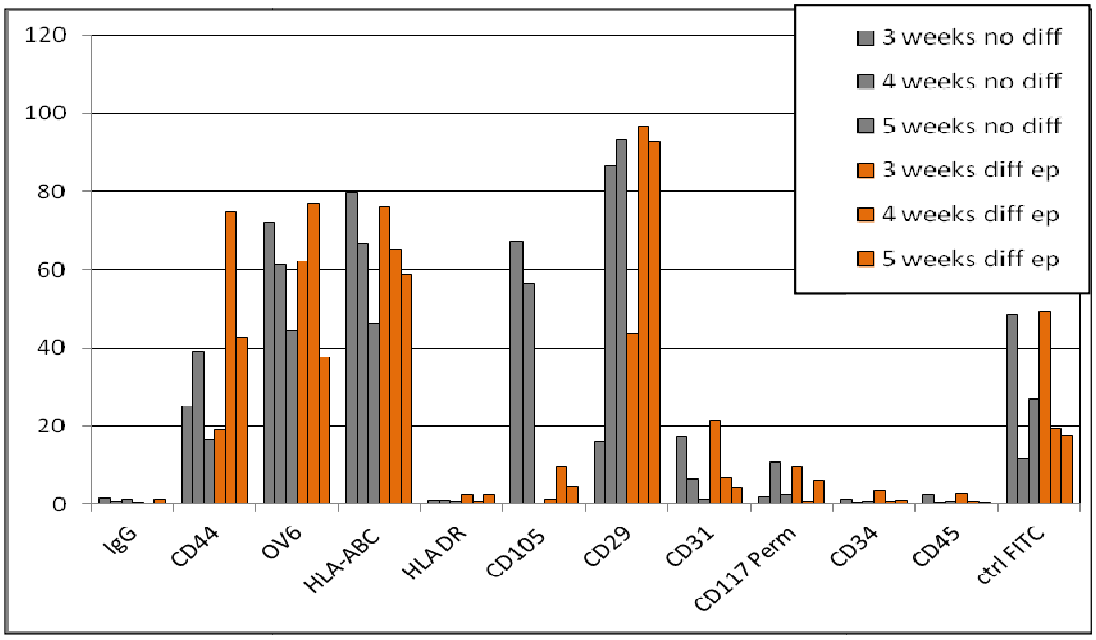


Figure 14: Flow cytometry analysis of expression of “core markers” typical of bone marrow derived mesenchynmal stem cells

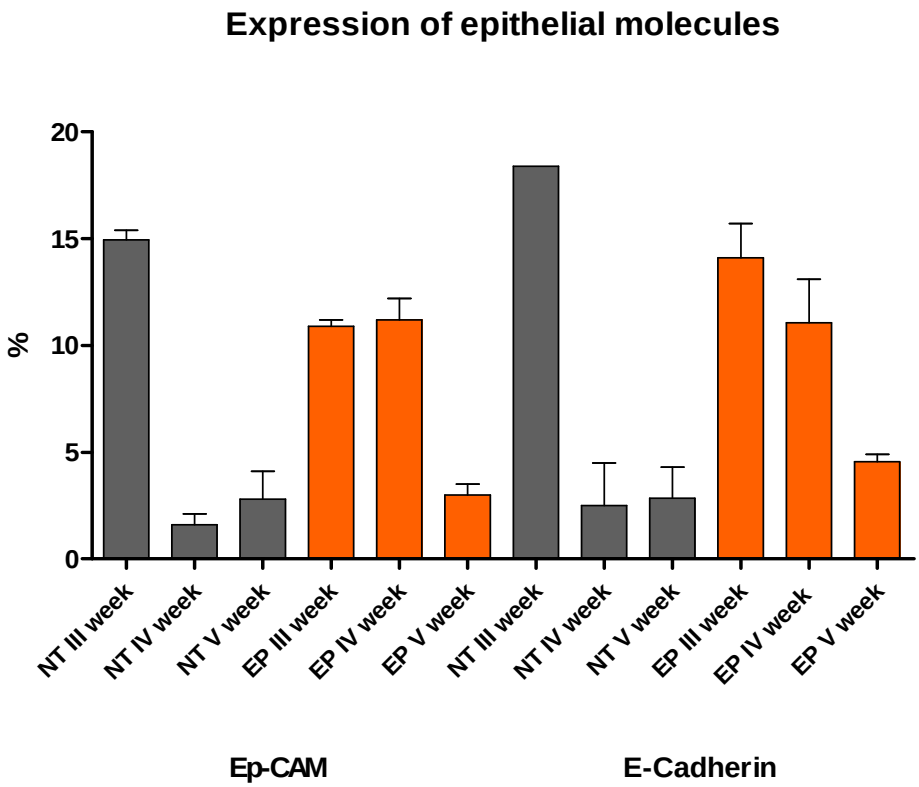
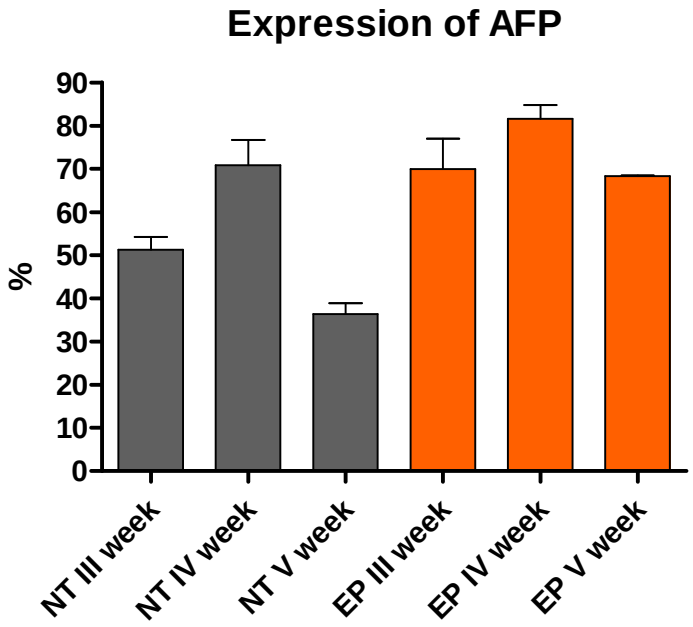
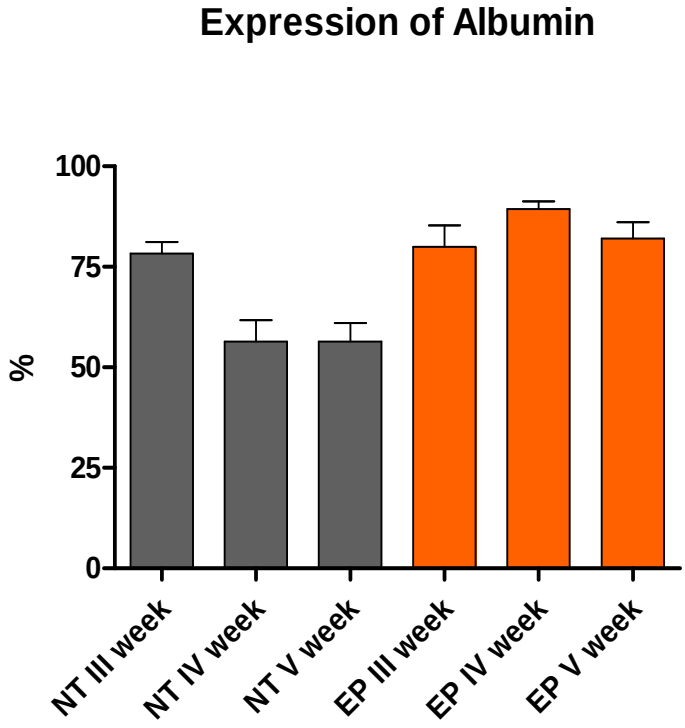


Figure 15: Flow cytometry analysis of expression of epithelial markers in undifferentiated and hepatocyte-differentiated cells at the considered time points, assessed by flow cytometry.



