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Wharton's Jelly Mesenchymal Stem Cells from Preterm Umbilical Cords

Isolation, Characterization and Potential Use as Extrahepatic Progenitors

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1. INTRODUCTION

1.1 STEM CELLS: DEFINITION AND GENERAL FEATURES

The term “stem cell” was coined by the end of the nineteen century in order to name some cells supposedly involved in the continuous tissue renewal, such as blood renewal.

During the last century uncountable scientific studies, as well as experimental evidences, have confirmed the existence of these cells, emphasizing their enormous and fascinating regenerative potential, so attracting on them a big interest of the scientific community for tissue engineering and other therapeutic applications.

A *stem cell* is defined as unspecialized, able to multiply and renew itself indefinitely, and able to generate differentiated cells in response to specific stimuli. In order to do that, a stem cell goes through an asymmetrical division that gives rise to daughter cells, one of which is identical to the “mother cell” (*self-renewal*) while the other one, in response to microenvironmental stimuli, will produce term differentiated cells. This last described differentiative capacity is called *potency:* it can depend on stem cell origin and it is one of the parameters commonly used to classify stem cells. Stem cells classification can fall within four categories: **Totipotent stem cells, Pluripotent stem cells, Multipotent stem cells** and **Unipotent stem cells.**

In mammals, only the zygote and its very early descendent cells can be defined *totipotent stem cells* because these cells are the only ones that possess the capability to give rise to all the specialized cells of the adult organism, and they can also

produce trophoblast derivatives (extraembryonic tissues and placenta) that support embryo growth.

As embryonic development proceeds, cells start losing the over mentioned potential, retaining the ability to generate all types of cells derived from three germ layers but not the extraembyonic tissues. These cells will be classified as *pluripotent stem cells*.

The last two stem cell categories (*multipotent stem cells* and *unipotent stem cells*) are tissue-resident stem cells that can respectively give rise to a certain number of cell types (e.g. hematopoietic stem cells) or to only one specialized cell type (e.g. melanocyte stem cells). In adult organisms, these cells play a role in supporting tissue renewal, regeneration and repair processes.

Depending on their residency, stem cells can be also classified into *embryonic, fetal, perinatal and adult stem cells*.

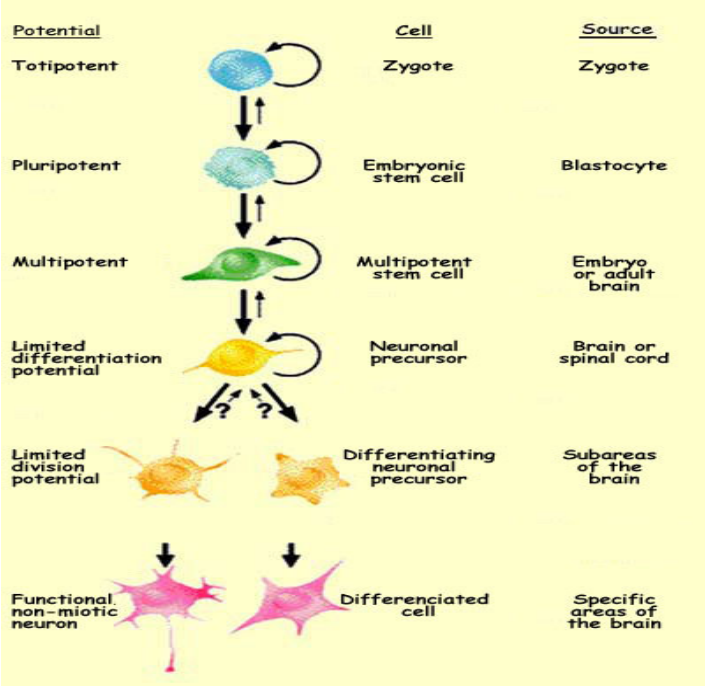


Figure 1: Stem cell potency (*www.stemcellresources.org*)

1.2 EMBRYONIC STEM CELLS (ESCs)

Embryonic stem cells derive from the inner cellular mass of the blastocyst, during early developmental stages.

Blastocyst appears as a spherical cyst composed by three main structures:

- *The trophoblast,* that represents the peripheral portion of the blastocyst, whose function is to interact with the maternal environment. During the development, it will give rise to the placenta

- *The blastocoel*, which is the fluid-filled central cavity of the blastocyst

- *The inner cell mass (ICM)*, a cluster of cells positioned at one end of the blastocoel which will give rise to all the embryonic tissues. ICM cells, that represent the source of the "embryonic stem cells", are able to self-renew indefinitely, maintaining a normal karyotype and high levels of telomerase activity and they can give rise to the three embryonic germ layers (ectoderm, mesoderm and endoderm) as well as to all cell types of an organism [1-2].

ESCs are derived after blastocyst inner cell mass collection. Human cells may be obtained from donated frozen supernumerary embryos that result from *in vitro fertilization* (*IVF*). The main restrictions concerning embryonic stem cell research are related to *ethical and safety issues* [3]. For the first point, it is still questionable whether a zygote or an early stage embryo can be defined as "the very beginning of a life" because they have the remarkable potential to develop into a human being. For this reason some persons consider the use of embryonic cells for research or clinical

application as morally unacceptable and the Catholic Church is against embryonic stem cell research.

In Europe stem cell research legislation and regulations are different from one country to another. In Italy, it is not possible to generate embryonic stem cell lines but it is permitted to use them for research only if they are imported [4].

The major safety issues concern teratoma formations. Teratomas are tumors made of a mixture of cells with different phonotypes that originate (*in vivo and in vitro*) as a consequence of a disorganized growth, due to the absence of correct environmental growth stimuli [5].

Based on the above mentioned reasons, alternative stem cell sources have been studied and proposed for stem cell-based therapies.

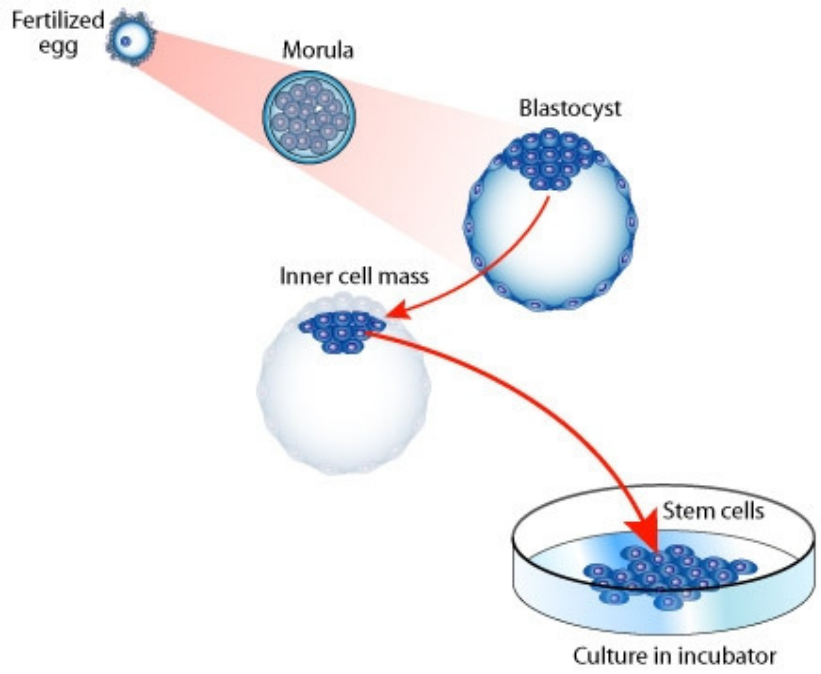


Figure 2: Illustration by Cell Imaging Core of the Center for Reproductive Sciences.

1.3 FETAL STEM CELLS (FSCs) AND PERINATAL STEM CELLS (PSCs)

Fetal stem cells, as indicated by the definition, are stem cells located in fetal tissues such as bone marrow, blood, liver, pancreas, kidney and skin. FSCs are collected from tissues and organs mainly deriving from therapeuthical abortions, after mothers' informed consent.

Although their development potential is more limited than pluripotent ESCs, their high plasticity (multipotency) and replicative potential, as well as the lack of tumorigenicity, have made them one of the possible candidates for regenerative medicine applications and clinical uses (cell therapy)[6].

Fetal stem cell clinical applications [7-9] have been proposed as an useful tool that could promote tissue regeneration, providing a short-term "*bridge"* to organ transplantation, or as an option to treat some diseases such as Parkinson's disease (human fetal dopaminergic neurons transplantation) [10-12].

Similarly to embryonic stem cells, FSCs isolation may give rise to ethical concerns but fewer restrictions are applied to their use. In Italy, there is no specific guideline regarding fetal stem cell research, but it is "generally regulated" by the law on organ donation and transplantation, after the mandatory informed consent is obtained.

The most promising and available source of fetal stem cells are *perinatal tissues* [13] *constituting the extra-embryonic structures of fetal origin*: *amniotic fluid, placenta and amniotic membrane, umbilical cord blood and umbilical cord matrix (Wharton's jelly).*

These structures are normally discarded after delivery and represent a precious source of stem cells, with high self-renewal potential and plasticity. Moreover, perinatal stem cells have a differentiation potential that could be considered slightly lower than ESCs, but higher than that of adult stem cells (ASCs).

Similar to embryonic stem cells, PSCs express pluripotency markers such as Oct-4, whose function is to prevent precursor cells from differentiating, thus regulating stem cell self-renewal [14-15]. Other pluripotency markers shared with ESCs are NANOG, SSEA-3, SSEA-4, Tra-1-60 and Tra-1-81 [16-19].

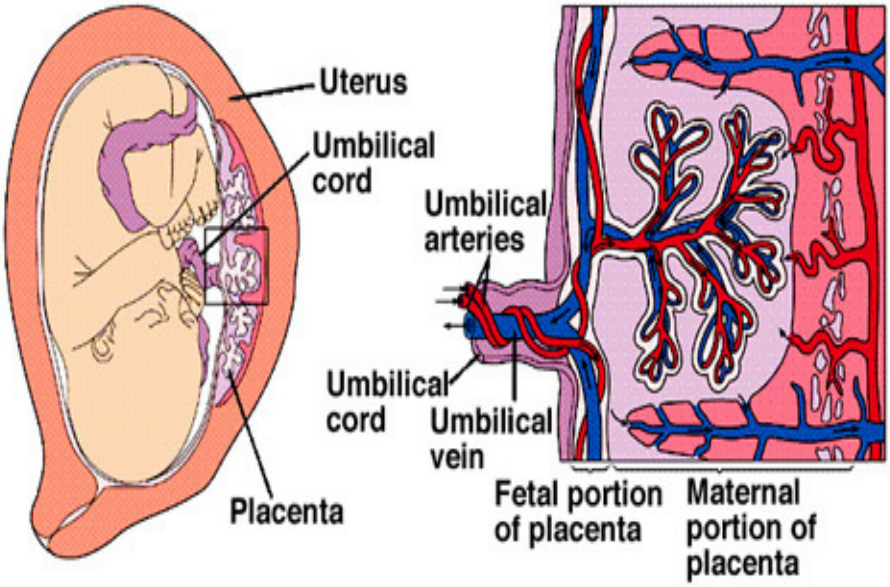


Figure 3: Schematic representation of Placental structures. *(Santrock J. W. Child Development,1998, , 8th edition, McGraw Hill)*

1.3.1 AMNIOTIC FLUID STEM CELLS (AFSCs)

Amniotic fluid is a protecting liquid that surrounds the fetus during pregnancy. It is composed by a heterogeneous cell population made of cells with variable capacity for self-renewal and differentiation, among which multipotent stem cells and terminally specialized cells (e.g. epithelial cells) could be indicated as the two opposite extreme examples. Amniotic fluid cells can be easily isolated from amniotic fluid specimens obtained, with informed consent signed by patients, for amniocentesis.

One of the most attractive AFSCs population isolated, was found to be positive for the antigens Oct-4, SSEA-4, Nanog and CD117 (c-Kit) and for the mesenchymal cell markers CD105, CD90 and CD73 [20, 192-194]. Their multipotency (toward mature tissues of all the three germ layers) and high proliferative capability [20-21], make them good candidates for regenerative medicine applications. Limitations about AFSCs use reside in their low isolation rate (c-Kit positive cells are approximately 1% of total amniotic fluid stem cells) [20].

1.3.2 PLACENTA AND AMNIOTIC MEMBRANE STEM CELLS

Placenta for many years has attracted the interest of scientists because of its important role as intermediate structure mediating the fetal-maternal exchange of nutrients and gas and because of its involvement in maintaining immune tolerance between the mother and the fetus.

During the last decade the attention of the scientific community has been directed to this organ as a source of progenitor stem cells for clinical applications. Placenta

consists of two portions: the chorionic plate (the fetal portion) and the basal plate (the maternal portion). Umbilical cord is attached near to the center of the fetal portion which is covered by fetal membranes (amnion and chorion).

Two major stem cell populations can be isolated from placenta: *chorionic mesenchymal stromal cells* and *chorionic trophoblastic cells* [22] showing variable differentiation potential (e.g.osteogenic, adipogenic, chondrogenic). Moreover, an additional progenitor cell population, co-epressing mesenchymal and hematopoietic cell markers, was isolated from the decidua parietalis (belonging to the maternal part of the placenta) and characterized.

The most promising progenitor cells isolated from term placenta are those isolated from the amniotic membrane, the *amniotic stem cells*. Amniotic membrane is made of three layers, the outermost consisting of mesenchymal cells (amniotic mesenchymal stromal cells, AMSCs, derived from the embryonic mesoderm), an intermediate membrane (lacking cellular components) and the innermost layer consisting of epithelial cells (amniotic epithelial cells, AECs, derived from the embryonic ectoderm). Both AMSCs and AECs populations express low levels of MHC (major histocompatibility complex) class I antigen and lack the expression of MHC class II molecules, suggesting their low immunogenicity [23-25]. This important property is also due to the expression of HLA-G, a non-classical MHC class Ib involved in the maintenance of tolerance between maternal immune system and fetus [26]. Approximately 10% of AECs express the stem cell markers Tra 1-60, Tra 1-80 and SSEA-4, and feature cellular plasticity [22,27-28]; conversely, amniotic mesenchymal stromal cells, possess a higher mesodermal differentiation potential

[29] compared to AECs. Since the beginning of the last century, whole amniotic membranes are used in clinic applications to treat burns and ulcers, as well as for corneal ulcerations [30].

1.3.3 UMBILICAL CORD and UMBILICAL CORD-DERIVED STEM CELLS

Umbilical cord is the extraembryonic structure connecting placenta and fetus during the development. It represents the vehicle through which nutrients and oxygen are transported from the mother to the fetus. The UC is formed within the first weeks of gestation by the enclosure of the vessels (one vein and two arteries) into a bulk of mucous connective tissue, named Wharton's jelly (WJ) and lined by the umbilical epithelium [31]. At term gestation, the umbilical cord appears as a 60-65 cm twisted tubular structure covered by an epithelium derived from the enveloping amnion and containing two arteries and one vein surrounded by the Wharton's jelly. Cord twisting may occur due to discordant vessels' growth. The main function of Wharton's jelly is to protect umbilical cord vessels from mechanical insults such as compression and torsion events. After birth, closure of the UC is an important and yet poorly understood process that safeguards against blood loss of the newborn.

Wharton's jelly is composed by proteoglycans, by collagen fibers and by heterogeneous, fibroblast-like cell populations, which were classically described as myofibroblasts. Miofibroblasts are not defined either as fibroblasts or as smooth muscle cells despite the fact that they have muscle-specific cytoskeletal filaments. The term of "myofibroblast" was assigned because these stromal cells are positive for vimentin [32], a marker typical of fibroblasts, and desmin [33], a marker of muscle cells. The other fibroblast-like cells populating the Wharton's jelly have instead

features similar to fibroblasts since they produce collagen and other extracellular matrix components.

Even if the stroma can be divided into three different zones (subamnion, Wharton's jelly and perivascular zone) and there are some differences between cells dispersed in these zones, the term 'Wharton's jelly cells (WJCs) is often extended to all umbilical stromal cells [31].

UMBILICAL CORD BLOOD STEM CELLS (UCSCs)

Umbilical cord blood is being explored for use in regenerative medicine applications because it represents a rich source of hypo-immunogenic stem cells (mainly hematopoietic stem cells, HSCs) characterized by properties of proliferation and expansion greater than those of HSCs isolated from adult bone marrow (BM), which currently still represents the major source of stem cells [34-36].

Another advantage of UCSCs use is that cord blood collection is not at all an invasive procedure, it is painless and free of risks for both mother and baby. Moreover, UCSC showed a reduced risk of graft-versus-host disease (GVHD) mainly due to the more immunologically immature (naïve) nature of T cells derived from cord blood with respect to those isolated from adult tissues and, consequently, also more tolerant to HLA tissue mismatching [37-39]. Limitations to cord blood applications are related with problematic engraftment kinetics compared to BM-derived cells.

UMBILICAL CORD MATRIX (WHARTON'S JELLY) STEM CELLS

Wharton's jelly mesenchymal stem cells (WJ-MSCs) are multipotent fibroblast-like cells involved in collagen and extracellular matrix component production. They were first isolated more than 10 years ago [40] and they were shown to provide secretion of mucopolysaccharides, glycosaminoglycans and extracellular matrix proteins that constitute the bulk of umbilical cord matrix. As stated earlier, WJ-MSCs have been isolated from three umbilical cord regions - the perivascular zone, the inter-vascular zone and the sub-amnion – showing rough differences in phenotype and differentiation potential depending on the WJ compartment from which they are derived [41-42].

During the last decade the **immunophenotype** of WJ-MSCs has been extensively studied by several researchers that have demonstrated an antigenic pattern resembling that of mesenchymal stem cells originated from other tissues.

In accordance with "minimal criteria" estabilished by *the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy* for defining mesenchymal stem cells, and similarly to bone marrow mesenchymal stem cells (BM-MSCs), WJ-MSCs express CD105, CD73 and CD90 antigens but lack the expression of endothelial (CD31) and hematopoietic markers (CD45, CD34) [44]. Both WJ-MSCs and BM-MSCs also express CD29, CD166, CD44, CD10, CD13 and HLA class I [45-49] but do not express HLA-DR [50-53].

Furthermore, these cells are positive for mesodermal markers such as α-smooth muscle actin and vimentin, and for antigens expressed by cells of different tissues (e.g nestin, connexins, stem cell factor), so allowing the hypothesis of an intrinsic

multilineage differentiation capability. Nestin is an intermediate filament protein first identified in rat neuro-epithelial stem cells. Subsequently it was found to be expressed by various precursor cells such as beta cell progenitors or hepatic oval cells [54-55]. Other neuronal markers expressed in WJ-MSCs are: glial fibrillar acidic protein (a neuroectodermal marker) and neuron-specific enolase [56-57].

Connexins are integral membrane proteins involved in intercellular gap junctions. In particular, connexins 40, 43 45 [58-59], typically found in cardiomiocytes, have been demonstrated by our laboratory to be expressed by WJ-MSCs.

Moreover, a possible hepatic differentiation potential was first indicated by the expression of cytokeratins (CK8 and CK18) and hepatocyte nuclear factor 4α (HNF-4α) [58] and then recently confirmed by our group (Lo Iacono et al. *manuscript in preparation*). HNF-4α is indeed a transcription factor necessary for the maintenance of hepatic functions [60-61], inducing the expression of many genes, some of which play an important role in lipids and glucose metabolisms [62-63]. HNF-4α is also involved in insulin expression and secretion activities in beta cells [64-65]. This is due to the sharing of a common embryonic origin between liver and pancreas, as both are derived from almost the same regions of the primitive endoderm. This spontaneous expression of all described differentiation markers strongly suggests that WJ-MSCs are prone to differentiate toward several cellular types.

WJ-MSCs differentiation capability.

In accordance with the minimum criteria defined by International Society for Cellular Therapy [44], they are able to differentiate into mesodermal cell types including chondroblasts, osteoblasts and adipocytes. Moreover, their ability to differentiate into muscle cells, cardiomyocytes, glial and endothelial cells has been demonstrated by several reports indicating umbilical cord matrix as a promising stem cell source for regenerative therapies.

