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HUMAN WHARTON'S JELLY MESENCHYMAL STEM CELLS (WJ-MSCs): CHARACTERIZATION AND PROPERTIES

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HUMAN WHARTON'S JELLY MESENCHYMAL STEM CELLS (WJ-MSCs): CHARACTERIZATION AND PROPERTIES

by

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Dedication

Dedicated to my wonderful love, my family and all people that supported me during this journey. Their love and effort have been precious and infinite.

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HUMAN WHARTON'S JELLY MESENCHYMAL STEM CELLS (WJ-MSCs): CHARACTERIZATION AND PROPERTIES

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In recent years, mesenchymal stem cells (MSCs) have attracted researchers' attention because of their promising prospective and potential application in regenerative medicine. MSCs are defined as immature cells with the ability to self-renew and differentiate into specialized cells belonging to different lineages. Due to their high proliferative potential, MSCs can be rapidly expanded *in vitro* when growing in adhesive plastic dishes containing a classical culture medium. In addition, they are able to differentiate into mature cell-types. Various parts of the umbilical cord (UC) have been considered a promising source of MSCs because they can be isolated in relatively high numbers when compared to other mesenchymal sources and they can be easily cultured or cryopreserved. Many other properties make UC cells a comparatively more desirable therapeutic agent. However, more research is needed in order to elucidate the biological features and specific markers expressed by specific UC MSCs named Wharton jelly mesenchymal stem cells (WJ-MSCs). WJ-MSCs are multipotent stem cells with many advantages as a potential source of MSCs; the tissue is readily available, cells are easy to grow in culture, they can be cryopreserved, and have great expansion capacity *in vitro*. In this study, we

have shown that WJ-MSCs possess MSC features and therefore can be considered as an alternative MSC source in regenerative medicine applications. Following isolation, culture (self-renewal ability), characterization and differentiation of WJ-MSCs according to three different cell phenotypes, we have investigated other stem markers and the immune-modulatory/anti-inflammatory properties of WJ-MSCs. This last investigation introduces a new concept in the utilization of WJ-MSCs in regenerative medicine where instead of replacing function of cells/tissue, stem cells could be used as adjuvant when co-transplanted with other cells, tissue or organs. The results of this research will give us a better understanding of typical MSCs markers from WJ-originated cells and additional peculiar markers still unknown.

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LIST OF ABBREVIATIONS

AF	Amniotic fluid
bFGF	Basic fibroblast growth factor
BGL	Blood glucose level
BM-MSCs	Bone marrow mesenchymal stem cells
BMP	Bone morphogenetic protein
CCE	Cornified cell envelope
CD	Cluster of differentiation
CK	Cytokeratin
COMP	Cartilage oligomeric matrix protein
CS	Carnegie stages
cTnT	Cardiac troponin T
Cx-43	Connexin-43
DC	Dendritic cell

DMSO	Dimetylsulfoxide
ECs	Endothelial cells
EGF	Epidermal growth factor
EPF	Early pregnancy factor
ESCs	Embryonic stem cells
FGF	Fibroblast growth factor
G-CSF	Granulocyte colony stimulating factor
GAGs	Glycosaminoglycan rich substance
GDNF	Glial-derived neutrophic factor
GFAP	Glial fibrillar acidic protein
GM-CSF	Granulocyte-macrophage colony stimulating factor
HA	Hyaluronic acid
HGF	Hepatocyte growth factor
HNF-4α	Hepatocyte nuclear factor 4 alpha

ICAM-1	Inflammatory cytokine-induced intercellular adhesion molecule-1
ICM	Inner cell mass
IDDM	Insulin –dependent diabetes mellitus
IDO	Indoleaminedeoxygenase
IEq	Islet Equivalent
IPGTT	Intra-peritoneal glucose tolerance test
ITS	Insulin-transferrin-sodium selenite
MHC	Major histocompatibility complex
MSCs	Mesenchymal stem cells
Neurog3	Neurogenin3
NO	Nitric oxide
NSE	Neuron-specific enolase
OSM	Oncostatin
PDGF	Platelet –derived growth factor