A



B

Figure16: A: Flow cytometry analysis of expression of alpha-fetoprotein (AFP), an early hepatic protein, by flow cytometry; B: expression of albumin, a late hepatic marker, by flow cytometry

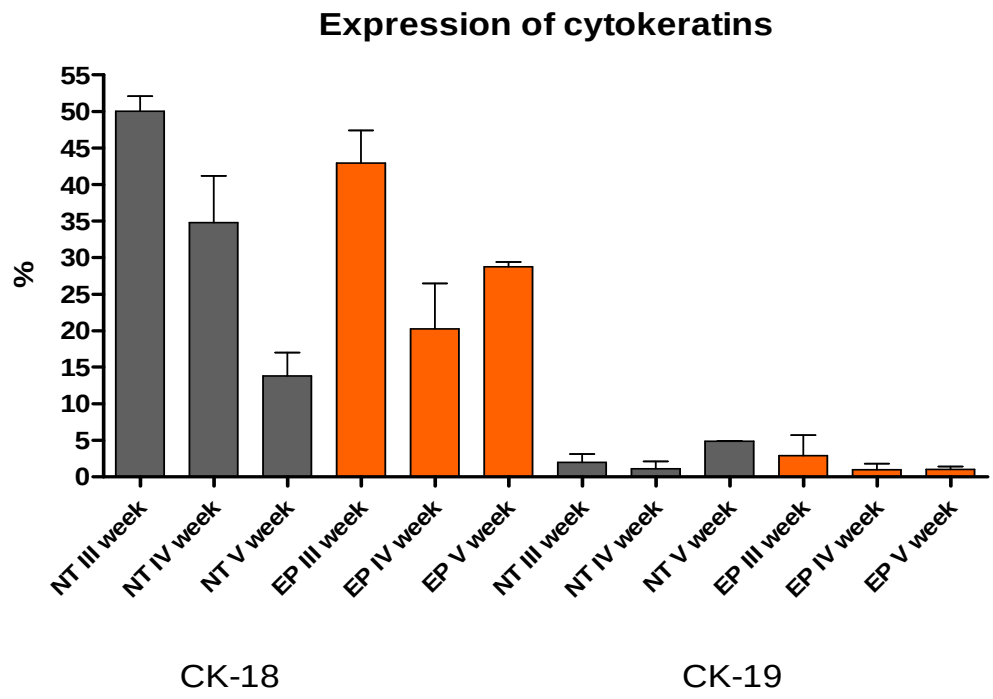


Figure 17: Flow cytometry analysis of expression of cytokeratins by flow cytometry in undifferentiated and differentiated cells.

7.8 Expression of hepatic molecules in differentiated WJ-MSC by ICC

Confirm of hepatic phenotype by differentiated WJ-MSC was further assessed by immunocytochemistry analysis.

The expression of CK-18 in undifferentiated and differentiated cells was also confirmed by ICC after both three and four weeks of differentiation (see figure 18).

Flow cytometry allowed demonstrating that control cells expressed albumin, but its concentration decreased at fifth week. Conversely, in hepatic differentiation induced cells, in all weeks, levels of expression of albumin protein were constantly higher than controls. As shown in figure 19, immunocytochemistry allowed further confirming these data.

Another hepatic marker evaluated by immunocytochemistry, was HNF-4α. This factor has an important role, in the conversion of parenchyma in epithelium during liver development and in maintaining the differentiated hepatic phenotype. In cells cultured in a standard growth medium, HNF-4α expression and nuclear localization decreased progressively with culture time, while in treated cells expression and nuclear localization were maintained at all the considered time points (see figure 20).

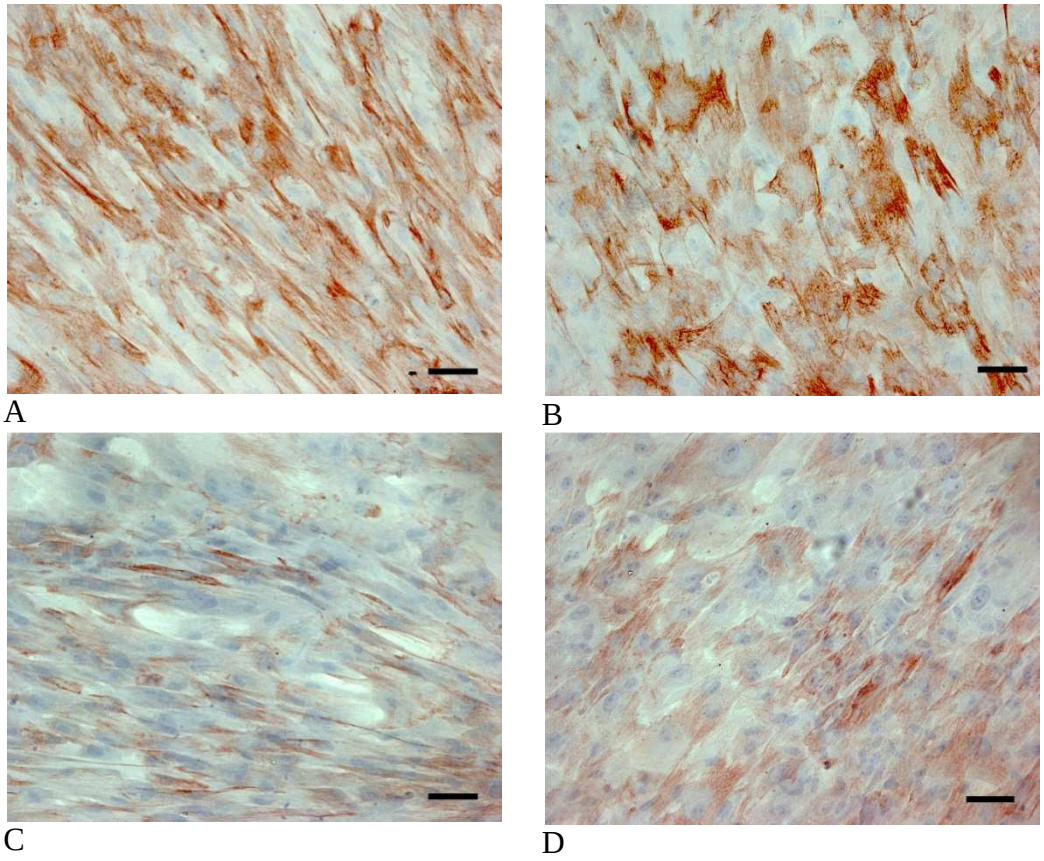


Figure 18 : Immunocytochemical analysis of expression of cytokeratin 18 in control cells (A-C) and hepatocyte-like cells (B-D) at 3 -4 weeks, respectively. A-B: 3 weeks; C-D: 4 weeks; Magnification 20x. Bar: 70µ