Cardiac differentiation: WJ-MSCs can be differentiated into cardiomiogenic lineages, as demonstrated by the acquisition of a specific morphology and by the expression of cardiac specific markers, such as connexin 43 and cardiac troponin I, following stimulation with 5-azacytidine for 21 days [49]. In addition, WJ-MSC therapeutic potential in cardiovascular tissue engineering application has been suggested [66].

Skeletal muscle differentiation: WJ-MSCs hve been also demonstrated to give rise to skeletal myocytes in response to myogenic culturing conditions [67].

Chondrogenic differentiation: Chondrogenic potential of WJ-MSCs was also reported by my research group, indicating the possible use of these cells as a future treatment for articular diseases [68-69].

Endothelial differentiation: WJ-MSCs can differentiate into endothelial-like cells, as demonstrated by both morphological and physiological changes [70].

Neural differentiation: WJ-MSCs can give rise to Schwann cells that, once transplanted into rats transected sciatic nerve were able to support *in vivo* functional recovery [71-72].

Hepatic differentiation potential: Recent data showed that WJ-MSCs can also undergo hepatocyte differentiation. Campard and al. demonstrated that these cells can differentiate into hepatocyte-like cells, by culturing them in a medium containing hepatic growth factors [73]. The initial cell population was positive for the expression of cytokeratins 8, 18 and 19 but negative for cytokeratin 7; the expression of α-1-antitrypsin, tryptophan 2,3-dioxygenase and Glucose-6-Phosphatase was also demonstrated but not the positivity for Hepatocyte specific antigen-1 (HepPar1), HNF-4α (Hepatocyte nuclear factor 4 alpha) or for the isoform 3A4 of the cytochrome p450.

The hepatocyte-like cells derived from the induction process increased the expression of some hepatic-specific markers such as tryptophan 2,3-dioxygenase, but remained negative for HNF-4 α and HepPar1, questioning the acquisition of a mature phenotype, and therefore highlighting the necessity of further improvements of the differentiation protocol [74]. Despite that, a functional maturation was achieved as demonstrated by several assays that quantify hepato-specific abilities such as urea production, Glucose-6-Phosphatase activity, CYP3A4 induction and glycogen storage capability.

Hepatic differentiation was also demonstrated *in vitro* by Zhang and co-workers [75] applying a one-step protocol. HGF and FGF-4 stimulation resulted in liver-specific

markers expression, such as albumin and alpha-feto protein, by treated cells. Moreover stored glycogen and uptake of LDL was also demonstrated

In vivo experiments strongly supported future application of WJ-MSCs for liver regenerative medicine. Published data showed the acquisition of hepatic markers expression by undifferentiated WC-MSCs, following transplantation, in liver of SCID mice following partial hepatectomy [73].

In addition, a supportive role of naïve Wharton's jelly mesenchymal stem cells in rescuing injured liver functions and in reducing fibrosis was also demonstrated by Tsai and colleagues in a rat model ofhepatic injury and fibrosis induced by CCl_4. WJ-MSC-infused rats showed reduced levels of serum transaminases, as well as a reduction of TGFβ1 and α-SMA if compared to control groups. These data nicely correlated with an overall reduction of liver fibrosis [76]. These data support the hypothesis that undifferentiated mesenchymal stem cells may favor the functional recovery of recipient livers, perhaps inducing the differentiation of endogenous liver cells and by enhancing fibrous matrix degradation [77].

Moreover, recent data from another work by Yan and collaborators demonstrated the effects of WJ-MSCs engraftment in livers of CCl_4-injured mice. Interestingly, compared with control group, mice that received WJ-MSCs administration showed a reduction of apoptosis together with an increase of hepatocytes proliferation [78]. Finally, Zhao and collaborators [79] showed that hepatocyte-like cells derived from WJ-MSCs following hepatic differentiation, expressed hepatic specific antigens and also maintained *in vitro* hypoimmunogenicity features (lack of HLA-DR expression).

These data are very interesting and demonstrated that the differentiative process did not interfere with the immunological features of these cells.

Immunomodulatory properties of WJ-MSCs

As demonstrated for mesenchymal stem cells isolated from different tissues (such as adipose and bone marrow), many scientific publications have also reported the expression of immunomodulatory and tolerogenic molecules by WJ-MSCs, such as not classical HLA class I molecules (HLA-E, HLA-F and HLA-G) [80-82], CD274 and CD276 (belonging to B7 family of molecules) [80, 83-84], as well as leukaemia inhibitory factor (LIF) [85]. In addition, the lack of expression of HLA-DR and the absence of CD80 and CD86 costimulatory antigens [58, 86] (commonly involved in acceptance or rejection of transplanted organs), strongly suggests the capability of these cells to regulate immune cells activation and proliferation.

In vitro analyses of MHC molecules expression by stem cells is of fundamental interest because it gives important suggestions regarding the way these cells could act once transplanted *in vivo*. As also commented in other sections of the present thesis, WJ-MSCs low immunogenicity is due to the absence of HLA-DR molecules whereas tolerogenic features can be mainly explained by the presence of B7-H3 molecules as well as by the expression of class Ib HLA molecules (HLA-E and HLA-G). HLA-G and HLA-E were initially characterized on extravillous trophoblast, where both act as molecules protecting fetus form maternal allorecognition during pregnancy. To date it is known that these molecules are also expressed in different districts and that they

exert multiple immunoregulatory functions by acting as ligands for inhibitory receptors present on NK cells and macrophages.

HLA-G exists as a membrane-bound molecule and as a secreted isoform (HLA-G5), thus exerting its immunomodulatory function through two different mechanisms. It has also been suggested that HLA-G could explicate their immunomodulatory function by activating T regulatory cells (Tregs), known to be key suppressors of effector responses to alloantigens [87].

1.4 ADULT STEM CELLS (ASCs)

Adult stem cells are classically defined as multipotent stem cells that are thought to reside in a quiescent state into specific areas of multiple tissues and organs after birth called “niches”, representing cellular microeviroments that provide specific stimuli necessary for survival, proliferation, migration and differentiation events.

ASCs primary function is to maintain tissue homeostasis and they are mainly involved in tissue repair processes following traumatic events or diseases of different nature. Even if they appear to have a more restricted differentiation potency and self renewal ability compared to embryonic or perinatal stem cells, there are many advantages that suggest their use over other cell categories. For example, ASCs do not form teratomas, there are no ethical concerns on their use and these cells can be easily used for autologous transplantation with no need of immunosuppression.

Conversely, depending on tissue origins, adult stem cells are sometimes difficult to be isolated (e.g. hepatic precursor cells) and expanded, resulting in a very low cellular yield and the limited differentiation potential imply a reduced applicative potential if

compared to embryonic stem cells. Moreover, these cells are very difficult to be maintained in culture in undifferentiated state.

Even if for many years ASCs were thought to have a tissue-specific differentiation capability, recently an increasing numbers of reportes have demonstrated that these cells are able to give rise to cells of different origins, introducing the concept of "*adult stem cell plasticity*", defined as the ability of these cells to trans-differentiate into cells of different lineages.

This phenomenon is still not entirely clear and can be explained by different potential mechanisms: cell fusion, trans-differentiation, de-differentiation or pluripotency [88]. During cell fusion events, stem cells acquire the phenotype of the tissue they are transplanted within; this phenomenon is not so common and has been reported for bone marrow stem cells transplanted into organs such as the liver [89]. Trans-differentiation occurs when silent genes involved into other differentiation programs are activated, transforming cells into different cellular types. De-differentiation is a phenomenon during which cells spontaneously lose differentiated features, acquiring a more immature state and, subsequently differentiate again into diverse cell lineages. Finally, the presence of pluripotent stem cells residing into different niches has been demonstrated in many tissues. Some examples of stem cell plasticity are represented by bone marrow hematopoietic stem cells trans-differentiation into muscle cells [90]; moreover neuronal stem cells and stem cells isolated form muscle tissues were able to reconstitute the hematopoietic system in rodents [91-92]. As an additional example, it has been reported that bone marrow derived cells were induced to differentiate into neuron-like cells [93-94].

The two major adult stem cell families are represented by Hematopoietic Stem Cells (HSCs) and Mesenchymal Stem Cells (MSCs), the both of them expressing embryonic stem cell markers such as OCT4, SOX2, NANOG, LIN28, SSEA-1, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81 [95-99]. Hematopoietic stem cells are the most studied stem cells and they also have been effectively and extensively used for patients with chronic myeloid leukaemia or after high-dose chemotherapy to restore blood cell production, remaining an effective treatment for patients with certain haematological diseases. Even if initially HSCs were thought to have a reduced plasticity (limited to blood and blood derived cells) a recent review summarizes many scientific publications demonstrating that these cells can differentiate into almost all cell types [100].

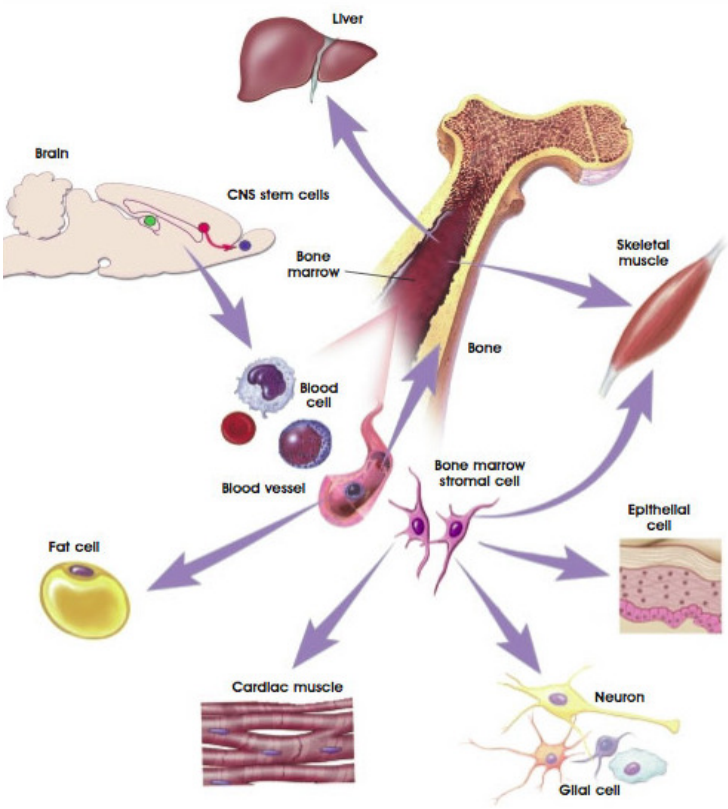


Figure 4: Examples of adult stem cells. *(Picture taken from http://stemcells.nih.gov).*

1.5 MESENCHYMAL STEM CELLS (MSCs)

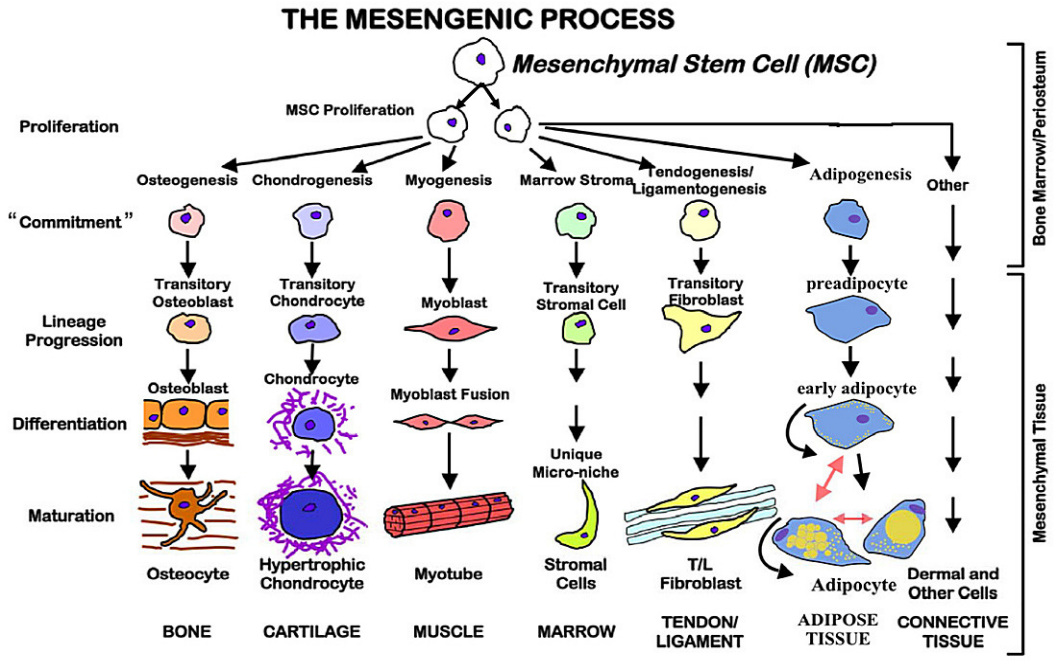


Figure 5: MSCs multipotent-capacity (*Tracey L Bonfield et al, Discovery Medicine April 15, 2010*)

Mesenchymal stem cells are multipotent progenitor cells originating from neural crest and mesoderm during embryogenesis [101-102] that reside within the stromal fraction of adult bone marrow where they are involved in non-hematopoietic stromal cell renewal. Even if MSCs reside in most tissues and can be easily derived from them, to date bone marrow represents the primary source of mesenchymal stem cells (bone marrow aspirates). These fibroblast-like cells in standard culture conditions grow adhering to plastic, are characterized by an extensive self-renewal capability and they also have a multi-lineage differentiation potential, being able to differentiate into mesodermal cell types such osteoblasts, chondroblasts and adipocytes [44, 103-104]. In addition, MSCs have also been demonstrated to have the potential to

differentiate into cells normally derived from endoderm or ectoderm (e.g. neuronal-like cells, cardiomiocytes or hepatocyte-like cells) [105-109] indicating an interesting versatility of these cells.

Because of all these properties, together with their hypoimmunogenicity (the absence or low level of expression of MHC class II antigens and costimulatory molecules such as CD80, CD 86 and CD40 limit immune-recognition) and immune-regulatory features, mesenchymal stem cells have often been considered an ideal cell source for stem cell based therapies and regenerative medicine applications, allowing therapeutic applications of both autologous and allogeneic MSCs in the clinical setting.

Tolerogenicity has also been associated with the expression of HLA-G antigen, a non classical major histocompatibility complex class I molecule, that represents a potent tolerogenic molecule reported to contribute to the regulation of immune responses in both physiological (e.g. playing a crucial role during fetal-graft tolerance via the maternal immune system [110]) and pathological conditions (such as cancer, multiple sclerosis and inflammatory diseases [111-113]).

MSCs immune-regulatory properties have been demonstrated on a wide range of immune system cells [114-119] by *in vitro* and *in vivo* experiments and this interesting and complex phenomenon, that it is still not fully understood, has been explained by direct cell to cell contact as well as by the action of several bioactive molecules and factors such as indolamine 2,3 dioxygenase (IDO), interleukin (IL)-6, nitric oxide (NO) and prostaglandin (PGE)-2 [119-122], and also the soluble isoform of HLA-G, named HLA-G5 [123].

MSCs for therapeutic applications:

A recent research of the clinical trials database (www.clinicaltrials.gov) showed 375 mesenchymal stem cells clinical trials all around the world (**Figure 6**), which make use of these cells to treat a wide range of pathological conditions or diseases (e.g. graft-versus-host disease, autoimmune diseases, bone diseases, liver and cardiovascular diseases). Most of the recorded clinical trials are in Phase I or Phase II. The results collected so far indicate that MSCs are mostly well-tolerated, with very few adverse effects; moreover, many of them showed the efficacy of mesenchymal stem cell infusions in treating stroke, liver cirrhosis and acute myocardial ischemia.

Patients, number	**Source of MSCs**	**MSCs Phenotype**	**Cells infused**	**Dosage**	**Adverse effects**	**Follow-up**	**Outcome**	**Reference**
Stroke, **16**	Autologous iliac crest	90% SH2	5×10^7 twice	2 doses, 2-week interval	None	To 5 years	HR for MSCs = 0.344	[125]
MI, **10**	Autologous iliac crest	CD73+ CD90+ CD105+	7.5×10^6 MSCs + EPCs	1 dose	None	6 months	LVEF 12% up	[126]
MI, **53**	Allogeneic unmatched 'prochymal'	CD105+ CD166+ CD45	0.5 to 5 × 10^6/kg	1 dose, intravenous	MSCs 5 pt Placebo 7	6 months	FEV1 up, LVEF up, arrhythmia down	[127]

ALS, **10**	Autologous iliac crest	CD29+ CD44+ CD90+ CD105+ CD166+	11.4 to 120 × 10^6	1 dose, thoracic spine	None	4 years	MSCs tolerated, SC scars	[128]
ALS, **19** MS, **15**	Autologous iliac crest	CD29+ CD73+ CD90+ CD105+ CD166+	ALS 5 × 10^7 MS 6 × 10^7 +/- ferumoxid	1 dose, intrathecal 1 dose, intravenous	None	6-25 months	ALSFRS stable, EDSS better	[129]
Refractory Crohn's Disease, **10**	Autologous iliac crest	CD73+ CD90+ CD105+	1 to 2 × 10^6/kg	2 doses, 1-week interval	Headache (3 patients) Allergy (1 patient)	14 weeks	CDAI fall (5 patients; > 70 in 3 patients);3 patients worse	[130]
Paediatric acute leukaemia, **8**	Haploid parent BM	Adherent CD phenotype ND	6 × 10^4 to 10^7/kg MSCs + UCBT	1 or 2 doses, 3-week interval	None	6.8 years, no chronic GVHD	All patients PMN+ at 9 to 28 days	[131]

Table 1: Examples of recent MSCs clinical trials [124]

Table 1 abbreviations: *ALS, amyotrophic lateral sclerosis; ALSFRS, ALS Functional Rating Scale; BM, bone marrow; CDAI, Crohn's disease activity index; Con, control; EDSS, Expanded Disability Status Scale; EPC, endothelial precursor cell; FEV1, forced expiratory volume in 1 second; GVHD, graft-versus-host disease; HCT, haematopoietic cell transplant. HR, hazard ratio; LVEF, left*

ventricular ejection fraction; MI, myocardial infarction; ND, not determined; PMN, polymorphonuclear leukocyte (surrogate for HSC engraftment); SC, spinal cord; UCBT, umbilical-cord blood transplant (unrelated).

Even if MSCs administration efficacy is not fully understood, it is widely accepted by the scientific community that four MSCs properties mainly contribute to their therapeutic/reparative effectiveness:

1) MSCs multipotency.

2) Mesenchymal stem cell hypo-immunogenicity and their immunomodulatory properties.

3) MSCs capacity to home to inflammatory or injured sites once injected in a host, irrespectively of the tissue (e.g lung or muscle tissue in mice [132-133]), in response to signals released by damaged tissues.

4) Anti-inflammatory properties associated with bioactive molecules secreted by mesenchymal stem cells in response to their local environment.

There are two main restrictions that limit the use of BM-MSCs: the invasive nature of the collection procedure and the progressive cell number decline from birth (where one mesenchymal stem cell is found per 10^4 cells) to adults, due to age-related alterations and replicative decline probably due to changes occurring at the level of the biological niche as a consequence of modified micro-environmental signals [134].

Consequently, more accessible sources of MSCs are desirable and these alternative stem cell sources could be represented by perinatal tissues, ready available and whose

isolated MSC are suggested to have a higher proliferative and differentiative potential over bone marrow derived ones.