PDX1	Duodenal homebox factor 1
PPAR-γ	Peroxisome proliferator-activated receptor gamma
RA	Retinoic acid
SOD3	Superoxide dismutase 3
STZ	Streptozotocin
TGF-β	Transforming growth factor beta
TID	Type I diabetes
UC	Umbilical cord
UCB	Umbilical cord blood
UCE	Umbilical cord epithelium
VCAM-1	Vascular cell adhesion molecule-1
vWF	Von Willebrand factor
WJ	Wharton's jelly
α-SMA	Alpha- smooth muscle actin

CHAPTER 1

BACKGROUND AND SIGNIFICANCE

STEM CELLS

Recently, stem cells have attracted researchers' attention because of their promising cell features in regenerative medicine. The term "stem cell" was adopted at the end of the nineteenth century as a theoretical postulate to describe their self-regenerative capacity (Ramalho-Santos and Willenbring, 2007). Normal tissues, with a limited life time, needs other cells in order to support the renewal of functional cell types for the organism's lifespan. There are two main criteria defining classic stem cell as stated by the International Society for Stem Cell Research, (ISSCR): 1) self-renewal: capacity to go through numerous cycles of cell division while maintaining the undifferentiated state and 2) potency: ability to differentiate into specialized cell types. However, stem cell function is regulated in a feedback mechanism and their excessive production could induce cancer (Moore and Lemischka, 2006).

In 1978, Schofield explained the "niche" microenvironment concept for the first time, where this microenvironment helps stem cells maintain tissue homeostasis and protects them against apoptotic stimuli (Schofield, 1987).

According to the differentiation ability (Figure 1.1), stem cells can be classified (Scholer, 2007) as:

- Totipotent stem cells: extra-embryonic tissue cells (e.g., zygote and the first embryonic cell division);
- Pluripotent stem cells: cell type derived from three germ layers (e.g., the blastocyst inner mass cells or umbilical cord stem cells - no from extra-embryonic tissues);
- Multipotent stem cells: some cellular cell types such as red blood cells, platelets, white blood cells, and adult nervous system cells;
- Unipotent stem cells: only one type of specialized cell.

In addition, stem cells can be classified in embryonic and somatic or adult stem cells, according to the origin of the tissue.

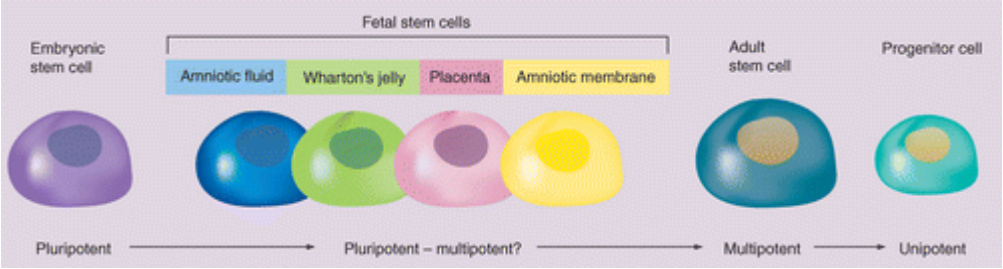

Figure 1.1: Stem cells differentiation ability

EMBRYONIC STEM CELLS

Embryonic stem cells (ESC) are derived from a limited embryo cellular population at the blastula or blastocyst stage. Blastocysts are made up of 50-150 cells and contain three specific structures: 1), trophoblast (cell layer around blastocyst), 2), blastocoel (blastocyst cavity) and 3), the inner cell mass (ICM) formed by 30 cells, defined as embryonic stem cells and located at the blastocoel end part. Generally ESCs are permanently diploid and immortal. They are able to maintain an undifferentiated state and they can later propagate and differentiate into cellular types derived from three embryonic germ layers (Bajada et al., 2008). Therefore, ESCs represent a potential source for several the treatment of various diseases. However, ESCs have some limitations: embryo cells need to be removed within 14 days of fertilization, the embryo graft can induce teratomas, and ultimately these grafts can induce immune reaction in the host recipient (Smith, 2001).