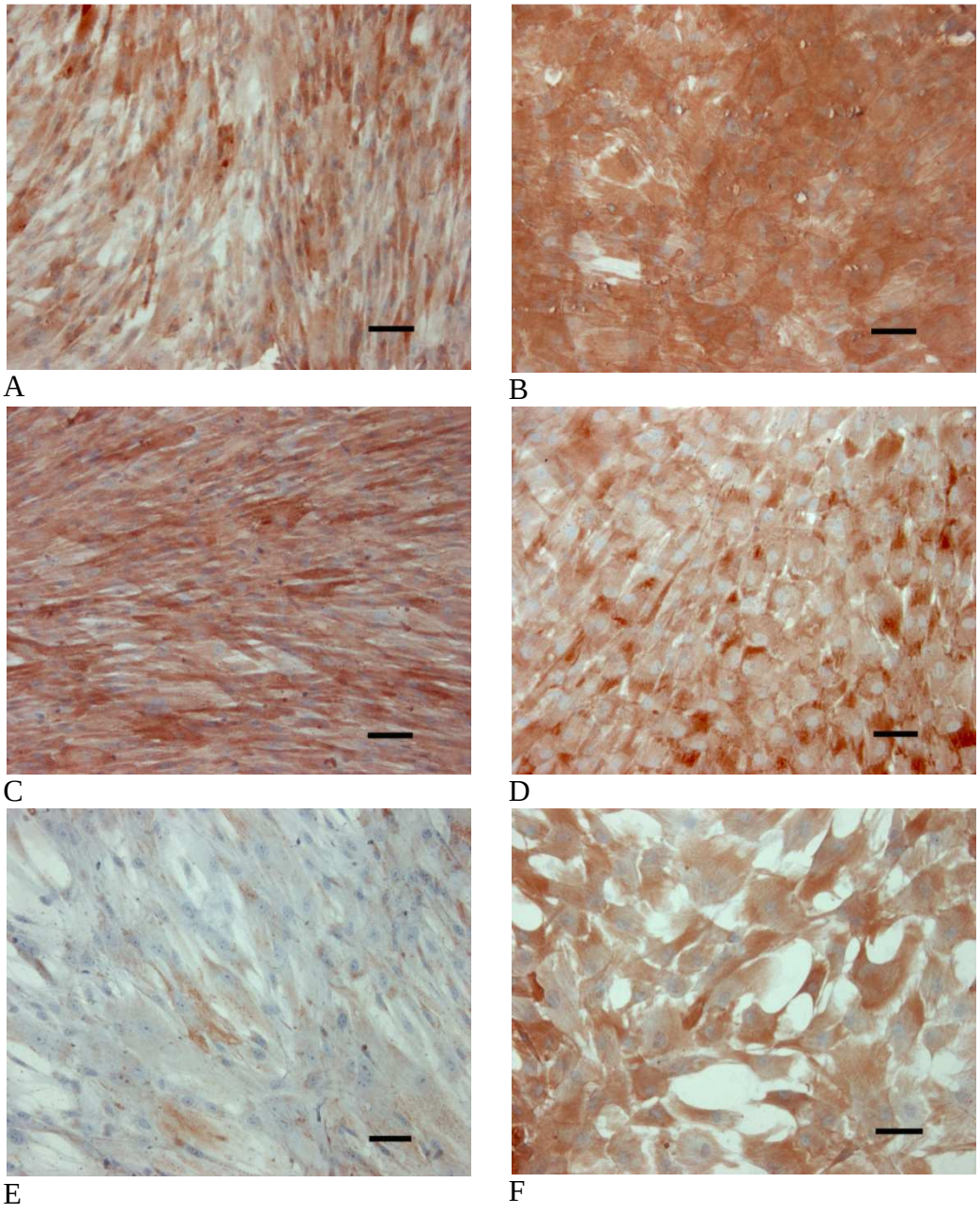


Figure 19: Immunocytochemical analysis of expression of albumin in control and treated WJ-MSC by ICC. A-C-E (control cells), B-D-F (hepatic differentiation). A-B: 3 weeks; C-D: 4 weeks; E-F: 5 weeks. Magnification 20x. Bar: 70µ

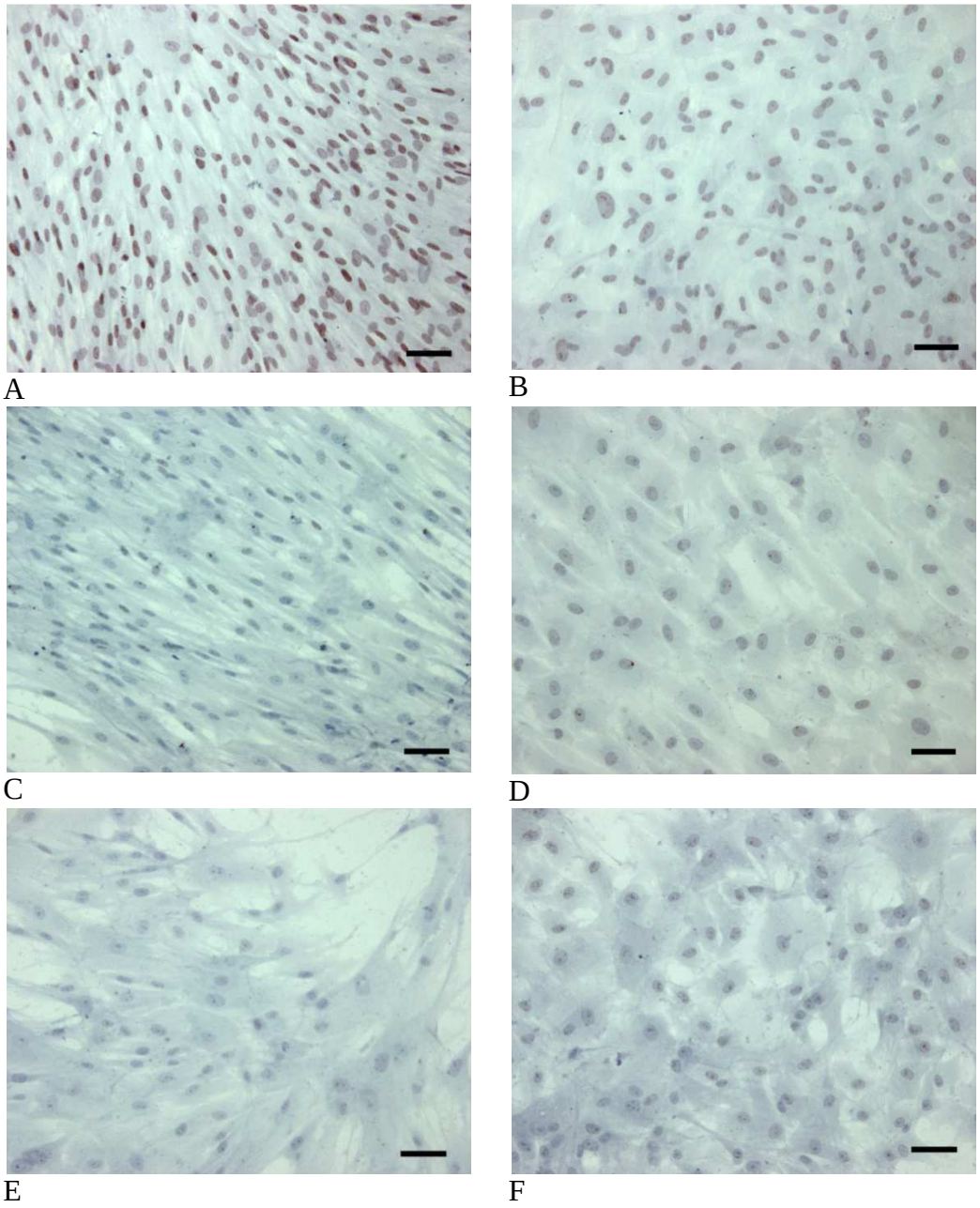


Figure 20: Immunocytochemical analysis of expression of hepatic nuclear factor-4α (HNF-4α) in control cells (A-C-E) at 3-4-5 week respectively and treated cells (B-D-F) at 3-4-5 weeks. A-B: 3 weeks; C-D: 4 weeks; E-F: 5 weeks. Magnification 20x. Bar: 70µ.

7.9 ICG staining

The acquisition of hepatic phenotype can also be confirmed by the presence of selective uptake of specific molecules. To this extent, both Ac-LDL and cardiogreen are widely used. It should be noted that Ac-LDL is also uptaken by endothelial cells, therefore Cardiogreen was chosen for this study. ICG (indocyanine green or cardiogreen) binds to the plasma proteins, in particular albumin, is taken up by hepatocytes and secreted into bile. For this reasons ICG staining may be used to assess liver function. As visible in figure 21, control cells appeared negative while hepatocyte-like cells exhibited a green cytoplasmatic staining.

7.10 Glucose-6-Phosphatase assay

Glucose 6-phosphatase assay was performed on control and hepatocyte-like cells to evaluate the presence and activity of one of main hepatic enzymes, involved into glucose metabolism. As visible in figure 22, cells cultured in a standard growth medium fail to form brown precipitates of lead sulphide. Conversely, hepatocyte-like cells, in all differentiation steps, exhibited a brown colouring due at the presence of precipitates.