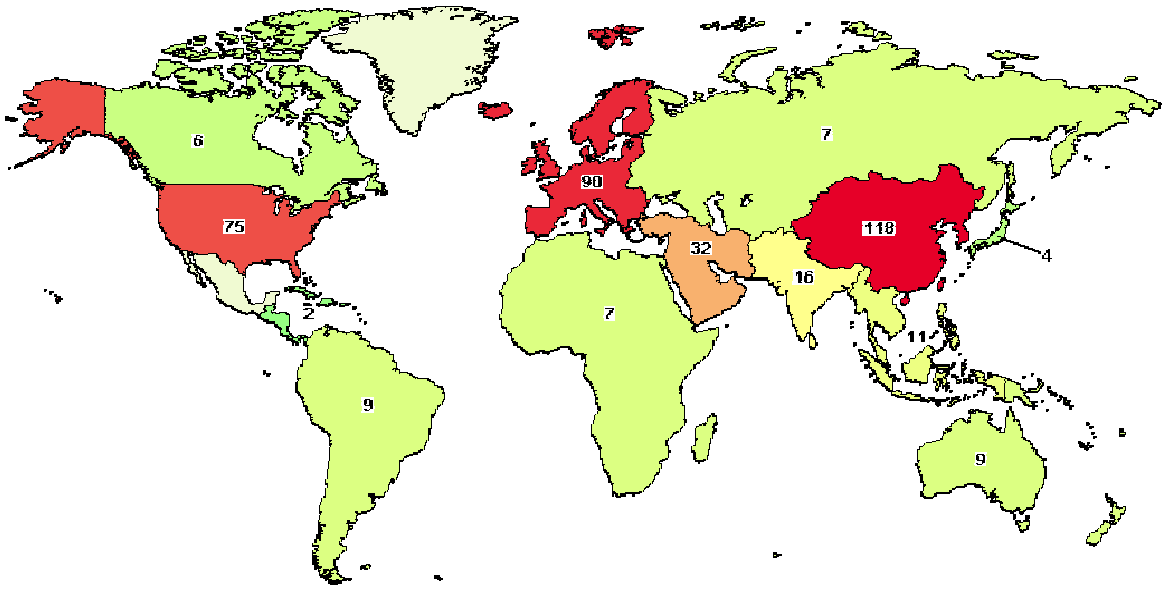


Figure 6: Mesenchymal stem cells clinical trials distribution worldwide (www.clinicaltrials.gov)

1.6 STEM CELLS AND HEPATIC REGENERATION

1.6.1 ANATOMY AND FUNCTIONS OF THE LIVER

The liver represents the largest solid organ of the human body and it plays a central role in metabolic homeostasis (lipid, carbohydrate and protein metabolism), participating in the synthesis, distribution and storage of many nutrients and metabolites. It is responsible of body detoxification and drug xenobiotic metabolism, via conjugation or metabolic modification processes.

As a gland, it exerts both exocrine function (bile production and secretion) and endocrine function (synthesizes chemicals and plasmatic proteins that are secreted directly into the blood regulating the functions of many other organs).

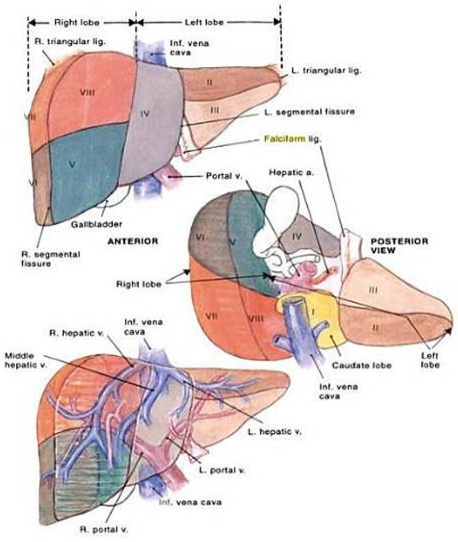


Figure 7: Liver Anatomy *(taken from "The Johns Hopkins Atlas of Human Functional Anatomy 4th Edition")*

The liver is located in the right and upper portions of the abdomen, beneath the right side of the diaphragm and under the right lower rib cage. The liver is mostly covered by the peritoneum and it has two surfaces: the *parietal surface*, and the *visceral surface*. The "classical description" of the liver anatomy considers the falciform ligament as dividing the parietal surface of this organ into a larger right lobe (that roughly represents 60-65% of the liver) and a left lobe. The visceral surface contains also the quadrate and caudate lobes [135].

Each hepatic lobe is composed of the *lobules* that traditionally have been considered as liver morphofunctional units (**Figure 8**). Each lobule resembles an irregular polygonal prism with a central vein in the middle of it and "portal spaces" at each corner.

In each lobule flat irregular plates of hepatocytes, separated by hepatic sinusoids, radiate from the central vein area towards periphery. The blood capillaries convey the blood centripetally from the periphery of the lobule (terminal branches of the hepatic artery and portal vein) to the central vein**,** while the bile flows centrifugally towards the portal areas.

Hepatocytes represent the main lobule cell type (80% of the total liver mass). They are large polyedric cells containing a big rounded nucleus (25% of these cells contain two nuclei), approximately 2000 mitochondria, abundant rough endoplasmic reticulum (albumin, prothrombin, fibrinogen and globulin synthesis) and smooth endoplasmatic reticulum (involved in detoxification or inactivation processes of various substances such as water-insoluble toxic bilirubin).

The remaining liver resident cells (20%) include Kupffer cells, Ito cells (fat-storing cells), stellate cells, natural killer (NK) cells, endothelial cells and cholangiocytes.

Kupffer cells are large mononuclear phagocytic cells derived from blood monocytes (representing approximately 70% of the total macrophage population of the body), involved in several processes such as bacteria and inflammatory mediator scavanging, phagocytosis of damaged or aged red blood cells and hemoglobin digestion [136]. A more modern perspective coniders the hepatic acinus to be the smallst functional comprising part of two adjacent hepatic lobules; the narrower ends are formed by two central veins, while the central wider portion is formed by two paortal areas. Even if the acinus better defines the hepatic functional unit, it is not so easy to be visualized by histology.

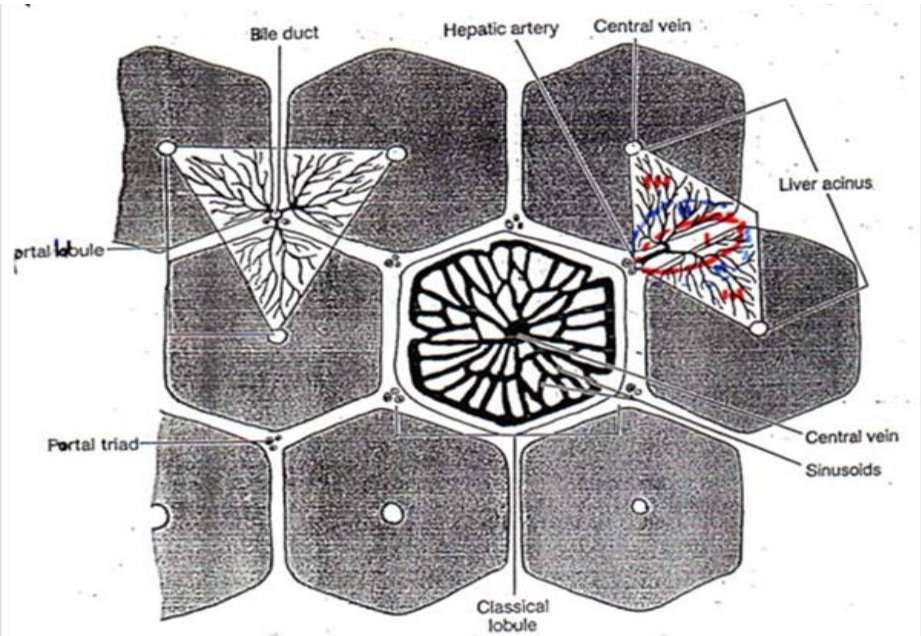


Diagram illustrating the defining characteristics of the classic liver lobule, portal lobule, and liver hepatic acinus of Rappaport. (Reprinted from Krause WJ and Cutts JH: *Concise Textbook of Histology*, 2nd ed. Baltimore, Williams & Wilkins, 1956, p 331.)

Figure 8*: The hepatic lobule* is roughly hexagonal in shape. The angles of the *hexagon* are called portal tracts (or portal areas). Hepatocytes are arranged in stacks of anastomizing plates, radiating from a centre of the lobule (central vein) towards the periphery. Hepatocyte plates are separated by an anastomizing system of sinusoids.

1.6.2 LIVER RIGENERATION

The extraordinary liver regeneration ability (after injury or upon surgical resection) has been known since classical times [43]. This phenomenon is made possible through proliferation of pre-existing mature hepatocytes that, in spite of their slow cell turnover rate, are able to regenerate hepatic tissue in response to tissue lesions. This important and complex regenerative process has to be considered as a compensatory process aimed to restore the number of functional hepatocytes necessary for the maintenance of body homeostasis.

Liver regeneration events that take place during the first 4-5 hours following partial hepatectomy (70% hepatectomy, HP) are often referred as "priming phase events", and they involve stress and inflammatory responses (cytokine production and release), transcription factor activation/generation (e.g. NFK-B, CIEBPP, STAT3 and AP-1) [137-141] extracellular matrix modifications (activation of metalloproteinase 9 within the first 30 minutes) [142-143] and regulatory events for cell-cycle entry (activation of almost 100 genes that directly or indirectly are involved in cell cycle entry and progression) [144]. The chronology of the very early signaling events after partial hepatectomy is summarized in **Table 2.**

Stat3 and NF-kB are important signaling molecules contributing to hepatocyte proliferation. Immediate early and delayed gene expression is not sufficient to reach DNA replication unless the hepatocytes progress through the cell cycle, progression sustained by growth factors such as transforming growth factor alpha (TGF-α) and hepatocyte growth factor (HGF) [145]. Once this restriction point is passed,

hepatocytes express cyclin Dl (at 6 hours there is clear evidence for of cyclin D1 activation) andcells are irreversibly committed to replication process [146].

When pathological conditions or extended hepatic injuries are so severe to compromise hepatocyte regenerative capability (as in chronic hepatopathy, viral hepatitis or hepatic steatosis), hepatic stem cells are activated. These cells (named *oval cells* in rats and mice) are liver progenitors characterized by ovoid nuclei and high nuclear/cytoplasmic ratio; these bipotential stem cells reside within the liver's terminal branches of the biliary tree (canals of Herring) and can give rise to bile duct cells or hepatocytes. In humans, oval cells were identified in several liver diseases such as hepatitis B-associated hepatocellular carcinoma, hepatoblastoma and in cholestatic liver diseases [147-149]. The lack of unique liver stem cell markers has posed significant challenges to the identification and isolation of the 'true' liver stem cell.

Oval cells express hepatocyte (albumin, alpha-fetoprotein, cytokeratins CK8 and CK18) and cholangiocyte (CK7, and CK19) markers and they also express some progenitor markers (OV-6 in rats, chromogranin A in humans) [150-153]. Moreover, these cells are positive for some hematopoietic stem cells markers (CD34, CD45, CD90, Sca-1, c-Kit, and flt-3) [150,154]. Under physiological conditions, these cells are few and quiescent; in response to hepatic injuries oval cells start to proliferate, modify their morphology and surface markers and start to differentiate to compensate liver mass loss.

Differently from rats, human bipotent progenitor cells express EPCAM (epithelial cell adhesion molecule), NCAM and do not express alpha-fetoprotein and albumin [155-163].

Multiple signaling pathways involving growth factors, cytokines, paracrine signals, and neuroendocrine factors occur simultaneously within the first 60 min after partial hepatectomy (PH). These include:
• Increase in urokinase activity (first 5 min)
• Translocation of Notch to the nucleus (15 min)
• Translocation of beta-catenin to the nucleus (5–10 min to 6 h)
• Decrease in HGF biomatrix stores (30 min to 3 h)
• Activation of the HGF receptor (within 30–60 min)
• Activation of the EGF receptor (within 30–60 min)
• Increase of HGF, Norepinephrine, IL6, TNFa, TGFb1 and hyaluronic acid in the plasma (1–2 h)
• Activation of AP1, NFkB, and STAT3 (30–60 min)
• Extensive gene expression reprogramming of hepatocytes within 30 min after PH

TABLE 2: Early signaling events activated followingpartial hepatectomy(from George K. Michalopoulos *Liver Regeneration* J Cell Physiol. 2007 November; 213(2): 286–300.)

1.6.3 THERAPEUTIC POTENTIAL AND CLINICAL APPLICATIONS OF CELL THERAPY FOR LIVER DISEASES

In spite of liver unique regeneration potential, in case of advanced end-stage liver diseases this capability is not enough to reestablish a sufficient liver mass and, consequently, to adequately restore liver homeostasis and hepatic functions.

To date, liver transplantation represents the only life-saving procedure for liver pathologies such as alcoholic liver disease, primary liver cancer and haemochromatosis, hepatitis C and B infections (that represent the major liver diseases); during the last decades it has been registered a dramatic increase of the number of human liver transplants but the demand greatly exceeds organ availability.

In U.S. 20,000 people wait for liver transplantation, but less than 1/3-1/4 of needed transplants are performed annually, therefore more than a hundred patients die yearly while on the waiting list.

Furthermore, it has to be said that liver transplantation benefits are hampered by many disadvantages, such an increased risk of infection, post transplant rejection, biliary conditions and a higher risk of developing type 2 Diabetes. All these issues have lead researchers to propose and develop new approaches as alternative therapies for intractable liver diseases; specifically, in recent years, scientific community has been giving increased attention to cell therapy to treat liver insufficiency or end-staged liver diseases, and that could be used as a bridge to liver transplantation.

Proposed approaches can be broadly divided into two categories: *transplantation of endogenous cells* (mature hepatocytes or hepatic committed stem cells) and *strategies*

involving extra-hepatic stem cells (mainly mesenchymal stem cells and hematopoietic stem cells).

Strong scientific evidences have demonstrated therapeutic efficacy of allogeneic hepatocyte transplantation, particularly to treat liver based metabolic disorders such as Crigler-Najjar Syndrome type I, ornithine-transcarbamylase deficiency and coagulation factor VII deficiency [157-160]. Moreover, Khan et al. completed a clinical trial based on human fetal liver stem cell transplantation in patients with end-stage liver cirrhosis, demonstrating the safety and efficacy of this procedure [161].

On the other hand the limited availability of human organs for hepatocyte isolation and short term effects of the therapy (from few days to few months for acute liver injuries) represent the major limits to this procedure.

Another alternative endogenous cell source for transplantation is represented by previouslydescribed hepatic progenitor cells. These cells can be easily isolated from liver of all donor ages and extensively expanded (more than forty population doubling) under serum free conditions, demonstrating a high expansion potential [162-164].

In India clinical trials involving fetal liver derived EpCAM ^{positive} stem cells have been conducted, resulting at 6-month follow-up into a significant decrease in the mean of MELD SCORE and minimal immunological issues have been proved. Notably, patients enrolled for the trials had an original life expectancy of 5-6 months [161, 165-166].

Preliminary results regarding ongoing clinical trials with hepatic stem cells (EpCAM+) and adult hepatocyte transplantation can be summarized as follow [167]:

Hepatic Stem Cells (isolated from fetal livers)	Cell number tested ranged between 100-150 milion
	More than 280 patients involved in trials
	No complication from transplanted procedure (delivery via hepatic artery)
	Immunosupression not required
	Engraftment efficiencies were around 20%
	Long term effects (more than 4 years)
	Significant improvement in MELD or Child-Pug Scores and in all measured liver functions

TABLE 3: Fetal stem cell clinical trials

Adult hepatocyte transplantation (cells isolated from neonatal, fetal or adult livers)	Number of cells transplanted: from few hundred million to billions
	Complications related with transplant procedure (delivery by vascular route into portal vein) such as emboli formation
	Immunosuppression required
	Significant improvement in measures of liver functions
	Transitory effects ranged from few month to few years for patients with methabolic disorders and from few days to few years for acute liver injuries

TABLE 4: Adult hepatocyte clinical transplantations

In a context in which it is still a matter of debate as to which adult stem cell population may be the most effective in the regeneration of injured liver tissues, hematopoietic and mesenchymal stem cells have emerged as two of the major candidates, because of their differentiation capability into hepatocyte-like cells *in vitro* (in presence of HGF, FGF and oncostatin M) and *in vivo* [89, 168-170].

It has been reported that hematopoietic stem cells (HSCs) can differentiate into hepatocyte in case of liver injuries/established liver fibrosis (animal models) [168-169, 171]. HSCs have also been used in clinical trials as a treatment for liver diseases. In two of them [172-173], the effects of whole bone marrow peripheral infusion and of CD34+ HSCs intrahepatic injection have been assessed in nine and five cirrhotic patients respectively. The results of these two studies indicate that bone marrow stem cell transplantation resulted in an increased hepatic function (e.g. significant improvement of albumin levels) in a number of cirrhotic patients; however, both analyses were conducted on patient groups that were too small to be representative of the population.

A clinical investigation involving a larger group of patients with end-stage liver cirrhosis (140 patients) was conducted in Egypt by Salama et al. [174]. Patients were randomized into two groups: a control group (receiving regular treatment) and a second group receiving granulocyte colony stimulating factor followed by autologous CD34 (+)/CD133 (+) hematopoietic stem cell infusion in the portal vein. The results obtained by this group were consistent with the data published by the other two groups in terms of significant improvement of albumin and bilirubin levels in the transplanted patients compared to the control group, as well as safety and tolerability of the procedure.

In the last few years MSCs, isolated from several tissues, have emerged as elective candidates for cell transplantation since they are abundantly resident in almost all tissues, can be easily isolated, and due to their multipotency and regenerative outcome in animal models, as well as their immunomodulatory properties.

MSCs capability to differentiate into hepatocyte-like cells was first described in 2004 [109] and MSCs trans-differentiation was subsequently demonstrated through several scientific reports, indicating the acquisition of hepatocyte-like features (described earlier) [173, 175-178].

To date, more than twenty clinical trials involving MSCs infusions (unfractionated bone marrow derived MSCs, bone marrow CD34/CD133 positive cells or MSCs pre-differentiated into hepatocyte-like cells) to treat cirrhosis or acute liver failure have been published (www.clinicaltrials.gov). Even if long term effects were not observed, safeness and liver function improvements have been reported.

In general it has to be said that so far many aspects such as cellular characterization (in some cases) or a better understanding of *in vivo* MSCs action that allow clinical improvements need to be better characterized, and randomized controlled studies are needed.

2. AIMS OF THE STUDY

Over the last decade, multiple evidences have highlighted the attractive therapeutic potential of perinatal mesenchymal stem cells making them promising candidates for tissue engineering and regenerative medicine applications. In particular, our group has demonstrated that multipotent Wharton's Jelly Cells (WJ-MSCs) can be easily isolated and successfully expanded *in vitro* from human umbilical cord. These cells have the potential to be committed towards different tissues and, recently, immunomodulatory features were also demonstrated by us and others.

Preterm cords can be made available from therapeutic abortions, constituting an additional cell source to obtain higher numbers of WJ-MSCs. Little is known about the phenotype and the differentiation potential of these cells.

Our hypothesis is that WJ-MSCs isolated from preterm umbilical cords, because of their more immature state, could have a greater differentiation potential than the ones isolated from term umbilical cords and may be proposed as additional stem cells that can be used for regenerative medicine applications.

The following objectives were designed to test the hypothesis:

Aim 1: Determine if preterm WJ-MSCs can be isolated and cultured using the protocols previously described by my group for mature umbilical cords.

Aim 2: Define a detailed phenotypic characterization of preterm WJ-MSCs using flow cytometry, immunocytochemistry and immunofluorescence analyses.

Aim 3: Evaluate mesenchymal multi-lineage potential of isolated cells testing their ability to differentiate into cells of mesodermal lineages (osteo-, condro- and adipo-genic potentials).

Aim 4: Induce hepatic differentiation by applying a two step protocol (an induction phase and maturation phase); confirm the acquisition of mature haepatic features by means of specific functional assays.

Aim 5: Evaluate if induced differentiation processes alter preterm WJ-MSCs immunomodulatory molecules expression.

3. MATERIALS AND METHODS

3.1 Isolation procedure and culture conditions:

Preterm umbilical cords were obtained following therapeutic abortions after mothers' informed consent, from *Reparto di Ostetricia e Ginecologia of Ospedale Civico of Palermo.* After the harvesting procedure, the cords were stored at room temperature into a sterile box containing Hanks' Balance Salt Solution (Lonza) supplemented with 2x Antibiotic and Antimycotic solution (Sigma). The cords were normally processed within 12 hours from collection.

To isolate Wharton Jelly Cells (WJCs), the immature cords were rinsed two times (10 minutes each) into fresh HBSS and typically cutted into 1 cm pieces (3-6 pieces depending on the cord length), avoiding tissue drying.

In order to expose the inner soft tissue, each piece was longitudinally and obliquely incised using a sterile scalpel. Furthermore, the jelly substance was gently scraped and each piece was placed into culture dish with Dulbecco's Modified Eagle's Medium (DMEM) low glucose w/glutamine (Sigma) supplemented with 10% fetal bovine serum (PAA), 1x non essential aminoacids (Sigma) and 1% antibiotic/antimycotic solution (Sigma) for cell expansion.