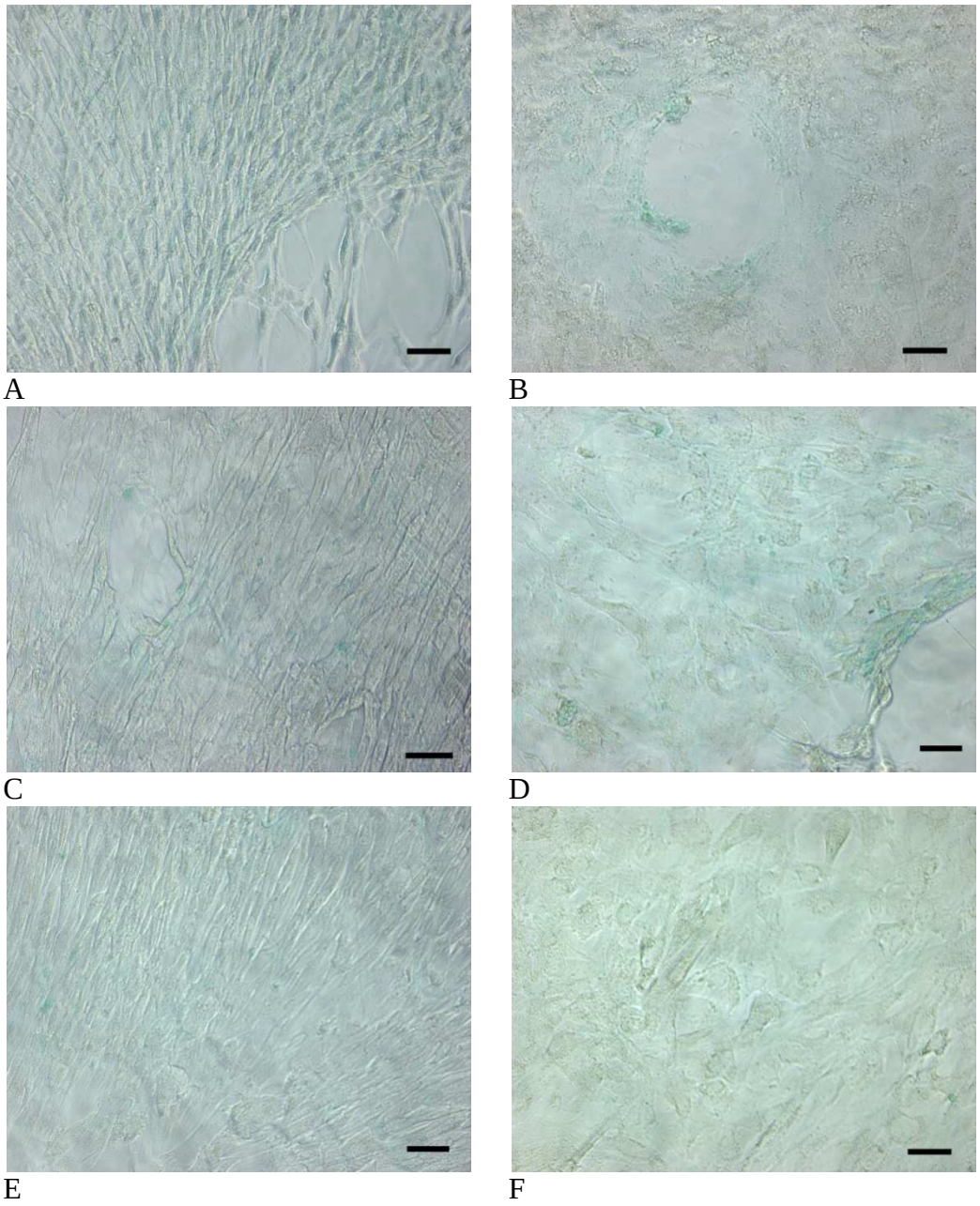


Figure 21: Microscopic demonstration of Cardiogreen uptake by Hepatocyte-like cells. Control cells were negative, while treated cells are positive. A-C-E: control cells at 3-4-5 week, B-D-F differentiated cells at 3-4-5 weeks. A-B 3 weeks; C-D 4 weeks; E-F 5 weeks. Magnification 20x. Bar: 70µ

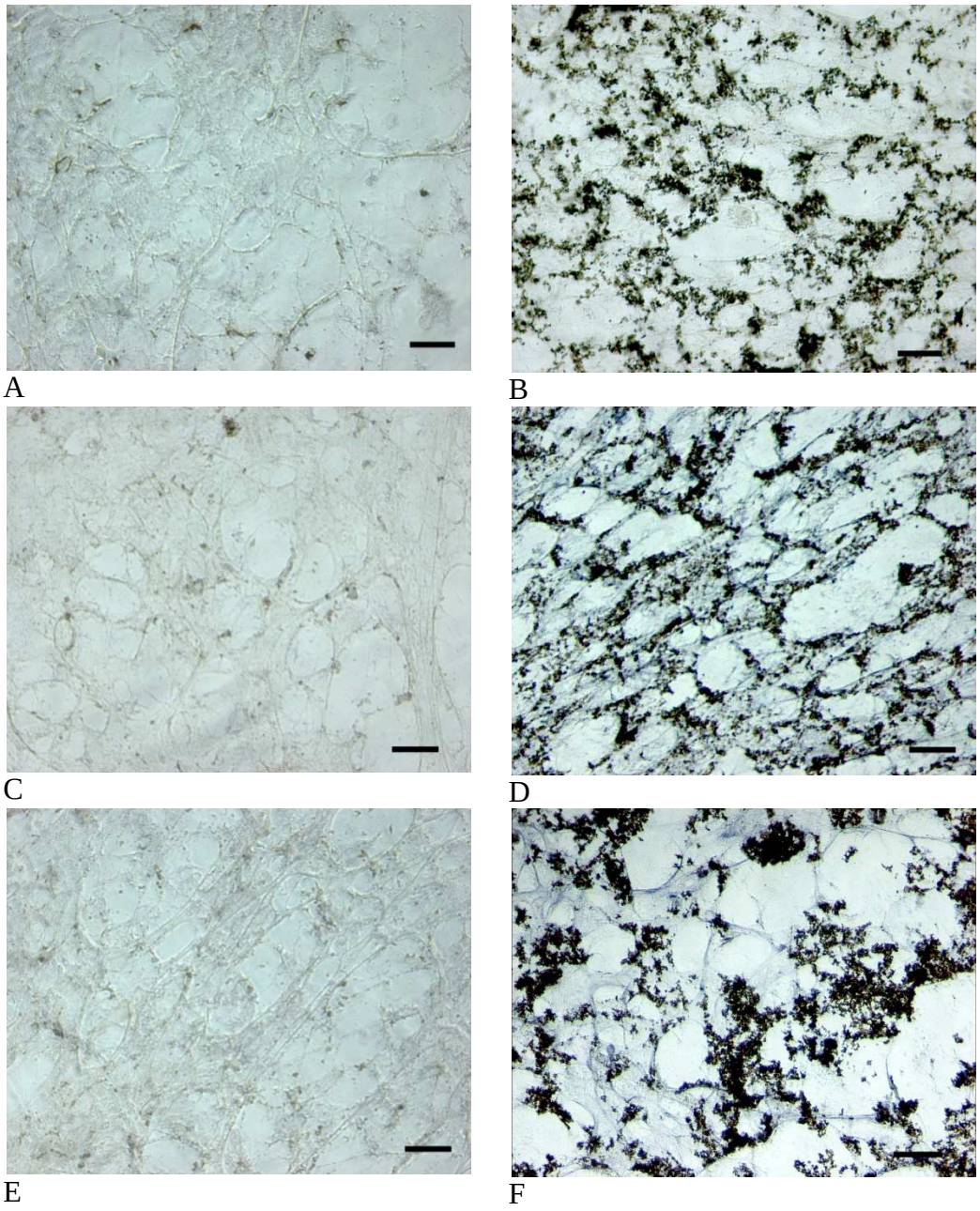


Figure 22: Microscopic demonstration of glucose-6-Pase activity in control cells (A-C-E) and hepatic differentiation-induced cells (B-D-F). A-B 3 weeks; C-D 4 weeks; E-F 5 weeks. Magnification 20x. Bar: 70µ

7.11 CYP 450 3A4 activity metabolic assay

Xenobiotics such as toxins and drugs are metabolized by cytochrome oxidase enzymes (kwon with term CYP 450) present in liver cells.

One of the most specific hepatic isoforms is CYP450 3A4, whose activity is widely recognized as sign of differentiation of precursor cells into mature hepatocytes. We evaluated its activity in response to a specific inducer (Rifampicin), with or without specific inhibition by ketoconazole in control and treated WJ-MSC.

The control cells, despite presence of rifampicin showed negligible CYP3A4 activity. On the contrary, cells cultured in hepatic induction medium with addition of the inducer, exhibited a significantly different activity in all tested time points. Furthermore, specificity of CYP3A4 activity was demonstrated by the significant decrease caused by the contemporary administration of the specific inhibitor ketoconazole. Similar results were obtained at all the considered timepoints: at three weeks (Figure 23) rifampicin significantly ($p=0.002$) induced enzyme activity with respect to control cells, and ketoconazole significantly ($p=0.002$) inhibited the same activity in differentiared cells; after four weeks (Figure 24), rifampicin significantly ($p=0.0079$) induced enzyme activity with respect to control cells, and ketoconazole significantly ($p=0.037$) inhibited the same activity in differentiated cells; after five weeks (Figure 25), rifampicin significantly ($p=0.0006$) induced enzyme activity with respect to control cells, and ketoconazole significantly ($p=0.027$) inhibited the same activity in differentiated cells

The expression of CYP3A4 was also confirmed by ICC, in control and hepatocyte-like differentiated cells at third and fourth week. As shown figure 26,

both control cell and differentiated cells express at high rates CYP 3A4 enzyme following rifampicin challenge. The marked differences between expression detected by ICC and enzymatic activity are object of further studies in our laboratory.

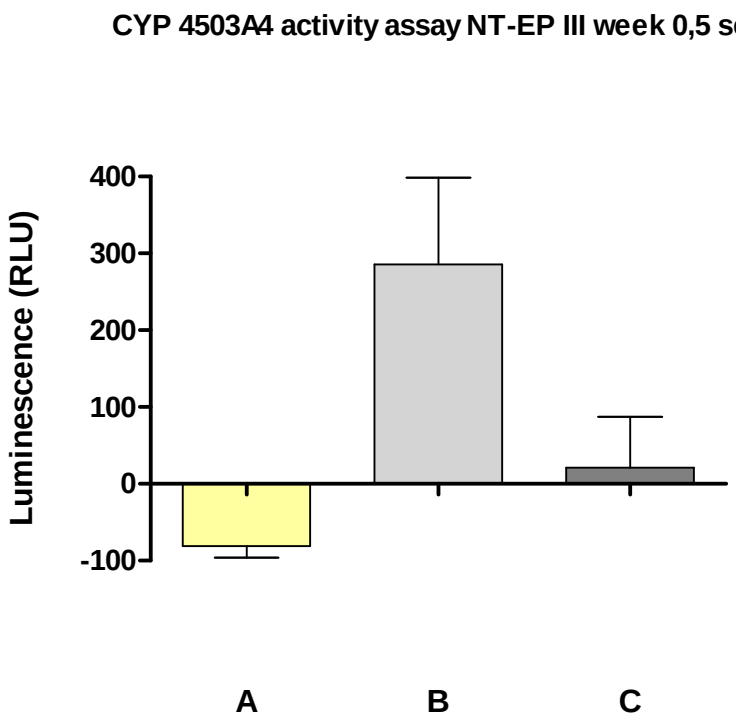


Figure 23: evaluation of CYP4503A4 metabolic activity in control and hepatocyte-like WJ-MSC at III week of differentiation. A: control WJ-MSC with rifampicin, B: hepatocyte-like WJ-MSC with rifampicin, C: kepatocyte-like WJ-MSC with rifampicin and ketoconazole. A/B p=0.002; B/C p= 0.002

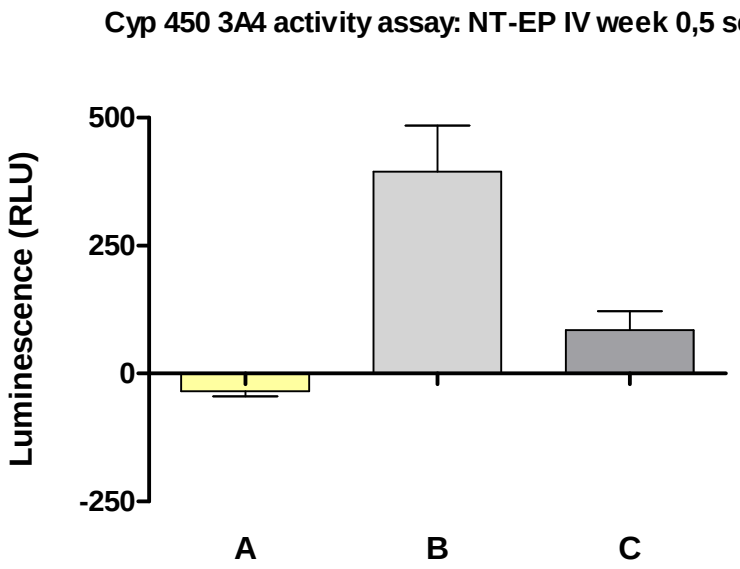


Figure 24: evaluation of CYP4503A4 metabolic activity in control and hepatocyte-like WJ-MSC at IV week of differentiation. A: control WJ-MSC with rifampicin, B: hepatocyte-like WJ-MSC with rifampicin, C: kepatocyte-like WJ-MSC with rifampicin and ketoconazole. A/B p=0.0079; B/C p= 0.037

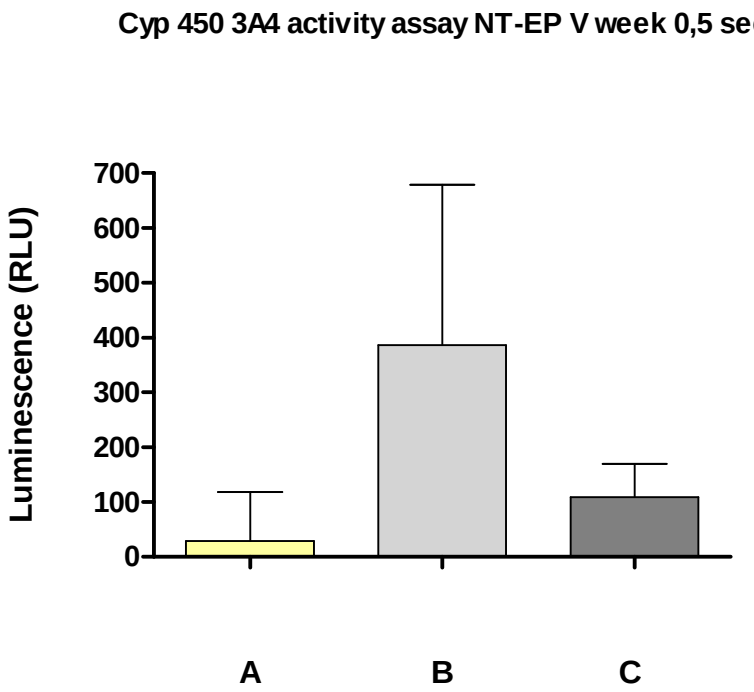


Figure 25: evaluation of CYP4503A4 metabolic activity in control and hepatocyte-like WJ-MSC at V week of differentiation. A: control WJ-MSC with rifampicin, B: hepatocyte-like WJ-MSC with rifampicin, C: kepatocyte-like WJ-MSC with rifampicin and ketoconazole. A/B p= 0.0006; B/C p= 0.027