The culture was maintained at 37ºC in a humidified atmosphere at 5% CO2. After 15 days the cord pieces were removed. At ~80-90% of confluence cells were passaged with TrypLE Select (Gibco) at 37 ºC and centrifugation at 1100 rpm for 5 minutes. The viability of the isolated cells was evaluated by trypan blue exclusion.

3.2 Immunophenotype profiling: FACS analysis, immunofluorescence and immunocytochemistry staining

In order to characterize the isolated cells, early passages of WJCs (typically P2 and P5) were used.

Flow cytometry analysis:

All flow cytometric acquisitions were performed on a BD FACS Aria II (Becton Dickinson) and data were analyzed using the software FACSDiva Version 6.1.2. Briefly, approximately 1 x 10^6 cells were used for each antibody staining. Fixation and permeabilization of cells were performed with the Cytofix/Cytoperm kit (BD Biosciences) according to manufacturer's instructions. Cells were investigated for the expression of molecules whose corresponding primary antibodies are listed in Table 5

Secondary antibodies (when required) and Isotype antibodies (*BD Biosciences)* were included as controls in each experiment.

Immunofluorescence (IF):

For immunofluorescence staining, cells were cultured in 8 well chamber slides (BD Biosciences). Once the cells reached confluence, each well was fixed according to antibody manufacturer's instructions. After being blocked in PBS 0.5% Tween 20, 3% bovine serum albumin (BSA) for one hour at room temperature, cells were incubated with the primary antibodies overnight at 4°C, rinsed with PBS, and then incubated with the appropriate fluorescence-conjugated secondary antibodies (Alexa

Fluor, Invitrogen, Carlsbad, CA) for 1 h at room temperature in the dark. Cells were mounted with Prolong Gold Antifade (Invitrogen), which includes diamidino-2-phenylindole (DAPI) for the nuclear counterstaining and stored in the dark at 4°C until fluorescence microscope analyses. Cell imaging was done with a microscope (Nikon Eclipse 50i, Melville, NY) coupled with a camera (Olympus XM10, Tokio, Japan) and Cell F software for image acquisition (Olympus). The primary antibodies are listed in Table 6.

Immunocytochemistry (ICC):

For Immunocytochemistry characterization, cells were cultured in 8 well chamberslides (BD Biosciences). Once the cells reached confluence, each well was singularly stained for the expression of albumin, B7-H3, B7-1, B7-2, CK18, CK19, connexin 40, connexin 43, connexin 45, c-Kit., GATA-4, HLA-E, HLA-G, nestin,, PSG1, vWF.

Briefly, cells were fixed with ice-cold methanol for 20 minutes, rinsed with PBS, and permeabilized with PBS 1x containing 0,1% Triton X-100. After a subsequent washing step with PBS, slides were exposed to 0.3% H_2O_2 in PBS and then blocked with blocking serum (included in Vectastain kit) according to the manufacturer's instructions. Next, the cells were incubated for 1.5 hrs at RT with various primary antibodies diluted in permeabilization buffer (The primary antibodies are listed in Table 6). Cells were then washed with PBS and incubated at room temperature for 30 minutes with the appropriate secondary antibody diluted in blocking solution. Immunostaining was performed with ABC *peroxidase staining* kit (VectorLab.) and

Dako / AEC Substrate-Chromogen. Cells were next washed with PBS and counterstained with hematoxylin to identify nuclei.

3.3 Adipogenic differentiation:

Adipogenic potential was evaluated after 21-day incubation in adipogenic medium 0.5 mM isobutylmethylxanthine (Sigma), 1 µM dexamethasone (Sigma), 10 µM insulin (Sigma), 200 µM indomethacin (Sigma), 10% FCS (PAA), 1x NEAA (non-essential aminoacids) (Sigma) and 1% antibiotic-antimycotic (Sigma), using Oil red O staining as described previously (La Rocca et al, 2009).

3.4 Osteogenic differentiation:

Osteogenic differentiation was induced on monolayer cells by adding osteogenic soluble factors to the regular culture medium (50 µM ascorbate-2-phosphate (Sigma), 10 mM β-glycerophosphate (Sigma), and 0.1 µM dexamethasone (Sigma). MSCs cultured in normal growth medium were included as controls. After three weeks, calcium deposition was assessed by Alizarin red S staining as described previously (La Rocca et al, 2009).

3.5 Chondrogenic differentiation:

Chondrogenic induction was performed in an alginate 3D system. Before encapsulation, the cells were pelleted by centrifugation and resuspended at a cell concentration of 3 million cells/ml, in 2.4% w/v solution of sterile filtered low

viscosity sodium alginate (Sigma). The solution was placed in a sterile syringe and gently sprayed (drop by drop) in a solution containing 100mM $CaCl_2$, causing gelation of the alginate as microspheres containing the cells. Groups of 4-5 microspheres where then cultured in standard medium or chondrogenic medium (DMEM supplemented with 1% FBS, 6.25 mg/mL insulin, 10 ng/mL transforming growth factor beta 1, 50nM ascorbate-2-phosphate, 1% antibiotic–antimycotic, and 1x NEAAs).

After three weeks, beads were collected into 50 ml conical tubes and fixed for 4 hours at room temperature with 4% paraformaldehyde in 0.1 M sodium cacodylate/10mM calcium chloride buffer. After fixation, the specimens were overnight rinsed at 4ºC in 50 mM $BaCl_2$ (barium chloride) to irreversibly cross-link the alginate matrix. Then the beads were dehydrated with a graduated ethanol series cleared with xylene, embedded in paraffin and 4 μm sections were prepared.

Histological assessment of chondrogenesis

Before proceeding with the staining protocol, the sections were deparaffinized and rehydratated. Hematoxylin and Eosin staining (H&E) for morphology analysis and Alcyan blue staining for proteoglycan detection were performed using standard procedures.

3.6 Hepatogenic differentiation:

Wharton's Jelly Cells were induced to hepatic differentiation by sequential addition of exogenous factors to cell culture medium.

For the first three weeks cells were cultured in 1% FBS DMEM low glucose w/glutamine supplemented with FGF-4 (10 ng/mL, Miltenyi Biotech), HGF (20ng/mL, Miltenyi Biotech), ITS (1x, Sigma), 0,1 μM dexametasone (Sigma), 1x NEAA (Sigma).and 1x Antibiotic-Antimicotic solution (Sigma). For the following two weeks oncostatin M (10 ng/mL, Prospec) was also added to the above-mentioned differentiation medium. Medium was changed three times per week and hepatogenic maturation was assessed at different time points (after three, four and five weeks) by flow cytometry, PAS staining, G6Pase assay, Indocyanine green (ICG) uptake and CYP3A4 assay. For each experiment samples cultured under standard conditions for the same timeframes were also included as controls.

Glucose-6-Phosphatase (G-6-Pase) assay:

Glucose 6- phosphatase is a key enzyme in the glucose metabolism involved in gluconeogenesis and glycogenolisys. In order to evaluate the activity of the enzyme, we used a protocol provided by Professor Etienne Sokal (University of Louvain, Belgium). After removing the medium, treated and control WJCs were incubated for 20 minutes with 0.1M Tris-acetate buffer (pH 6.5) containing 2.08 mM glucose-6-phosphate and 2.4 mM lead nitrate at 37ºC, in a fully humidified atmosphere containing 5% CO_2.

Active G-6-Pase hydrolyzed Glucose-6-Phosphate to free Glucose and inorganic Phosphate, thus inducing intracellular $Pb(NO_3)_2$ formation and precipitation. Cells were gently washed three times with water and then incubated for few seconds with ammonium sulfide 1% to convert lead nitrate into lead sulfate (brown precipitates).

Sulfide solution was removed and cell images were taken after an additional rinse with water.

Cytochrome-p450-3A4 (CYP3A4):

Xenobiotic detoxification is one of the main functions of the liver. This process is performed by a monooxigenase, known as CYP 450, an enzyme belonging to a superfamily of proteins that are involved not only acting in the first phase of methabolism of substances such as such as chemicals, alcohols and toxin but they also play an important role in the synthesis of steroid hormones. In the liver exist several isoforms of these enzymes, such as CYP450 3A4 and CYP450 2B6. Cytochrome P450 Isoform 3A4 activity was assessed by the luminescent-based assay P450-Glo (Promega) following manufacturer's instructions.

Rifampicin (25 μM) and Ketoconazole (10 μM) were respectively used as CYP3A4 inducer and inhibitor. Fresh compounds were added at 24 hours and cytochrome activity was measure in control and differentiated cells after. 48 hours after the treatments by Promega *GloMax* 96 Microplate *Luminometer*

Indocyanine *green (ICG) uptake:*

Cardiogreen or indocyanine green (SIGMA) is a water soluble cyanine dye that forms non covalent fluorescent complexes with some plasma proteins, mainly with albumin. Indocyanine green linked with albumin is rapidly taken up by the liver and then excreted unchanged into bile. Briefly, confluent cells were incubated for 15 minutes

at 37ºC with a solution of ICG in culture medium (1mg/mL, Sigma). After three washes with PBS, cells were microscopically observed and imaged.

Periodic Acid-Schiff staining:

Periodic Acid Schiff staining (PAS-staining) is a staining method that allow the detection of polysaccharides such as glycogen and mucopolysaccharides. The periodic acid oxides the diol functional groups these sugars, creating aldehydes. The aldehydes subsequentely react with Schiff reagent generating a purple-magenta colour. WJCs grown in 8-well chamber slides were fixed in 4% paraformaldehyde (in ethanol) for 1 minute at room temperature and glucose metabolism was analyzed by glycogen Periodic Acid-Schiff (PAS) (Sigma Aldrich) according to manufacturer's instructions prior to microscopic examination and imaging.

TABLE 5: List of antibodies used for flow cytometry analyses

Antigen	Clone	Conjugated	Dilution	Manufacturer
Albumin	188835	uncon-jugated	1:400	R&D Systems
AFP	189506	unconjugated	1:800	Cedarlane
CD10	HI10a	APC	1:20	Becton Dickinson
CD11b	ICRF44 (also known as 44)	PE CY7	1:20	Becton Dickinson
CD13	WM15	APC	1:20	Becton Dickinson
CD14	MφP9	APC CY7	1:20	Becton Dickinson
CD29	mar-04	APC	1:20	Becton Dickinson
CD31	WM59	FITC	1:20	Becton Dickinson
CD34	581	FITC	1:20	Becton Dickinson
CD44	G44-26 (also known as C26)	APC	1:20	Becton Dickinson
CD45	2D1	PerCP	1:20	Becton Dickinson
CD54	HA58	APC	1:20	Becton Dickinson
CD68	Y1/82A	PE	1:20	Becton Dickinson
CD71	M-A712	APC	1:20	Becton Dickinson
CD73	AD2	APC	1:220	Miltenyi Biotec GmbH, Bergisch Gladbach, DE
CD90 (Thy-1)	5 E10	FITC	1:400	Becton Dickinson
CD105	SN6	FITC	1:20	Abcam, Cambridge, MA
CD106	51-10C9	APC	1:20	Becton Dickinson
CD117	YB5.B8	PE	1:20	Becton Dickinson
CD166	3A6	PE	1:20	Becton Dickinson
CD276	FM276	APC	1:220	Miltenyi Biotec GmbH
CD309	89106	APC	1:20	Becton Dickinson
CK18	C-04	FITC	1:400	Abcam, Cambridge, MA
CK19	RCK108	PE	1:400	Santa Cruz Biotechnology, Santa Cruz, CA
EpCAM	EBA-1	FITC	1:20	Becton Dickinson
E-Cadherin	36	FITC	1/2000	Becton Dickinson
HLA ABC	W6/32	FITC	1/6000	Abcam Cambridge, MA
HLA DR	L243 (G46-6)	PerCP	1:20	Becton Dickinson
HLA-G	87G	PerCP	1:20	eBioscience Inc., San Diego, CA
HLA-E	3D12HLA-E	APC	1:20	eBioscience
IgG1	G18-145	FITC	1:20	Becton Dickinson Biosciences, San Jose, CA
IgG2	PC10	PE	1:20	Becton Dickinson
Oval cell	OV-6	unconjugated	1/2000	Santa Cruz Biotechnology
Vimentin	VI-RE/1	PE	1:20	Abcam, Cambridge, MA
STRO-1	MM-30	APC	1:20	Biolegend, San Diego, CA

TABLE 6: List of antibodies used for Immunocytochemistry and Immunoflourescence assays.

Antigen	**Host**	**Manufacturer**	**Dilution (IF)**	**Dilution (ICC)**
Albumin	Mouse	Santa Cruz	N/A	1:100
B7-1	Mouse	Santa Cruz	N/A	1:50
B7-2	Mouse	Santa Cruz	N/A	1:800
B7-H3	Rabbit	Santa Cruz	1:50	1:300
Connexin 40	Rabbit	Santa Cruz	1:50	1:100
Connexin 43	Rabbit	Santa Cruz	1:50	1:100
Connexin 45	Rabbit	Santa Cruz	1:50	1:100
Cytokeratin 8	Mouse	Sigma	N/A	1:200
Cytokeratin 18	Mouse	Sigma	N/A	1:800
c-Kit	Rabbit	Epitomics	1:20	1:50
GATA-4	Rabbit	Santa Cruz?	1:100	1:100
Nestin	Mouse	Santa Cruz	1:100	1:100
HLA-E	Mouse	Santa Cruz	N/A	1:100
HLA-G	Mouse	Santa Cruz	N/A	1:100
PSG-1	Mouse	Santa Cruz	N/A	1:100
Von Willebrand factor	Mouse	Santa Cruz	N/A	1:100

4. RESULTS

4.1 ISOLATION AND EXPANSION OF MESENCHYMAL CELLS FROM PRETERM UMBILICAL CORDS

For the present study, eight specimens were used, which correspond to a gestational age ranging between 15 to 22 weeks.

The isolation protocol applied resulted in the derivation of homogeneous populations of cells with fibroblastoid morphology with plastic adherence. Only for one of the eight umbilical cords used we were not able to derive a cellular population, due to a traumatic abortion procedure. Otherwise, no other subjective differences between the samples did influence the isolation procedure or the subsequent culturing phase. A detailed list of the preterm cords used for this study is summarized in **Table 7**.

Fibroblast-like cells exiting from tissue pieces were visible after about 4-5 days following tissue processing. The morphology of such cells strikingly resembled that of mesenchymal stem cells of different tissue origin. As shown in **Figure 9**, preterm WJ-MSCs exhibit a large nucleus containing a variable number of nucleoli and a cell body that is larger compared with term WJ-MSCs. As observed earlier for full-term WJ-MSCs, also for preterm ones, at confluence the cells changed their shape, assuming a tapered and elongated morphology with arrangement in strictly packed parallel rows.

Primary cell cultures were rapidly expanded up to nine-ten passages, without losing their proliferative potential. Adherent cells at passage two and five were chosen for the subsequent characterization by flow cytometry, ICC and IF.

SAMPLE CODE	GESTATIONAL AGE	FOETAL PATHOLOGY	CELL PASSAGE
MES 11_13	21 weeks	Trisomy 21	p9
MES 11_14	21 weeks	Multiple Pathologies (Cystic Hygroma..)	p10
MES 11_15	16 weeks	Trisomy 21	N/A
MES 11_16	15 weeks	Trisomy 21	p9
MES 11_17	20 weeks	Spina Bifida	p10
MES 12_01	21,5 weeks	Spina Bifida	p9
MES 12_02	22 weeks	Bilateral ventriculomegaly	p7
MES 12_03	16 weeks	Cardiac insufficiency	p7

TABLE 7: Pretterm umbilical cords used for the present study.

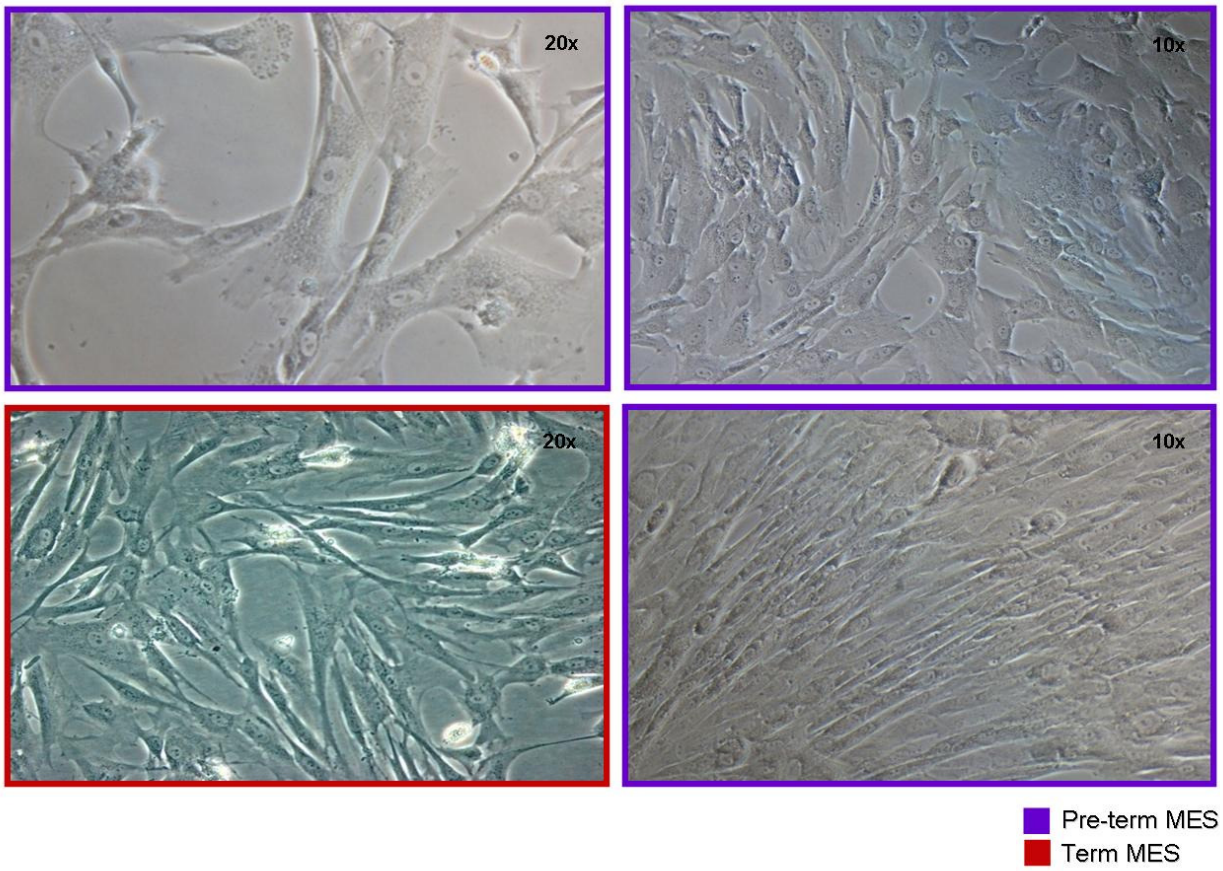


Figure 9: Phase contrast images showing morphological features of preterm cord derived WJ-MSCs (violet) compared to WJ-MSCs isolated from term umbilical cords (red).

4.2 PHENOTYPICAL CHARACTERIZATION OF UNDIFFERENTIATED WJ-DERIVED CELLS FROM PRETERM UMBILICAL CORDS BY FLOW CYTOMETRY, IMMUNOFLUORESCENCE AND IMMUNOCYTOCHEMISTRY ANALYSES.

Culture-expanded cells derived from preterm umbilical cords (p2 and p5), were analyzed by flow cytometry, immunofluorescence and immunocytochemestry, showing a phenotype that is overall similar to that of MSCs derived from other tissues.

To date, an exclusive set of antigens that could be used to uniquely identify mesenchymal stem cell population is still lacking, and MSCs are described as being positive for the expression of several mesodermal antigens and negative for the expression and hematopoietic and endothelial markers.