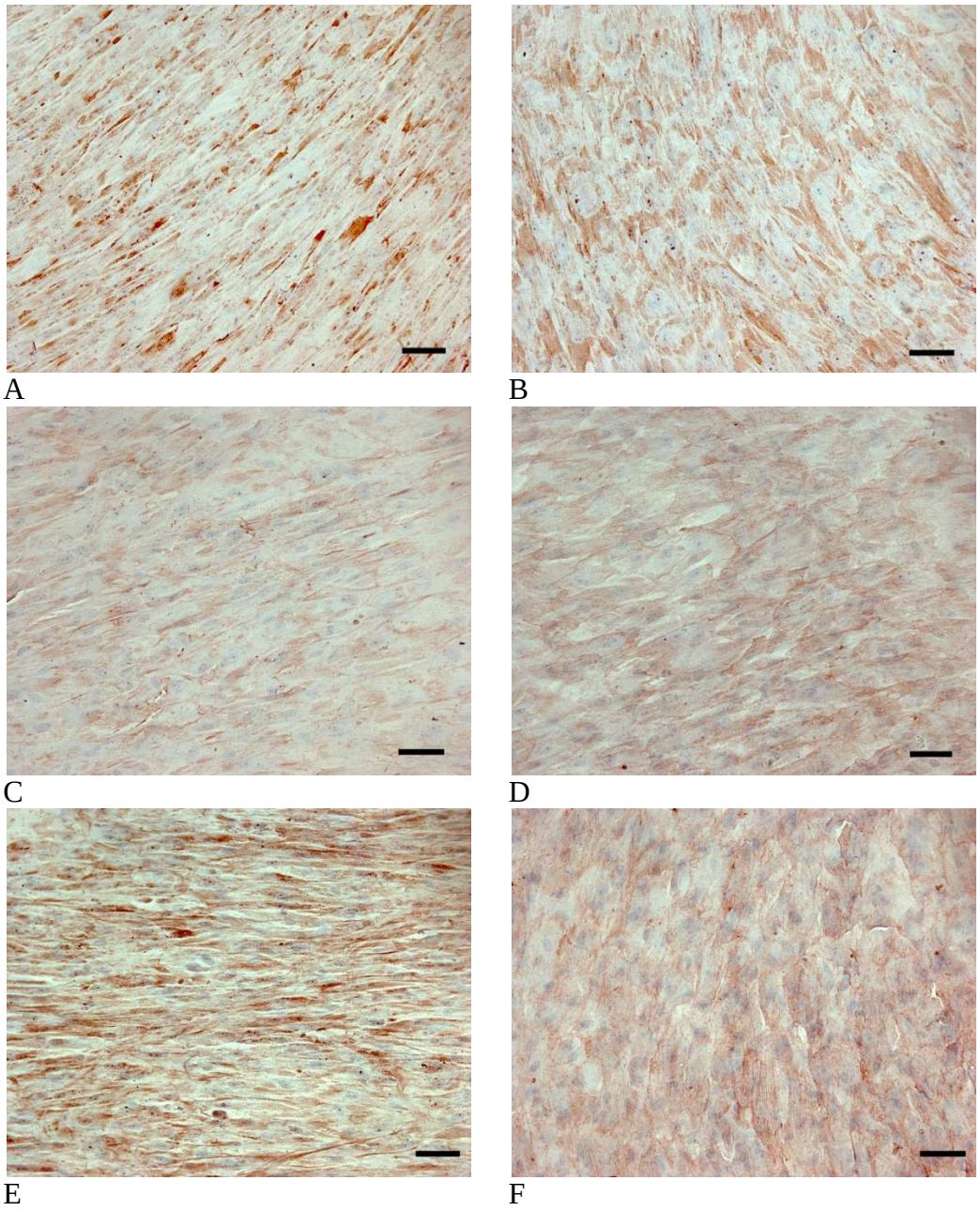


Figure 26 : Immunocytochemical analysis of expression of CYP 3A4 by ICC in control cells (A-C-E) at 3- 4 week and hepatocyte-like differentiated cells (B-D-F) at 3-4 week.). A-B: 3 weeks; C-D: 4 weeks; E-F: 5 weeks. Magnification 20x. Bar: 70µ

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7.12 Expression of immuno-modulatory molecules by undifferentiated and differentiated WJ-MSC through flow cytometry analysis

Evaluation of immune-modulatory molecules expression pattern is very important to apply these cells in regenerative medicine.

One of the main limits of cellular therapy is rejection by recipient host: knowing the behavior in vitro of cells candidates for transplant may help us to understand mechanisms underlying interplay of immune system of the host and engrafted cells.

Flow cytometry analysis was performed on undifferentiated cells and cells and differentiated towards hepatocytes. In both conditions, cells expressed canonic molecules of major histocompatibility complex of I class, HLA-ABC but did not express HLA-DR (see figure 27).

Moreover, control and hepatocyte-like cell exhibited expression of HLA-E and HLA-G, molecules involved into tolerance mechanism induced in NK and from mother towards fetus.(see figure 28-29).

We further investigated the expression of further members of the B7 family, namely B7-H1, B7-H3 and B7-H4 for all of these molecules, literature reports highlighted co-inhibitory roles in addition to the known co-stimulatory ones. It is known that B7-H1 and B7-H4 are expressed by BM-MSC [212], while no literature data exist on B7-H3 expression in MSC. We previously demonstrated that of these three markers, only B7-H3 (CD276) is expressed in naïve WJ-MSC (data not shown). Now, we extend this analysis to the differentiated progeny of WJ-MSC. As visible in figure 30, CD276 is amply expressed at the protein level in both untreated and hepatocyte-differentiated cells This may constitute a further point in the characterization of differentiated cells not only on the basis of the expression of desired markers of the mature cytotype, but also for the maintenance of the immunomodulatory properties of naïve cells, which may further promote the reparative action of these cells if used in regenerative medicine applications.

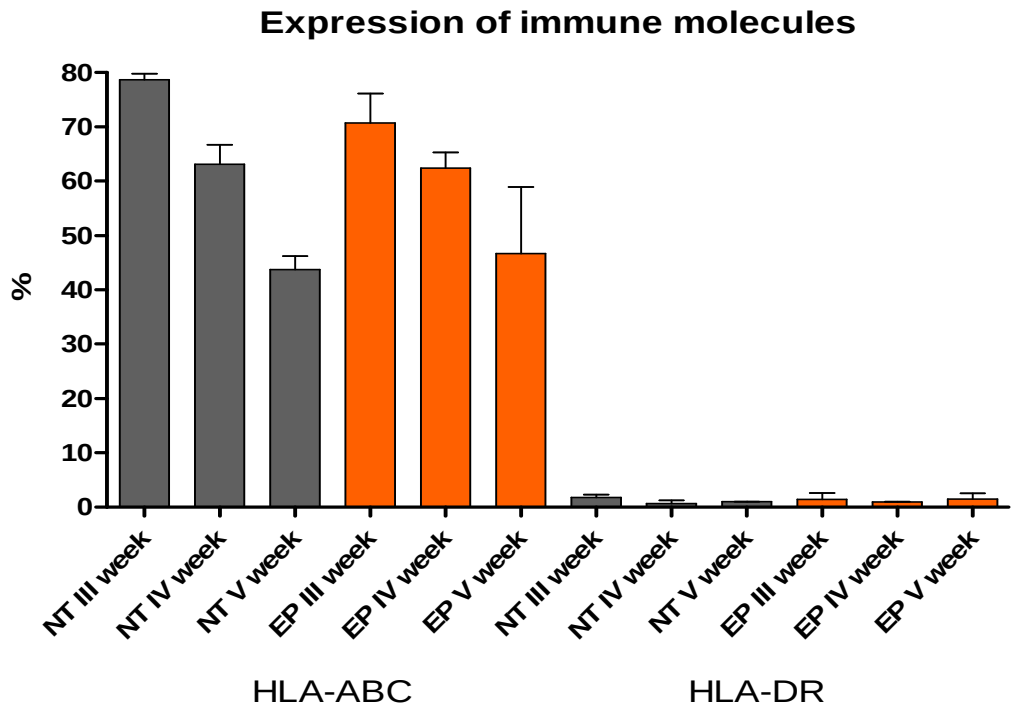

Figure 27: Flow cytometry analysis of expression of major histocompatibility complex molecules of the first and second class assessed by flow cytometry.