In order to provide an ample characterization of preterm MSCs antigenic profile, we defined a panel of 45 antibodies used to identify antigens that, based on their expression in different mature tissues derived from the three germ layers, or their functions, were grouped into five categories:

- Mesodermal antigens (11)

- Hematopoietic markers (4)

- Endothelial antigens (3)

- Immunomodulatory antigens (11)

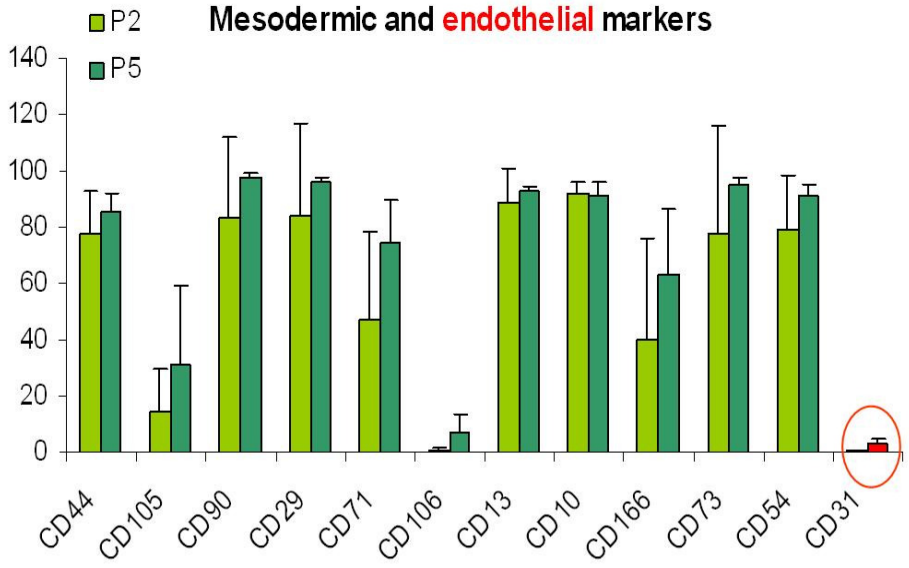
- Hepato-specific antigens (6)

- Other antigens (10)

Flow cytometry immunophenotyping showed that, regardless the foetal pathology associated with the umbilical cord and independently from the gestational age of the foetus, all primary cell cultures were markedly positive for *mesodermal antigens* (CD29, CD44, CD73, CD90, CD105, CD71, CD106, CD13, CD10, CD166, CD54 , **Fig. 10-A**) and negative (or weakly positive) for typical *hematopoietic* (CD45, CD34, CD14, CD68, CD309, **Fig. 10-B**) and *endothelial markers* (CD31 and Von Willebrand factor, the last evaluated by Immunocytochemistry, **Fig. 10-C**).

Considering the results altogether, more than 90% of the overall cell population at passage five expressed CD90, CD29, CD13, CD10, CD73 and CD24 antigens, whilethe expression of CD105 was more sample-dependent, with marked individual differences. This is not surprising since even if CD105 is one of the most commonly reported markers to be expressed in MSCs, a variable or low expression of this molecule has already been reported by others [reviewed in 179] and this variability may also be related to the lower gestational age of our samples.

A



B

HEMATOPOIETIC MARKERS (%)

	CD45	CD34	CD14	CD68	CD309
p2	0,4	0,1	4	0,2	0,4
p5	2	0,6	4	0,7	1,5

C

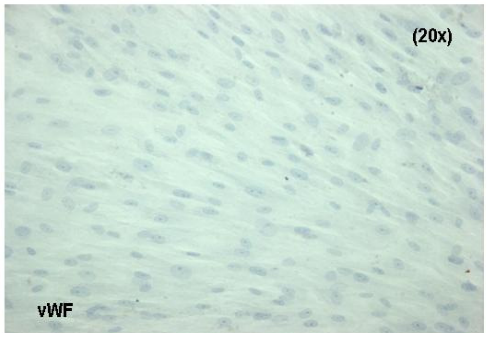


Figure 10: Preterm WJ-MSCs do express mesodermal antigens and lack both hematopoietic and endothelial ones. Flow cytometry data (A) showed that for most of the markers associated to the MSCs phenotype, WJ-MSCs were positive, while lacking hematopoietic markers (B). ICC was used to demonstrate the absence of vWF, a marker of mature endothelial cells (C). Magnification: 20 x.

Wharton's jelly MSCs are known to prominently express also a number of *immunomodulatory molecules*, which were correlated to the low immunogenicity of these cells both *in vitro* and *in vivo*. In particular, undifferentiated WJ-MSCs were characterized for the expression of markers such as class I and class II MHC and the expression of the costimulatory molecules CD80 (B7-1) and CD86 (B7-2), which is required for the development of T cell responses. The definition of an immunomodulatory profile is very important because it gives important indications, that could be very helpful to define the properties of these cells as possible candidates for *in vivo* cellular therapy applications.

As shown **in Figure 11**, all of the investigated preterm WJ-MSCs populations did express type I MHC (HLA-ABC) at high levels, while lacking expression of type II MHC (HLA-DR). In addition, molecules belonging to the type Ib MHC (HLA-E and HLA-G), were also demonstrated to be expressed in our cells, albeit at lower levels with respect to classical type I MHC. In addition, no expression of CD80 or CD86 was detectable in any of our cell lines, thus constituting a favourable phenotype with regard to lymphocyte stimulation ability. More interestingly, preterm WJ-MSCs did express high levels of CD276 (B7-H3), which is another member of the B7 family. The function of B7-H3 is still unknown but it has been postulated that it may exert immunoprotective and tolerogenic functions, attenuating peripheral immune responses through co-inhibition.

The expression of high levels of class I MHC is very important because these molecules can act “protecting” mesenchymal stem cells from certain NK cell mechanisms of deletion (e.g. NK cells kill tumor cells that have downregulated class

I) [180]. The absence of HLA-DR expression (<1%) indicates a reduced immunogenicity through the control of alloantigen expression [181], giving MSCs the possibility to escape recognition by alloreactive CD4+ T cells. In addition, preterm WJ-MSC do not express CD80 and CD86, two of the co-stimulatory molecules required for effector T cell induction [182]. The absence of co-stimulatory molecules is a significant observation. It implies that any residual engagement of the T cell receptor on Th cells would result in anergy and contribute to tolerance rather than allogeneic responses.

As stated above, we investigated the expression of two non-classical type I HLAs that are HLA-E and HLA-G, evaluated by flow cytometry and ICC. The results obtained indicated expression of HLA-E (the average value at passage 5, evaluated by flow cytometry, is 15%, in accordance to the ICC results that are here not shown) and low levels of HLA-G, which varied with respect to the different cell lines in a subjective fashion (**Fig. 11A**). The expression of HLA-E and HLA-G has been linked to the immunomodulatory ability of MSCs, and it constitutes part of the immunomodulatory machinery of the embryo and placenta to modulate the immune response of the mother towards the semi-allogeneic fetus.

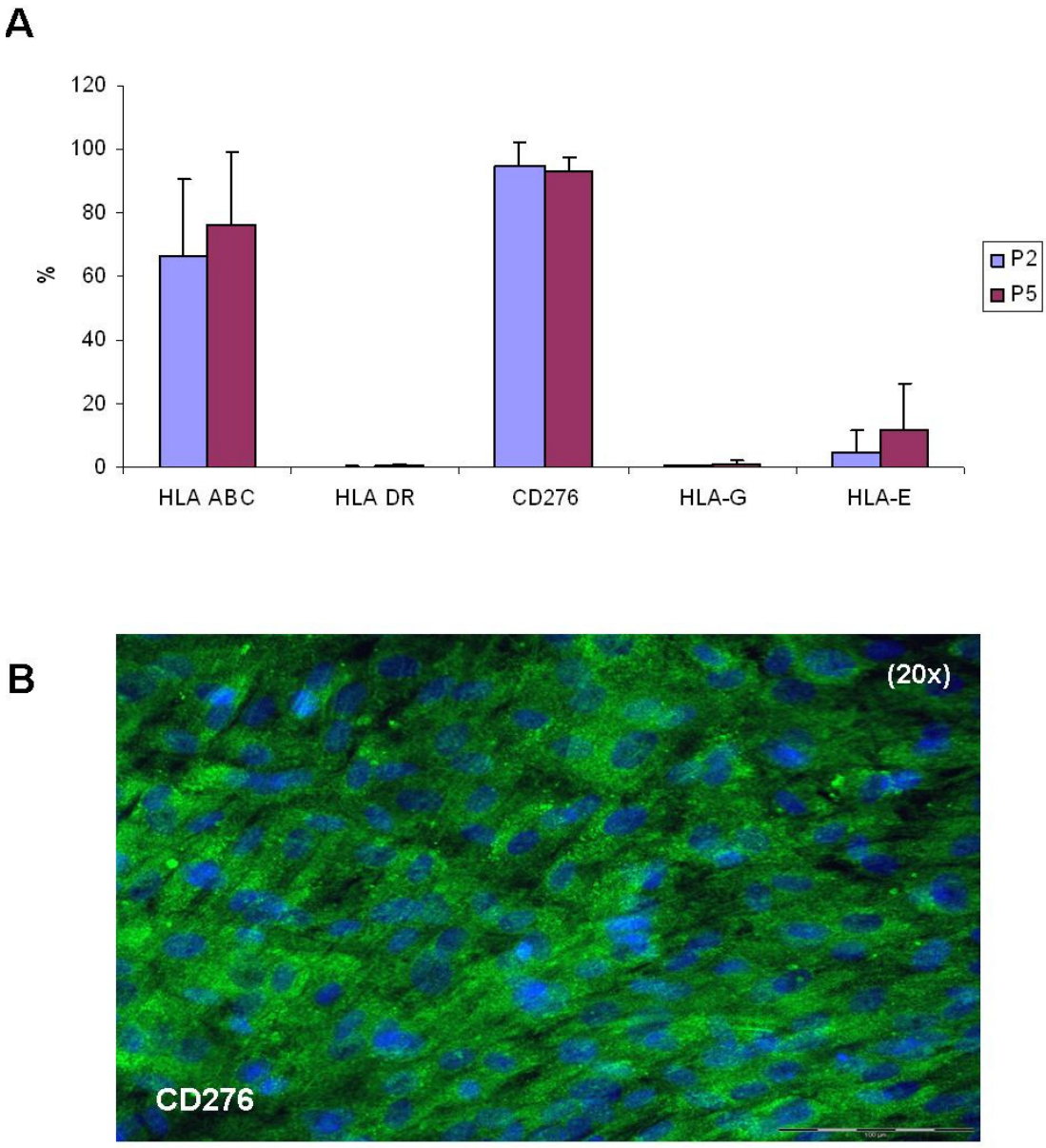


Figure 11: Immunomodulatory molecules expressed by preterm WJ-MSCs were assessed by flow cytometry (A) and immunofluorescence (B).

Single labeling experiments have also shown the presence of tissue-specific markers in undifferentiated preterm WJ-MSCs such as *Nestin (***Figure 12-B***)*, *GATA-4* and *c-Kit* (shown respectively in **Figure 12 A and 12C***,* connexins (*Connexin 40, Connexin 43, Connexin 45* **Figure 13 A-C** *),* and other antigens known to be expressed in "*hepatic* progenitor cells and mature hepatocytes".

Nestin is an intermediate filament first characterized in neuroepithelial stem cells whose expression was subsequently demonstrated also in progenitor cells of different origins such as hepatic progenitor cells, pancreatic endocrine progenitor cells and also in embryonic-derived progenitor cells. Based on all these observation, it was postulated that it represent a marker expressed in mutipotent stem cells [183].

Connexins are transmembrane proteins extensively expressed in cardiac tissue at the level of gap junctions. In particular, Connexin 40 and 45 are involved in cardiac morphogenesis and electrical impulse transmission [33], whereas Connexin 43 is also expressed in the bone marrow, thymus, spleen, and other lymphoid tissues [32,184].

GATA-4 is a zinc finger transcription factor (TF) expressed in endoderm derived tissues (such as liver and lung) and also in heart [185]. As demonstrated by ICC and IF (**Fig.12 A**), this TF is localized in the cytoplasm and perinuclearly indicating an inactive state in undifferentiated cells.

C-Kit (also called CD117) is a proto-oncogene, which encodes for the receptor for Stem Cell Factor (a growth factor). It is expressed by many cellular types such as hematopoietic stem cells, basal cells of the skin, melanocytes and germ cells. Its expression was also demonstrated in WJ-MSCs isolated from term umbilical cords,

whereas bone marrow derived MSCs lack the expression of this marker (reviewed in Anzalone et al, 2010 [186].

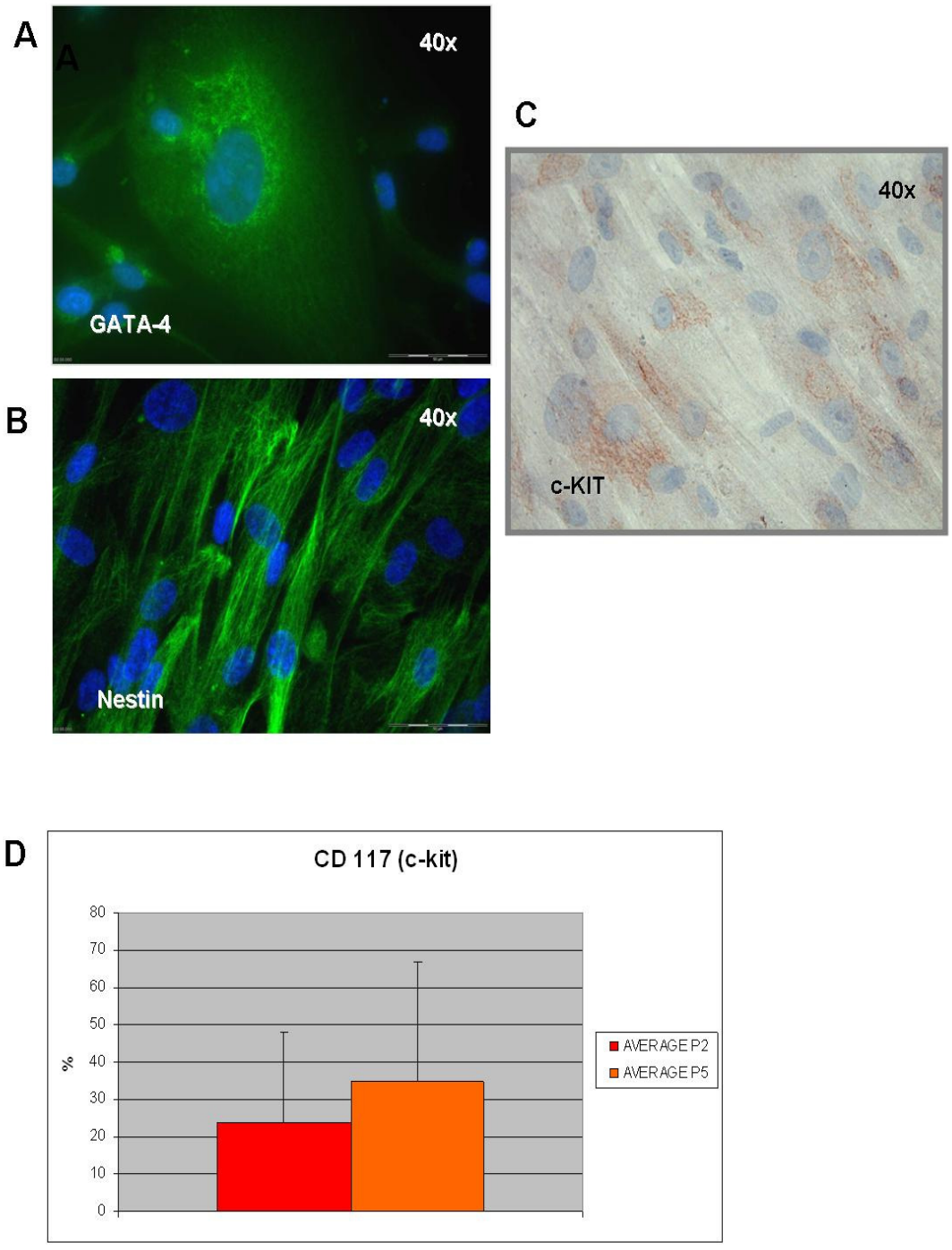


Figure 12: Expression of tissue-specific markers in WJ-MSCs: GATA-4 and Nestin (A,B) are amply expressed by WJ-MSCs, while c-Kit is present in a significative fraction of cells (C), as shown also by flow cytometry data at passages 2 and 5 (D).

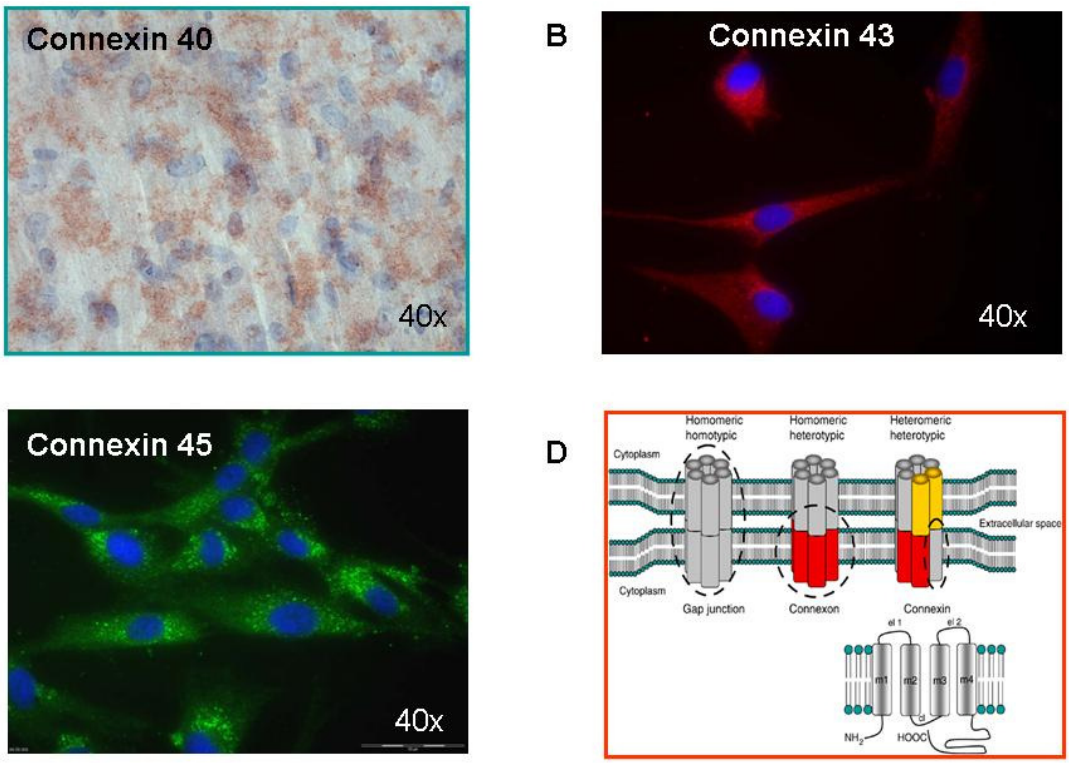


Figure 13: Undifferentiated preterm WJ-MSCs express high levels of connexins 40, 43 and 45 (A-C). These transmembrane proteins are typically expresses by cardiomiocytes and are part of gap-junction channels (D).

Moreover, we investigated the expression of *hepato-specific markers* by flow cytometry and ICC. WJ-MSCs express high levels of alpha-fetoprotein, albumin and cytokeratins, namely CK8, CK18 and CK19 **(**Figure **14 A-D).** The expression of hepatic markers by umbilical cord cells isolated by Wharton's jelly of mature umbilical cords was also reported by us and others [73, 80] liver specific antigen expression was also published by other groups investigating phenotypic expression and multilineage differentiation capabilities of mesenchymal stem cells isolated from different origins such as adipose-derived MSCs [156].

All of these markers (together with c-Kit receptor) were found to be co-expressed by oval cells (figure **14 E**), bipotential hepatic stem cells that play a major role in liver regeneration after injuries, giving rise to hepatocytes (CK8+, CK18+, albumin+, AFP+) and duct cells (CK19), thus restoring liver mass.

The observed expression of liver-specific markers suggests that preterm WJ-MSCs may have an intrinsic ability to differentiate into hepatocyte-like cells.

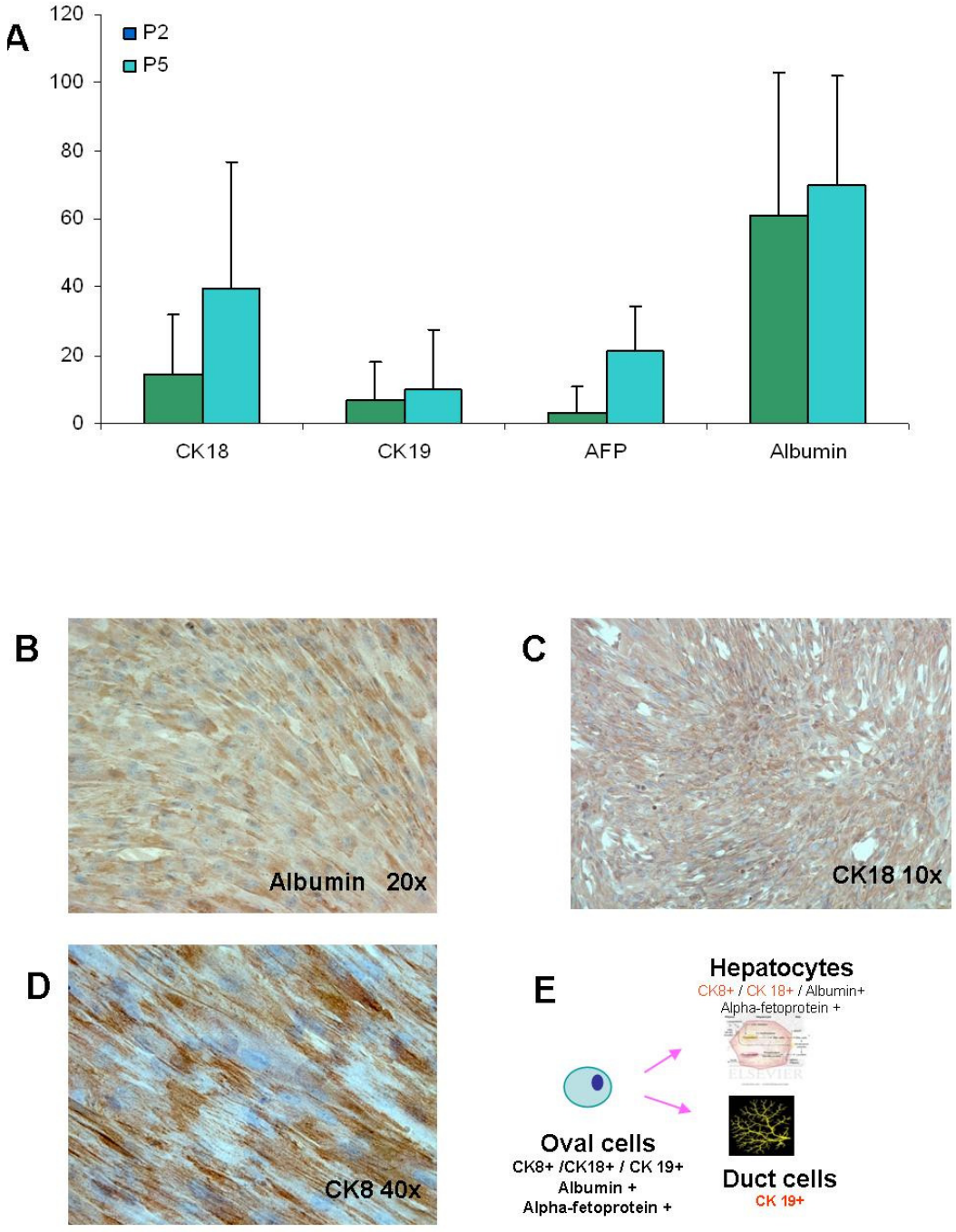


Figure 14: Undifferentiated WJ-MSCs express markers of bipotent hepatic stem cells named oval cells (E) such as CK8, CK18, CK19, albumin and α-fetoprotein (AFP), as assessed by cytofluorimetric (A) and immunocytochemistry analyses (B-D).

c-Kit	+++
CK8 (*)	+++
CK18 (*)	+++
vWF	-
B7-1	-
B7-2	-
Connexin 40 (*)	+++
Connexin 43 (*)	+++
Connexin 45 (*)	+++
NESTIN (*)	+++
GATA-4	+++
B7-H3 (*)	+++
HLA-E (*)	+
HLA-G (*)	-
Albumin (*)	+++
PSG-1	-

(*) data were cross-confirmed by *Flow Cytometry*
(*) data were cross-confirmed by *Immunofluorescence*

Table 8: Global results of the semiquantitative evaluation of multiple markers in WJ-MSCs, assessed by ICC, and cross confirmed by flow cytometry and immunofluorescence analyses.

4.3 *IN VITRO* DIFFERENTIATION TOWARDS THE MESODERMAL LINEAGES: ADIPOGENIC, OSTEOGENIC AND CHONDROGENIC DIFFERENTIATION

To confirm the stemness of the isolated cells, we performed the classical tri-lineage differentiation experiments, to test their ability to differentiate towards adipocyte, chondrocyte and osteocyte lineages, by applying standard protocols.

4.3.1 ADIPOGENIC DIFFERENTIATION

Adipocyte differentiation begins with an accumulation of small lipid-rich vacuoles into the cytoplasm of MSCs starting to be committed to pre-adipocytes. Subsequently, these cells turn into mature adipocytes containing larger fat droplets derived from small lipid vesicles fusion events. Adipogenic induction has been performed in monolayer cultures using a previously described protocol based on the use of multiple specific inducers.

After three weeks of induction, adipogenic differentiation occurred in our cells as demonstrated by the visible accumulation of lipid droplets in the cytoplasm of treated cells, with respect to control ones, highlighted by the use of Oil-red-O dye. (**Figure 15**)

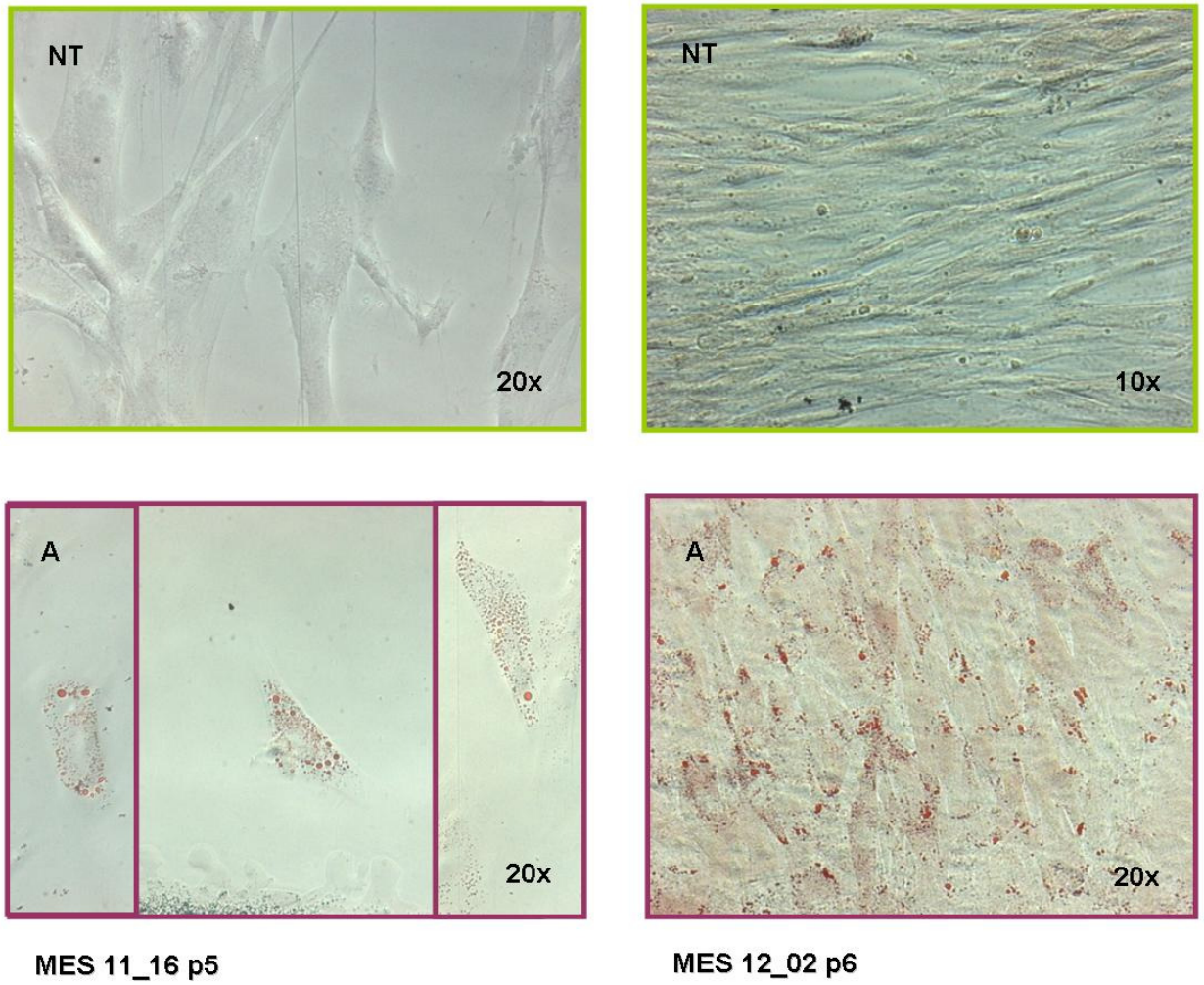


Figure 15: Microscopic demonstration of adipogenic differentiation of WJ-MSCs. Differentiated cells accumulated neutral lipid vacuoles in the cytoplasm, which were stained by Oil red O dye (lower panels). Control cells (upper panels) did not show any staining.

4.3.2 OSTEOGENIC DIFFERENTIATION

Bone is a specialized connective tissue consisting of cells surrounded by an extracellular matrix (ECM) containing both inorganic salts (mainly hydroxyapatite and calcium carbonate) and proteins (collagen fibers and non-collagen proteins). Osteoblasts are mesenchymal-derived cells specialized for the synthesis and the secretion of both the inorganic and organic constituents of the ECM. WJ-MSCs were subjected to osteogenic differentiation for three weeks in a specific medium, and then the deposition of a mineralized extracellular matrix was assessed by Alizarin Red staining. This dye specifically marks extracellular calcium deposits highlighting osteogenesis. As visible in panels in **Figure 16,** differentiated cells showed high levels of extracellular calcium deposits indicating a successful osteogenic differentiation, with respect to controls cultured for three weeks in standard growth medium.

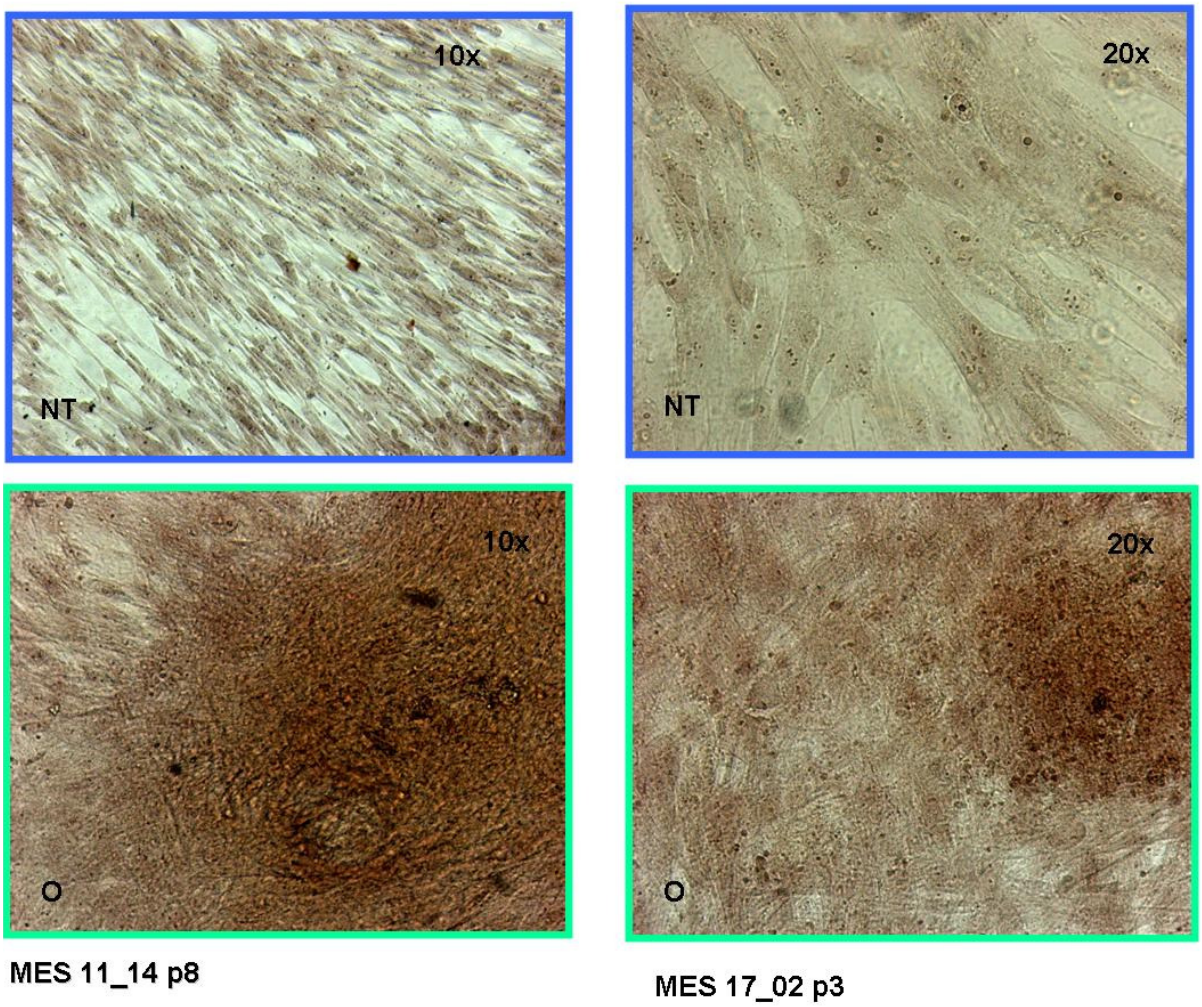


Figure 16: Microscopic demonstration of osteogenic differentiation of WJ-MSCs. Differentiated cells deposited a calcium-enriched extracellular matrix, which was stained by Alizarin red S dye (lower panels). Control cells (upper panels) did faint staining.

4.3.3 CONDROGENIC DIFFERENTIATION

MSCs were also described to be able to differentiate into chondrocytes. Chondrogenic differentiation was achieved under standard differentiation conditions in a three-dimensional system (alginate beads) mimicking the physiological milieu.

After 21 days differentiation, cells were uniformly distributed into the matrix and MSCs changed their shape, assuming a rounded morphology with large nuclei stained with nuclear fast red, in both control and treated conditions (**Figure 17**, arrows). Treated beads showed an increased level of glycosaminoglycans (GAG) into the bulk of extracellular matrix, as demonstrated by the intense blue staining obtained via Alcian blue dye. This was not visible into the parallel control beads (**Figure 17**).

This indicates that *in vitro* the cells acquired a matrix synthesis activity of specific cartilage molecules, such as those expressed by chondroblasts during development.

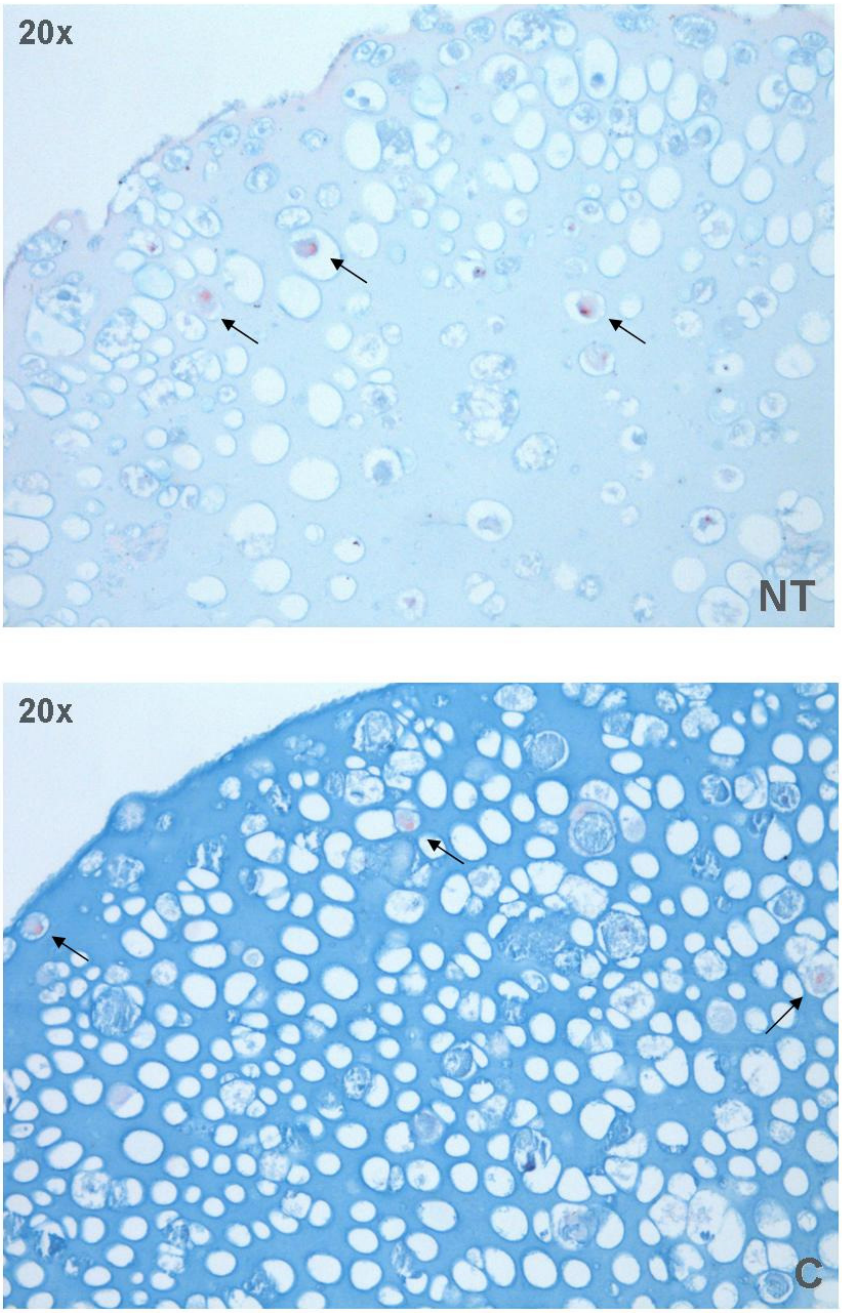


Figure 17: Microscopic demonstration of chondrogenic differentiation of WJ-MSCs. Differentiated cells secreted glycosamminoglycans in the extracellular spaces, which were stained by Alcian Blue dye (lower panel). Control cells (upper panel) did not show any staining. Arrows indicate cell nuclei counterstained with nuclear fast red.

4.4 *IN VITRO* DIFFERENTIATION POTENTIAL OF WJ-MSCs TOWARDS HEPATIC LINEAGE

Hepatic differentiation was achieved by applying a two step protocol developed by my group [Lo Iacono et al, manuscript in preparation] consisting of a "pre-inductive step and a maturation step" that mimics *in vitro*, in a time dependent manner, some of the inductive events that take place during the foetal liver morphogenesis.

During the pre-inductive step 80% confluent cells, grown on plastic supports, were cultured for three weeks in a medium supplemented with 1% FCS, antibiotic/antimycotic, non essential aminoacids, HGF (hepatocyte growth factor), FGF-4 (fibroblast growth factor-4), dexamethasone, and insulin-transferrin-selenite (ITS). Subsequently, cells were grown in a maturation medium containing oncostatin M (OSM) in addition to all the over mentioned factors. FGF-4, HGF and oncostatin M are cytokines that exert a crucial function in liver morphogenesis, being involved during different developmental stages and also playing diverse roles in hepatic differentiation: the first two early activating a cascade of events that lead to hepatic cell differentiation whereas OSM is fundamental for the maturation and the maintenance of hepatic differentiated phenotype.

After 3, 4, and 5 weeks cellular modifications, both at the morphological and functional level, were investigated respectively by microscopy, ICC and functional assays.