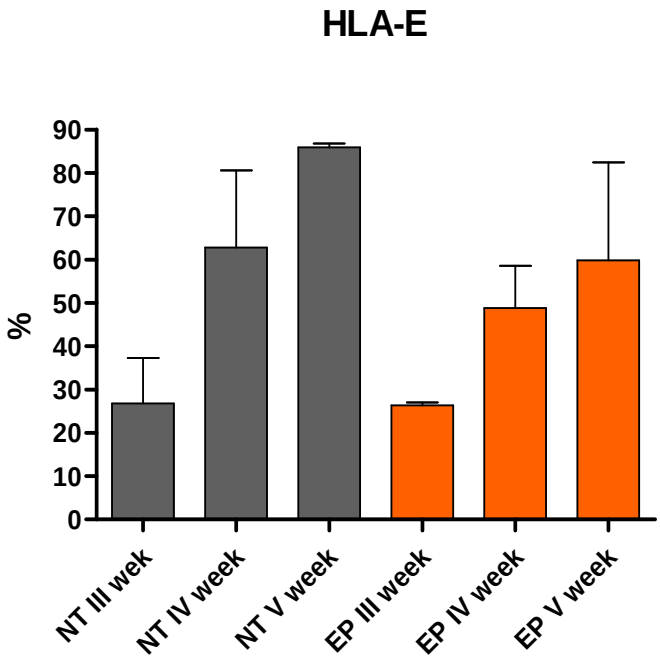

Figure 28: Flow cytometry analysis of expression of non –canonical molecule of major histocompatibility complex of I class in undifferentiated and treated WJ-MSC.

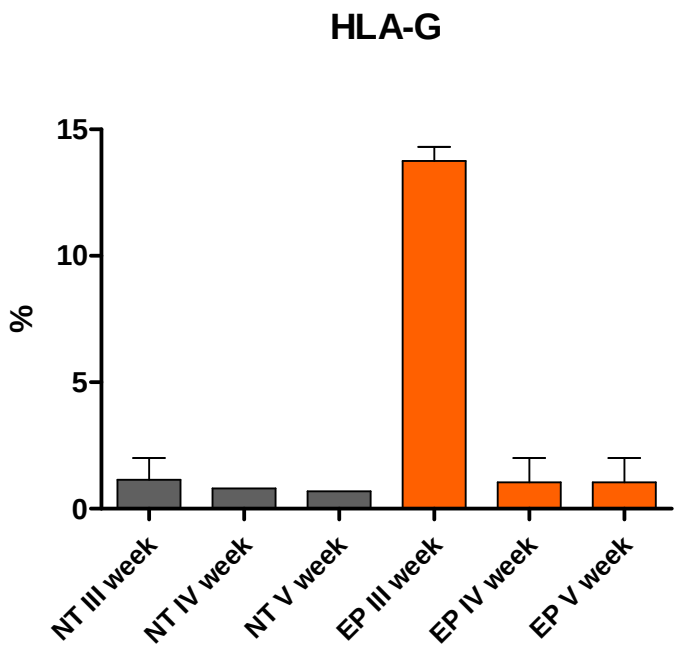

Figure 29: Flow cytometry analysis of expression of non –canonical molecule of major histocompatibility complex of I class in undifferentiated and treated WJ-MSC

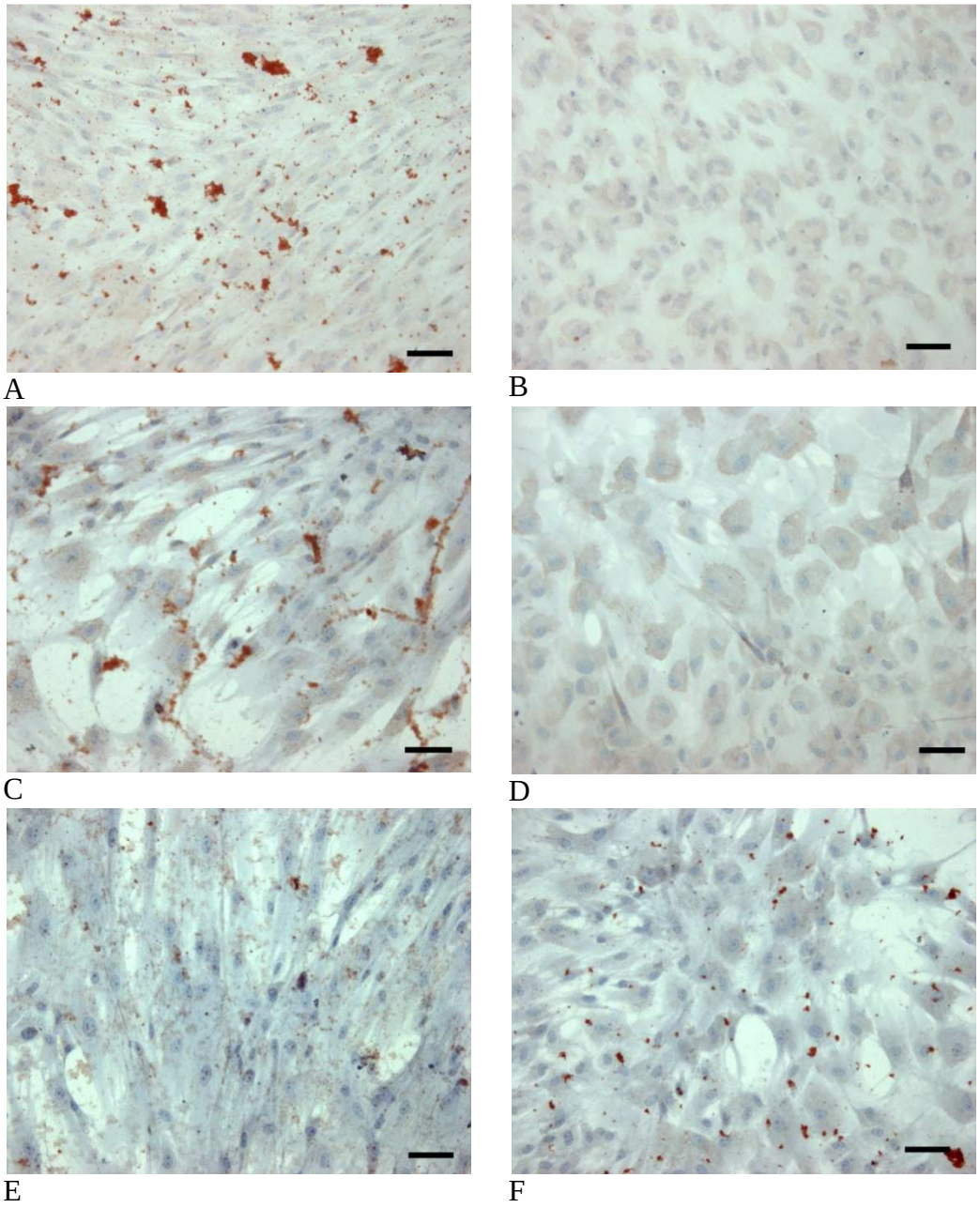

Figure 30: Immunocytochemical analysis of expression of B7H3 (CD276) by ICC in control and treated cells. A-C-E: control cell at 3-4-5 week, B-D-F: hepatic differentiation induced cells. A-B: 3 weeks; C-D: 4 weeks; E-F: 5 weeks Magnification 20x.Bar: 70µ.

8. DISCUSSION

In the last years, many advances were made on the biology of stem cells in vitro for future clinical applications and in particular for regenerative medicine. Certainly, a complete characterization of stem cell may help researchers to better understand in vivo data, mechanisms of interactions between microenvironment and stem cells.

Stem cells can originate from adult, embryonic and extra-embryonic tissues. While embryonic stem cells may be favorably viewed for their intrinsic pluripotency, studies demonstrated that use of embryonic stem cells in vivo can generate teratomas considering their undifferentiated state. Safety for human is the main parameter to be considered in all experiments. For this reason many reports highlighted the application of several differentiation protocols on adult stem cells.

My project was based on the characterization and evaluation of endoderm-related differentiation potential of Wharton's jelly cells. In the world millions of people suffer of liver pathologies, and despite organ transplant is considered the main therapeutic approach for end-stage diseases, cellular engraft should be a valid alternative therapy. The Wharton's jelly, a mucous tissue, is the main component of extracellular matrix of umbilical cord. In this tissue there are mesenchymal stem cells (WJ-MSC) with fibroblast-like morphology.

In our work, we initially evaluated the phenotype of these cells, and then we tried to differentiate WJ-MSC into hepatocyte-like cells.

Previous studies, carried out by our research group, demonstrated that these cells proliferate for several passages in vitro and showed a stable karyotype. Moreover, molecular analysis demonstrated expression of hepatic/endodermal-specific markers as GATA-4, GATA-5, GATA-6 and HNF-4α (hepatic nuclear factor4α). Therefore these data allowed hypothesizing the possibility to induce in these cells a hepatogenic differentiation.

A key feature of MSC is the capability to undergo self-renewal, maintaining their replicative potential together with their undifferentiated state. In this respect, the RT-PCR highlighted expression of Nanog and Oct3/4A, Sox2, responsible for maintaining a long-term self-renewal and the undifferentiated state in ESC. The presence of these factors in undifferentiated WJ-MSC was demonstrated for the

first time by our research group, and it constitutes a molecular confirmation of the clonogenicity experiments previously performed. In literature, several works recognized the presence of ABC carriers in adult and embryonic stem cells.
Molecular analysis showed expression of ABC carriers such as ABCG2 and MDR-3, responsible of drug resistance phenotype in WJ-MSC.
In addition, we wanted evaluate expression of some tissue-specific markers such as ISL-1, BAAT, NKX2.5 to further estimate the differentiative capability of these cells. In particular, to support the idea that WJ-MSC could generate endodermal cellular types we analyzed the presence of some hepatic transcripts as AFP, A1AT, BSEP, G6PASE, TDO2, several HNFs, and epithelial cytokeratins (CK-8, 18,19). 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. This initial effort guided us through the choice of reliable markers which should be induced in these cells subjected to hepatic differentiation. The expression of cytokeratins by WJ-MSC should indicate their ability to differentiate toward epithelial cytotypes. In particular, 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 population. This parameter has been further evaluated in our experiments aiming to differentiate WJ-MSC toward hepatocytes.
A key feature of mesenchymal stem cells is hipoimmunogenicity, this factor could be very important for applications in regenerative medicine.
Mechanisms underlying this immune-property are several: inhibition of T-cells proliferation and dendritic cells differentiation, anergy, secretion of soluble factors and tolerance induction. In particular, we demonstrated by molecular analysis, that WJ-MSC expressed a various histocompatibility major complex non canonical molecules such as HLA-G, HLA-E, HLA-F1 and HLA-F3.
These factors are implied into tolerance mechanism induced by NK and by mother toward fetus. Studies highlighted that HLA-G bind two major inhibitor receptor of natural killer (KIR1 and KIR2) blocking NK killing process [213]. In addition, our umbilical cells showed expression of CD80 but not of CD86. CD80 bound to

CD152 has an inhibitory role while CD86 bound to CD28 shows a role in the activation T-cells proliferation. Some researchers highlighted that in absence of CD86, the CD80 mostly exercise inhibitor effect through the link with CD152 [214].

Therefore, these B-7 co-stimulatory molecules appear to be involved into immune tolerance development together with HLA-G.

CD66F , a glycoprotein expressed by placental cells, induces secretion of anti-inflammatory cytokines and abolish antigen-induced T-cell proliferation. Undifferentiated WJ-MSC also expressed the transcript factors glycoprotein. In conclusion, all these data supported the idea that WJ-MSC could induce immune tolerance in the host but obviously further studies need to be performed in vitro to translate them successively in vivo experiments.

After characterization of cells in the undifferentiated state, WJ-MSC were subjected to the hepatic differentiation protocol that mimed hepatogenesis process. For three weeks the cells were cultured in a proprietary medium supplemented with HGF (hepatocyte growth factor), FGF-4 (fibroblast growth factor-4), dexamethasone, and ITS. For another 2 weeks, the cells were cultured in another medium composed by all factors previously described with addition of OSM. After 3-4 weeks time points, the cells appeared with polygonal morphology similar to the hepatocytes, The control cells, indeed, cultured in a standard growth medium maintained fibroblastic-like morphology.

To confirm the acquisition hepatic differentiation we performed PAS staining on treated and control cells. This stain highlighted the presence of glycogen. The treated cells were positive to the stain presenting reddish coloration and blue nuclei, while control cells were mainly negative to this stain.

RT-PCR analyses were used to determine the expression of selected gene by untreated and differentiated cells: we have observed that undifferentiated cell expressed some hepatic differentiation such as albumin, HNF4α, CK18, CK19, CK8. After 3 weeks of treatment in an hepatic medium, the cells started expressing other markers such as GYS2L and HNF-6. After 4 weeks of treatment the cells switched off the expression of CK19, and expressed BSEP, a protein

responsible to transport of bile salts. After 5 weeks, cytokeratins switch, and all markers previously expressed were maintained.

Flow cytomerty analysis demonstrated that differentiated WJC-MSC still expressed some of the markers of undifferentiated MSC as CD29, CD44, HLA-ABC, while lacking expression of hematopoietic markers such as CD34, CD45, and HLA-DR.

Expression of early and late hepatic molecules such as AFP and albumin was confirmed also in differentiated cells with an increase seemingly due to differentiation increase in albumin expression was also confirmed by immunocytochemistry analysis. Cytokeratins switch was confirmed also by flow cytometry analysis.

Finally, the expression of HNF-4α was evaluated in control cells and in hepatocyte-like cell at different timepoints by ICC. Nuclear expression of this factor was visible in all steps of hepatic differentiation.

Confirmation of hepatic phenotype acquisition by WJ-MSC was showed by indocyanine staining and functional assays where we evaluated metabolic activity of main hepatic enzymes.

Indocyanine or cardiogreen is molecule that forms fluorescent compounds when bound to the plasmatic proteins, in particular albumin. Hepatic differentiated cells were positive to the stain, with cells exhibiting green granular cytoplasm, due to cardiogreen uptake. Control cells, were mostly.

Glucose metabolism and xenobiotic detoxification are the main liver functions.

Glucose-6-phosphatase is an enzyme involved during gluconeogenesis and glicogenolysis. We performed an assay to evaluate the metabolic activity of this enzyme, demonstrating that hepatocyte-like cells, in all different hepatic differentiation steps, showed presence of lead sulphide precipitates as consequence of enzymatic activity, unlike control cells.

CYP 450 3A4 metabolic assay was performed in presence of an inducer and an inhibitor of CYP enzyme both in control and treated cells. Hepatocyte-like cells in presence of the inducer exhibited significantly elevated activity of CYP 4503A4 when co-incubated with the inhibitor ketoconazole, the same cells expressed significantly lower levels of enzymatic activity.

In conclusion we demonstrated that WJ-MSC, induced with a protocol resembling the succession of inductive processes occurring in vivo during hepatogenesis, showed morpho-functional characteristics of hepatocyte-like cells, expressed novel endoderm-restricted and hepatocyte-restricted genes and enzymes involved in some typical hepatic functions such as detoxyfication of xenobiotics, glycogenolisys, glycogensynthesis and gluconeogenesis.

Final objective of all in vitro experiments is translation in vivo, and knowing the phenotype of WJ-MSC could be more important to understand the behavior of these cells in recipient host. For these reasons, we evaluated immune property of these cells also after hepatic differentiation. WJ-MSC induced to differentiate towards hepatocyte like-cells expressed some immune-modulating molecules involved in tolerance mechanism, previously characterized in WJ-MSC at undifferentiated state.

Therefore, obtained data confirmed the possibility by these cells to establish immune tolerance or to have simply an immune privilege, suggesting their possible role for clinical applications.

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Acknowledgements

The course of this thesis has been possible thanks to Professor Giovanni Zummo, director of the department of Sperimental Biomedicine and Clinical Neuroscience (BIoNeC), section of Human Anatomy, thanks to Professor Francesco Cappello and a special thanks to Professor Felicia Farina.
I would like to thanks Dr. Rita Anzalone and Dr. Giampiero La Rocca who were my teachers and my guides during these three years of research.
In particular, I sincerely thank all my colleagues with whom I shared good and bad times. I hope that the relationship has been created with them in these years, will be maintained over time