After three weeks of induction, cells exposed to hepatogenic differentiation medium

showed a *morphological transition*, from a typical fibroblastic-like shape to a polygonal-like morphology that closely resembles that of hepatocytes, suggesting an active maturation process toward a hepatocyte-like phenotype (an example is indicated in red colour in **Figure 18**). Moreover, cytoplasm granules were observed in hepatic differentiating cell as indicated by black arrows in **Figure 18.**

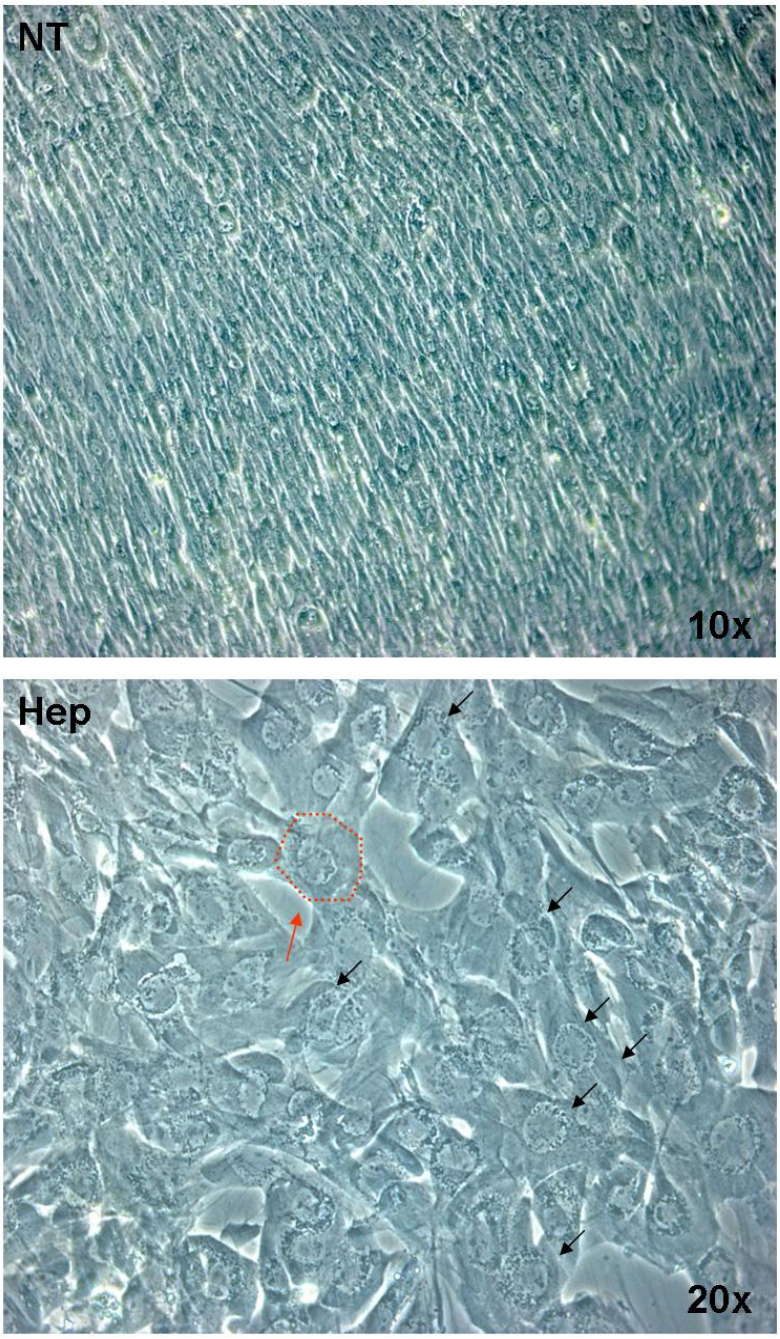


Figure 18: Phase contrast micrographs showing the morphological transition of WJ-MSCs subjected to hepatic differentiation. While control cells (upper panel) retain the fibroblastoid morphology during culture, hepatocyte-like cells assume a polygonal shape (highlighted by red arrow) and show the presence of granulation in the cytoplasm (black arrows)

4.4.1 PERIODIC ACID SCHIFF (PAS) STAINING

After three weeks of culture in standard growth medium, PAS staining revealed low levels of glycogen deposits in undifferentiated cells, whose expression was maintained constant up to the 5^{th} week (**Figure 19**, upper panels). The presence of glycogen in the cytoplasm of Wharton's jelly native mesenchymal stem cells, together with cytofluorimetric and immunocytochemistry data revealing the expression of alpha fetoprotein, albumin, and cytokeratins 8 and 18, indicate that among the WJ-MSCs population there are some cells that express basal levels of hepatic markers, thus suggesting an intrinsic predisposition to differentiate into hepatic-like cells.

Hepatic induction after three, four and five differentiation weeks resulted in an evident increase of glycogen deposits, clearly indicating an enhancement of the expression and the activity of the enzyme Glycogen synthase (**Figure 19**, lower panels).

We next wanted to evaluate whether the evidence of mesenchymal–hepatic transition, revealed by phenotypic changes in response to differentiation stimuli, was associated with the acquisition of other mature hepatic function.

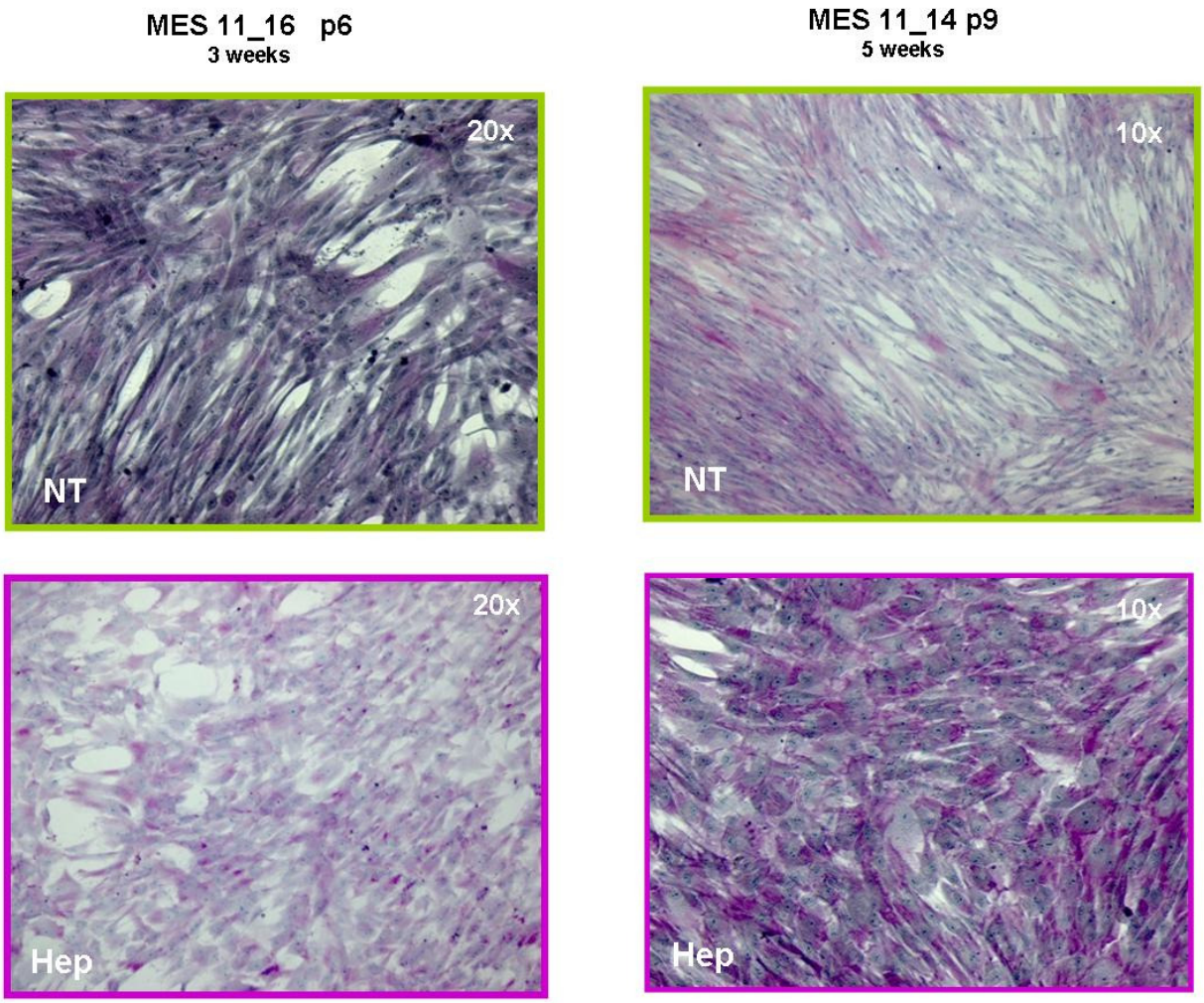


Figure 19: Microscopic demonstration of glycogen accumulation by PAS staining. Control cells (upper panels) grown in standard medium, show moderate staining, while differentiated cells (lower panels) show more intense staining.

4.4.2 CYTOCHROME P450 (CYP3A4) ACTIVITY QUANTIFICATION

Liver represents the major site of xenobiotic metabolism, a process consisting of multiple biochemical transformations aimed to prevent the accumulation of toxic substances globally indicated as “xenobiotics” (e.g. pharmacological compounds and toxins) transforming them into inactive metabolites. Hepatocytes contain a wide range of enzymes to process xenobiotics, some of which belong to the cytochromes P450 family: monooxigenases that catalyze the first phase of the metabolism of many xenobiotics.

Isoform 3A4 of cytochrome p450 (CYP 3A4) represents the most abundant cytochrome of liver and its activity is considered to be one of the most important parameters used to demonstrate the acquisition of hepatic maturity by precursor cells.

We therefore decided to measure CYP3A4 activity in control and treated WJ-MSCs in response to Rifampicin (its specific inducer), with or without the addiction of its specific inhibitor ketoconazole, by using a luminometric assay.

The obtained results, shown in **Figure 20**, indicate no major differences in cytochrome activity in both control and treated cells after four weeks of hepatic induction. On the contrary, cells cultured in hepatic induction medium for five weeks exhibited an enzymatic activity induced by Rifampicin that is markedly higher than the one measured in correspondent control cells. Moreover, the activity of the enzyme decreased following the incubation with Rifampicin and Ketoconazole, thus confirming the specificity of CYP3A4 activity measurement. Homogeneous results were obtained at five weeks in all considered samples. These data, albeit not reaching the statistical significance, strongly suggest that WJ-MSCs acquire the ability to

metabolize xenobiotics in an inducible manner following the applied differentiative stimulus.

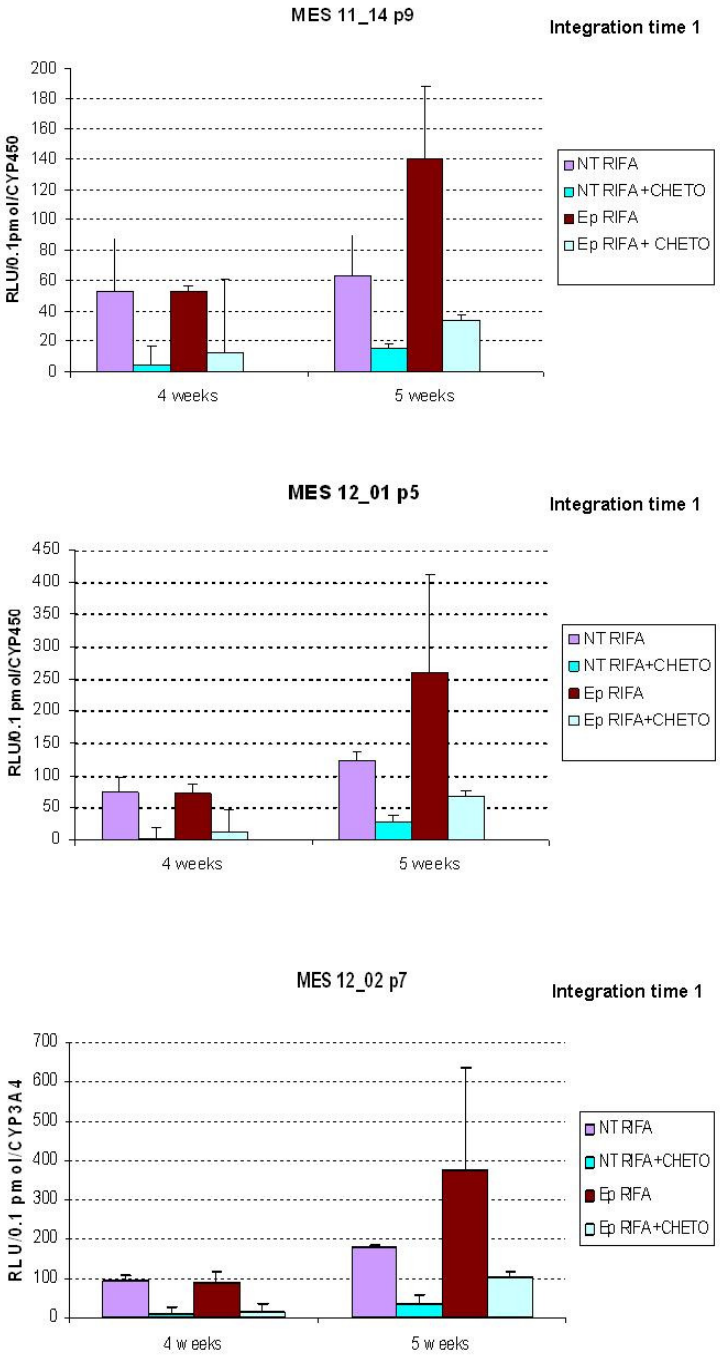


Figure 20: Evaluation of CYP3A4 induction following rifampicin challenge in undifferentiated and differentiated WJ-MSCs at different time points. The activity of the enzyme was specifically inhibited by the addition of ketoconazole to culture medium.

4.4.3 GLUCOSE-6-PHOSPHATASE (G-6-Pase) ASSAY

We also investigated glucose-6-phosphatase activity in both undifferentiated and hepatocyte-like cells. G-6-Pase is one of main hepatic enzymes involved in glucose metabolism. As shown in **Figure 21**, at all considered time points, hepatocyte-like cells exposed to the enzyme substrate were able to form lead sulfate precipitates, with their characteristic black-brown colour, indicating an intense G-6-Pase activity compared to that observed in control cells at the same time points.

4.4.4 INDOCYANINE GREEN (ICG) UPTAKE

Another parameter which may be evaluated to demonstrate the acquisition of a hepatocyte-like phenotype is the uptake of indocyanine green, also referred to as cardiogreen. Cardiogreen is an organic anion that is selectively taken up by hepatocytes and that is able to bind plasma proteins, and particularly albumin. It is clinically used because its uptake, storage, and excretion by the liver give useful indication of hepatic function.

As visible in **Figure 22**, control cells did not show any green colour, whereas hepatocyte-like cells exhibited intense green cytoplasmatic staining indicating dye internalization.

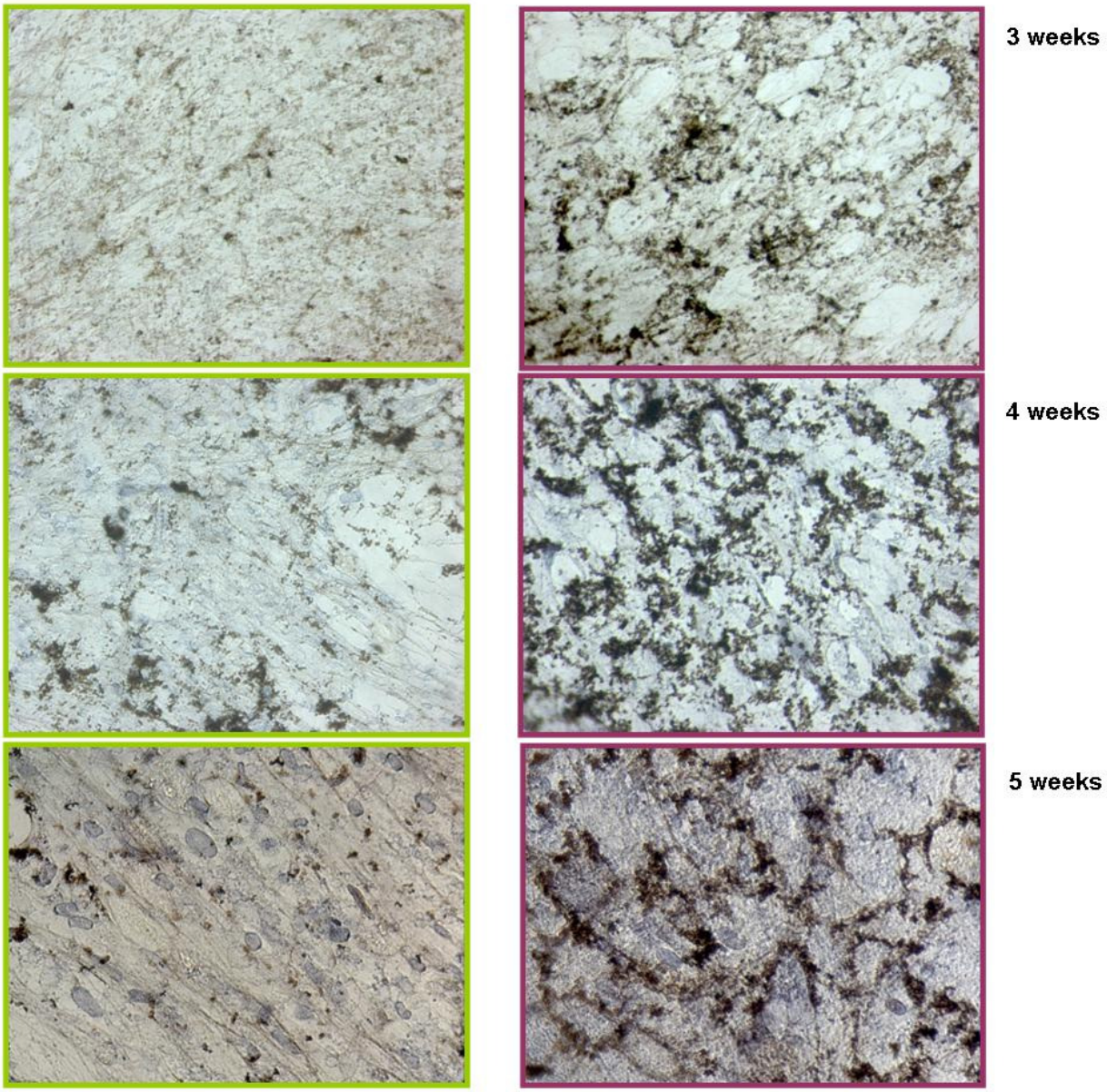


Figure 21: Microscopic demonstration of G6Pase Activity, assessed by a colorimetric enzymatic assay, showing production of intracellular lead nitrate precipitates at higher levels in differentiated cells (right panels) with respect to undifferentiated ones (left panels). magnification: 20x.

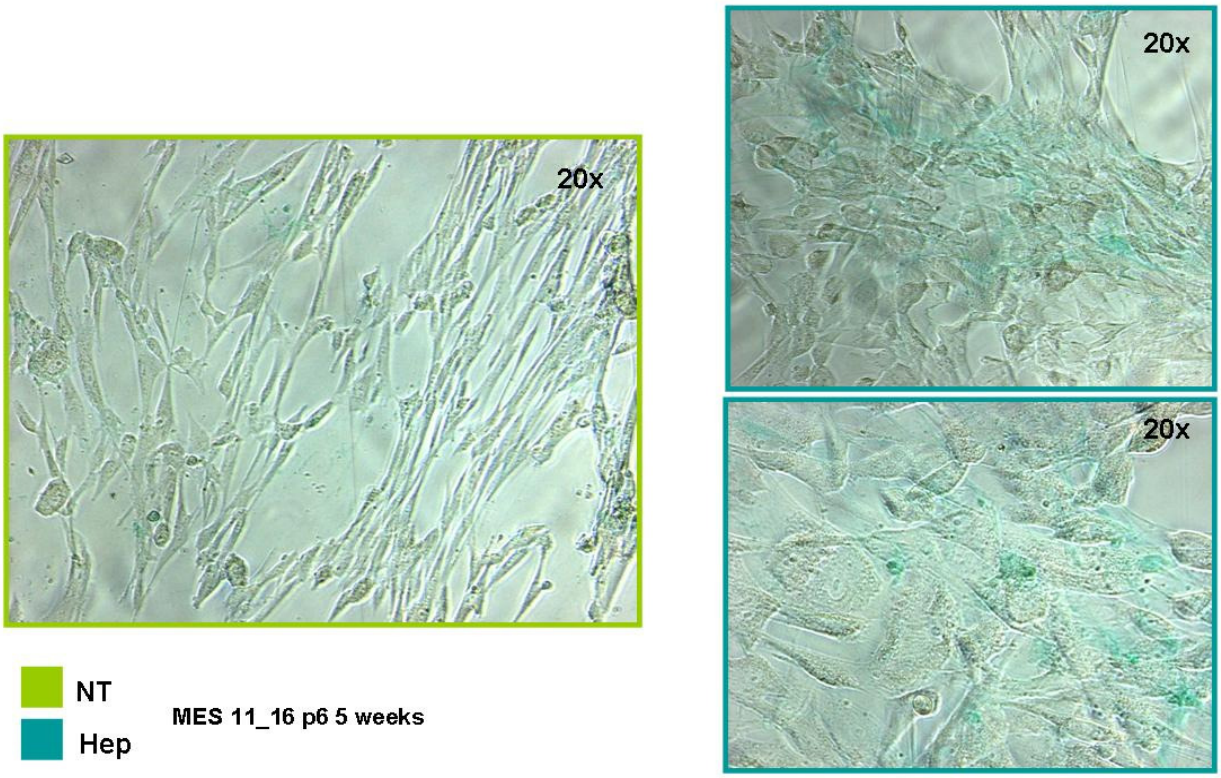


Figure 22: Microscopic demonstration of ICG uptake by differentiated cells (right) with respect to control ones (left).

4.4.5 IMMUNOMODULATORY ANTIGENS EXPRESSION IN UNDIFFERENTIATED AND DIFFERENTIATED WJ-MSCs

As previously discussed, one of the most attractive features of mesenchymal stem cells is their hypoimmunogenicity, since these cells may inhibit allogeneic lymphocytes proliferation *in vitro*, and are also tolerogenic when infused *in vivo* in immunocompetent hosts. This has been linked to the peculiar phenotype of these cells, which for example lack expression of major histocompatibility complex class II molecules, and which are also able to produce secreted molecules known to interfere with the immune function at various levels. Recent literature data pointed out the occurrence of immunomodulatory molecules also in the differentiated progeny of stem cells, in particular for WJ-MSCs and hAECs. Since, as shown above, we have demonstrated in our cells the expression of other interesting imunomodulatory molecules such as B7-H3, also involved into immune tolerance, we wanted to further evaluate whether preterm WJ-MSCs may also maintain the expression of immunomodulatory molecules.

Therefore, by flow cytometry analyses on undifferentiated and differentiated preterm WJ-MSCs, subjected to adipo- osteo- and hepato- differentiation inductions, we assessed the levels of the immunomodulatory molecules and the variations with respect to the naive cells.

As shown in **Figure 23**, and confirming the results recently obtained with WJ-MSCs derived from mature cords, preterm WJ-MSCs subjected to both osteogenic or adipogenic differentiation did maintain the expression of B7-H3 and HLA-E at levels comparable to the control cells. More interestingly, also cells subjected to the hepatic

differentiation (**Figure 24**) showed the maintenance of the immunomodulatory molecules expression, thus suggesting the possibility that hepatocyte-like cells may also be better tolerated when infused *in vivo*.

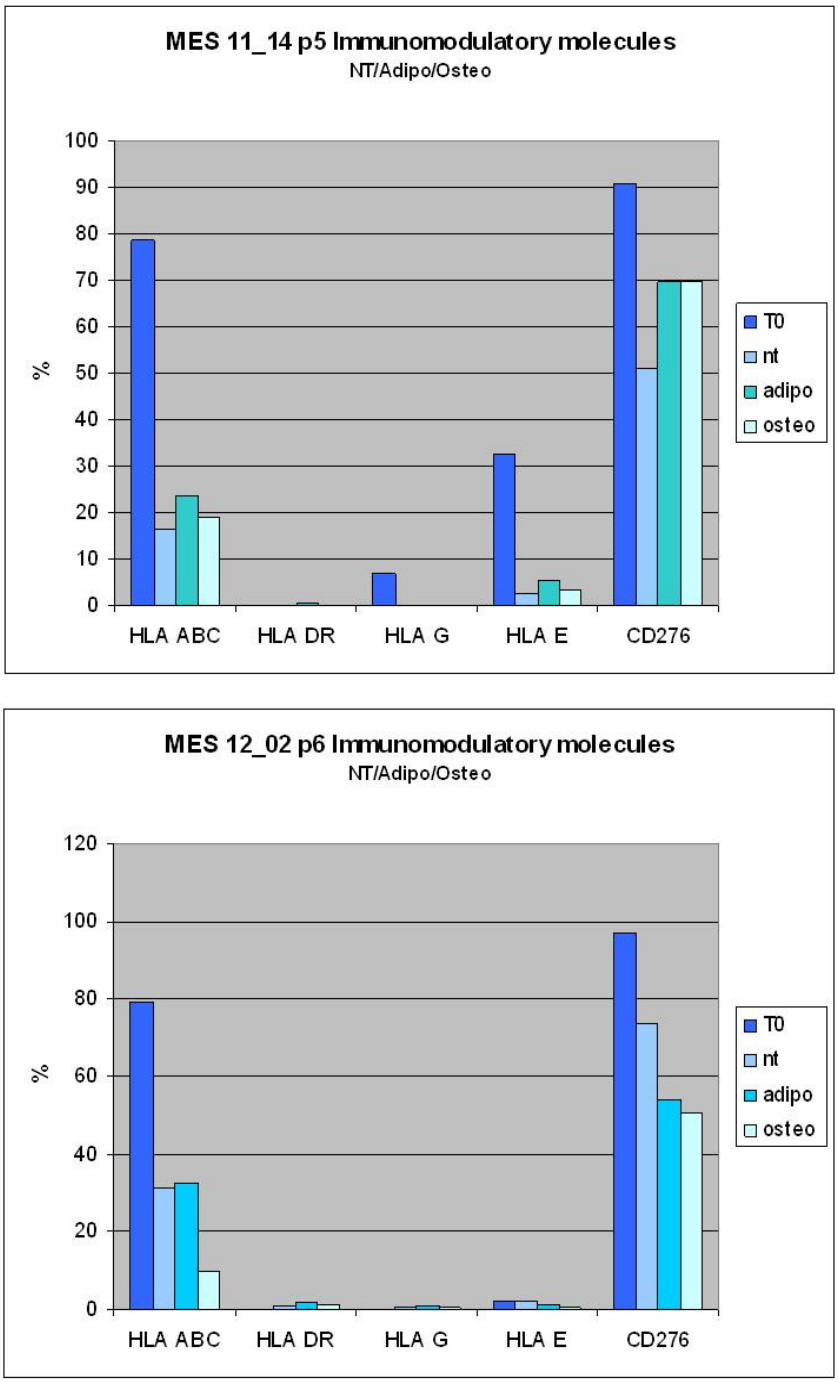
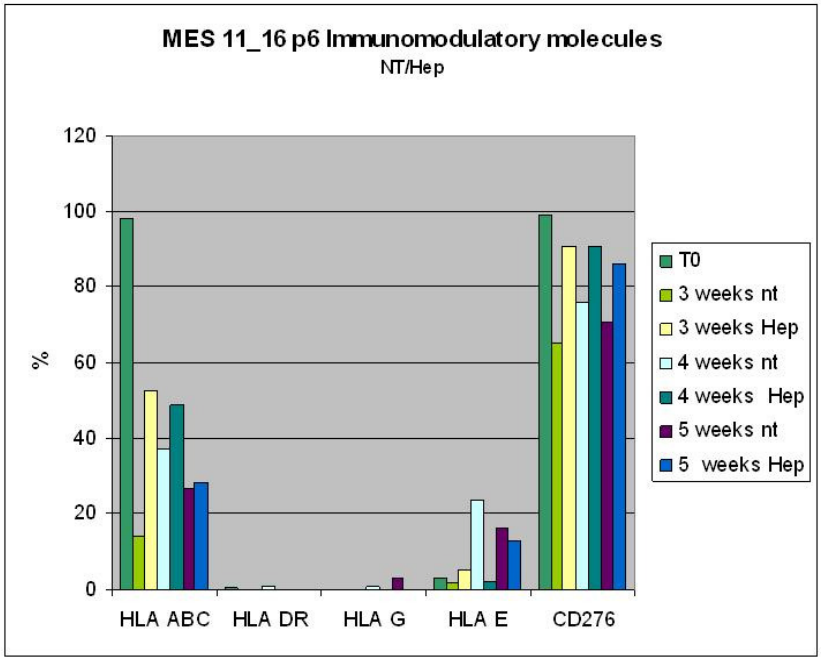


Figure 23: Immunomodulatory molecules expression following adipogenic and osteogenic differentiation protocols in treated (adipo and osteo) and control cells (nt).



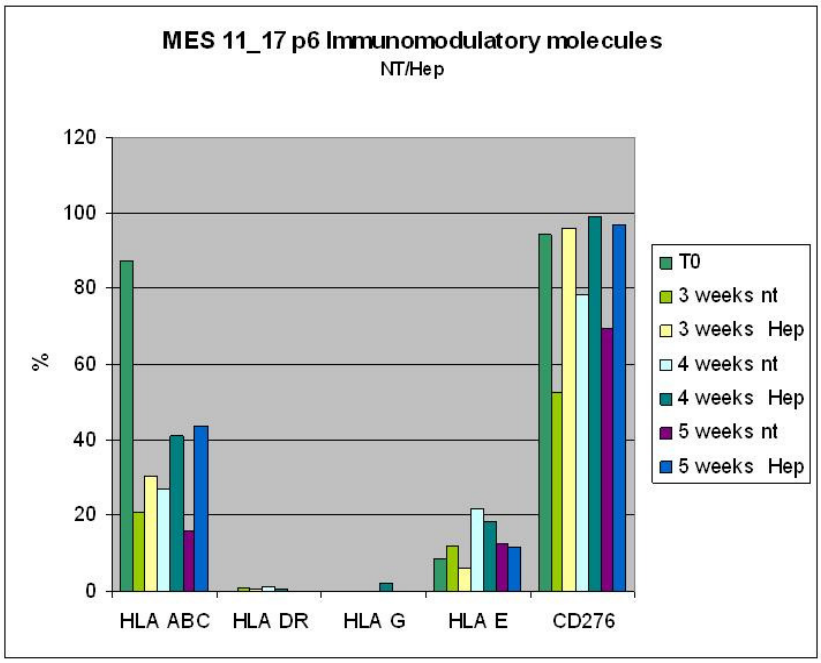


Figure 24: Immunomodulatory molecule expression following hepatogenic differentiation in treated (Hep) and control cells (nt)

5. DISCUSSION

Stem cells research is a field that over the last three decades has been characterized by a rapid and tremendous expansion representing, without any doubt, the future of biomedical research and applicative medicine which, hopefully one day, will make stem cell administration one of the possible therapeutic treatments to cure several diseases and pathological conditions.

The large collection of scientific data obtained so far from *in vivo* models, and above all, the promising results collected from the increasing number of clinical trials that, in particular, make use of adult stem cells such as bone marrow derived mesenchymal stem cells and haematopoietic stem cells, have created a general optimism about future stem cell applications.

Contextually, stem cells have been demonstrated to be a useful tool to screen *in vitro* the toxicity of pharmaceutical compounds, as well as an interesting model to study some of the mechanisms underlying cancerogenesis and tumour progression (cancer stem cells). Hopefully the comprehension of these complex phenomena, in the future, will allow the formulation of new drugs that will be more effective than the ones currently used to treat different types of cancer.

Regardless all these evidences, there are still a huge amount of questions that have to be answered, and also many problems associated with the use of stem cells have to be addressed.

There are several issues associated with each stem cell category including ethical concerns (*embryonic/fetal stem cells*), the possibility to give rise to teratomas (*embryonic stem cells*) and in some cases the invasiveness of stem cell collecting

procedure (*bone marrow mesenchymal stem cells*) as well as some limitations of stem cell intrinsic properties such as the ex vivo expansion and differentiation potentials (*adult stem cells*).

During the last decade the interest of the scientific community has been focused on a particular group of tissues indicated as "perinatal tissues" which include foetal annexes such as placenta, amniotic membranes and umbilical cord.

Several reports have demonstrated many advantages regarding the use of these cells over embryonic and adult stem cells, suggesting that perinatal stem cells may represent the most promising stem cell source for regenerative medicine and for the treatment of various diseases.

During the last years my research group has been studying mesenchymal stem cells isolated from Wharton's jelly, the umbilical cord mucous connective tissue containing a primitive mesenchymal stromal stem cell compartment. The results obtained so far demonstrated that WJ-MSCs *in vitro* display a high self renewal, clonogenicity, a stable karyotype and, in response to specific stimuli, they can be differentiated into adipo-, chondro- and osteo-like cells.

Moreover, WJ-MSCs can be induced to undego hepatogenic differentiation applying a two step protocol developed by my group that mimics the main differentiation events which take play *in vitro*. Interestingly, the obtained hepatocyte-like cells were also demonstrated to retain the expression of some immune-modulatory molecules involved in tolerance mechanism, previously characterized in naïve WJ-MSCs [80].

Another advantage over BM-MSCs is that WJ-MSCs expansion potential is higher and these cells *in vitro* can also proliferate more rapidly than bone marrow-derived

ones. In addition, the number of progenitor cells present in umbilical cord matrix is the highest of the human body (1/133-1/1609 clonogenic cells in WJ versus 1/10000-36000 of BMSCs, 8.2±0.6 per 10^8nucleated cells in UCB or the foetal circulation and less than 1/ 10^6 mononuclear cells in adult whole bone marrow (Reviewed in [187]).

Recent reports suggested that stem cells potency diminishes as function of the age of donor tissue, with ESCs showing the higher potency and adult stem cells being more restricted. Therefore, WJ-MSCs are in a developmental position which may ensure their differentiation into multiple adult populations, without the limits listed for ESCs. Following this concept, we hypothesized that Wharton's jelly mesenchymal stem cells isolated from umbilical cords at an earlier developmental stages (such as preterm umbilical cords made available from therapeutical abortions), may feature a higher degree of *stemness* in terms of differentiation potential.

To date there is lack of literature data describing the phenotypic profile of these cells, or their differentiation potency. The only preterm umbilical cord available data were included in a patent application filed some years ago [189], containing the description of an enzymatic method, based on collagenase type I digestion, that allowed mesenchymal stem cells isolation from first trimester umbilical cords. The patent application includes a partial description of some of the cellular surface antigens characterized on these cells by immunocytochemistry, as well as the displayed osteogenic and adipogenic differentiation potentials. The partial nature of the information contained in this application, together with the need to better characterize the potential of these cells for regenerative medicine applications, further justified this research project.

The main objectives of my research project were to phenotypically characterize mesenchymal stem cells isolated from preterm umbilical cords and to investigate their differentiation potential in order to evaluate if these cells could also represent an additional stem cell source that could find a future application in regenerative medicine. Moreover, an additional task was represented by the characterization of the immunomodulatory molecules expressed by these cells both in the undifferentiated and differentiated states.

Cells isolated by plastic adherence from preterm umbilical cords showed a fibroblast-like morphology, with a cell body that is more expanded if compared with the corresponding cells isolated from term umbilical cords. Phenotypical analyses (including flow cytometry, ICC and IF) demonstrated collectively that these cells were mainly adherent to the MSCs phenotype, with the relevant expression of key molecules (such as CD117, nestin, albumin, alpha-fetoprotein, connexins) which might indicate their differentiative ability towards ectoderm-, mesoderm- or endoderm-derived mature tissues. In addition, this initial phase also allowed to demonstrate that preterm WJ-MSCs do express many immune-related antigens, which were also characterized recently in the mature counterpart, such as HLA-E, CD276, and at some extent, also HLA-G. This, together with the expected negativity for type II MHC and positivity to classical type I MHC, strongly suggests a hypoimmunogenic and immunomodulatory phenotype for these cells.

WJ-MSCs immunomodulatory features (as for T-cell anergy induction) can be explained by both cell-cell interactions and bioactive molecule secretion; moreover, it has been demonstrated that the expression of surface antigens such as HLA-G and

HLA-E (type Ib MHC molecules) as well as the presence of CD276 are responsible for tolerogenic features of WJ-MSCs. In particular, the protein encoded by CD276 gene (demonstrated to be highly expressed in both preterm and term WJ-MSCs) is named B7-H3: it belongs to the immunoglobulin superfamily and it is thought to participate to the regulation of T-cell-mediated immune responses.

In order to demonstrate the adherence to the mesenchymal stem cells features, we then evaluated the differentiation of these cells toward mesodermal lineages (adipocytes, condrocytes and osteoblasts) in response to specific culture conditions. As expected, preterm WJ-cells gave rise to adipo-, osteo- and chondro-like cells , thus providing a formal proof of their stemness.

Then, we addressed the question of whether preterm WJ-MSCs could undergo hepatogenic differentiation as previously demonstrated for mesenchymal stem cells isolated from the matrix of mature umbilical cords.

Hepatic differentiation of stem cells is currently a “hot topic” because of the need of alternative approaches to treat end stage liver diseases. In fact, despite liver transplantation is the most effective treatment for acute or chronic liver failure resulting from a variety of causes, to date the number of donor organs available for transplantation is not sufficient if compared with the number of patients in the waiting lists, most of which die waiting for a transplantation procedure. For this reason many research groups have moved to evaluate alternative therapeutical strategies that at least could act as *bridge therapies* for patients waiting liver transplantation.

Achievement of hepatic differentiation was evaluated by morphological and functional analyses, after applying a two step differentiation protocol that mimics some of the main events that take place during hepatogenesis *in vivo*.

The acquisition of mature hepatic features was confirmed by PAS staining (that showed low glycogen levels in naive cells, which accumulation was significantly increased after differentiation events) and hepatocyte specific functional assays such as Cytocrome p 450 (isoform CYP3A4) activity, Glucose 6-Phospatase assays as well as Indocyanine uptake. Overall, our data showed that cells differentiated from preterm WJ-MSCs were able to respond to CYP3A4 inducers, with an increase in the activity of the enzyme which was also abolished using a specific inhibitor. Moreover, glucose-6-phosphatase activity was demonstrated in differentiated cells with respect to control ones, so as for the indocyanine green uptake. These functional assays constitute reliable proofs of the acquisition of a mature hepatocyte-like phenotype by these cells.

Finally, we wanted to verify if preterm WJ-MSCs induced to differentiate towards mesodermal and endodermal cells expressed the immune-modulatory antigens involved in tolerance mechanism, previously demonstrated in untreated cells. Our results indicated that the expression of immunomodulatory antigens remained almost unvaried in all the considered differentiated cell populations.

Overall, the data obtained during the PhD project provided novel and useful information on the features of preterm WJ-MSCs, which can be reliably isolated from abortion foetuses, and present a number of key features for their use in regenerative medicine approaches. In particular, these cells appear to express the same

immunomodulatory molecules which have been characterized in the mature counterpart (i.e. isolated from term cords) and which may provide an effective advantage when transplantation in an immunocompetent host is attempted. In addition, these features are maintained also following the application of several differentiation stimuli. This is an important feature since multiple literature data suggest that the engraftment potential of pre-differentiated cells may be superior to that of undifferentiated ones in certain settings. Therefore, it may be imagined that hepatocyte-like cells derived from preterm WJ-MSCs may be used as a bridging therapy for end-stage liver diseases, since they can provide mature hepatocyte functions, coupled to key immunomodulatory activities. This may be relevant also in the restoration of physiological conditions in an organ severely impaired by the underlying disease. One of the effects of these cells *in vivo* may be the reactivation of local progenitors, and therefore the stimulation of the self-reparative mechanisms that physiologically take place in liver at higher levels with respect to other organs. In our opinion, the data obtained in this project may be complemented by the development of an *in vivo* model of end-stage liver disease to verify the effectiveness of our hypothesis.

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