ADULT STEM CELLS

Adult stem cells are responsible for the tissue structure functionality and support the cell repair process. Adult stem cells can be isolated from tissue samples, they can self-renew and proliferate under elevated cell passage numbers, and they also are associated to a moderate risk of teratoma formation. Recently, it has been shown that adult stem cells can generate other cell lineages that are different from the cell derivation. For example, hematopoietic derived bone marrow stem cells can be differentiated into muscle after transplant (Wakitani et al., 1995); bone marrow cells can repopulate the liver after transplantation or can be differentiated into cells that express neuronal markers (Mezey et al., 2000; Brazelton et al., 2000). This type of cell ability is known as plasticity and may refer to the whole or part of cell fusion events (Blau et al., 1983).

MESENCHYMAL STEM CELLS

Mesenchymal stem cells (MSCs) constitute various cell populations. They were first observed and described at the bone marrow stromal level thirty-five years ago (Friedenstein et al., 1976). Later, Pittenger and colleagues described MSCs isolation and multi-potency from human bone marrow (Mackay et al., 1998). MSCs from bone marrow were considered the primary source to obtain stem cells as based on the numerous well-documented studies.

MSCs are star-shaped, mononuclear cells that are in direct contact with each other via cytoplasmic processes. MSCs are defined as immature cells with the ability to self-renew and differentiate into specialized cells that are permanently tissue-specific. They have a fibroblastic-like morphology with less cytoplasm and mitochondria, and an underdeveloped Golgi apparatus (Netter, 1987). Due to their high proliferative potential, MSCs can be rapidly expanded *in vitro* when growing in adhesive plastic dishes containing a classical culture medium. Also, they are able to differentiate into three cell lineage types: osteocytes, chondrocytes and adipocytes. The stromal component is a typical MSC feature. MSCs can be found in adipose tissue, muscle, amniotic fluid and in the umbilical cord matrix. Despite the initial success in regenerative medicine, bone marrow cells, have some disadvantages. Among them, only a very small fraction (0.05-0.001%) of the entire bone marrow cell is represented by MSCs and their collection requires a painful and invasive procedure. For this reason, scientists continue to search for alternative mesenchymal cell sources (Jeschke et al., 2011).

In recent years, the umbilical cord (UC) has been considered a promising new source of mesenchymal stem cells because they can be isolated in relatively high numbers when compared to bone marrow-MSCs and can be maintained in culture or even cryopreserved (La Rocca, 2011).

Many other properties make these cells a more efficacious therapeutic agent as compared to bone marrow MSCs. For instance, UC is usually discarded after birth and its use does not involve ethical restrictions. UC-MSCs have low immunogenicity and they can also modulate immune functions by producing several cytokines, growth factors and missing co-stimulatory molecules (Weiss et al., 2008). Human UC cells share many of

the surface phenotype markers of MSCs. They both can be cultured in a plain dish and can easily adhere to plastic flask and they are multipotent. Under suitable stimulation, UC-MSCs can differentiate *in vitro* into adipocytes, osteoblasts, chondrocytes, hepatocytes, cardiac cells and neurons (Weiss and Troyer, 2006; Cheng et al., 2010).

OTHER SOURCES OF STEM CELL

PLACENTA DERIVED STEM CELLS (PDSCs)

The placenta is an extra-embryonic tissue that represents a valuable source of stem cells with numerous applications. PDSCs are a type of stromal mesenchymal cells, which belong to the corium and trophoblast lineage both of which have variable plasticity. MSCs derived from placenta appear to be more efficient than BM-MSC in terms of support and cell maintenance.

PDSCs have immune-modulatory properties with Nanog and Oct3-4 inducible expressions. This shows the typical wide range of the MSCs differentiation capacity, which includes neuronal and adipogenic differentiation in *in vitro* models as well as heart valve generation when stem cells are implanted into biodegradable scaffolds. Experimental data has shown that PDSCs have the ability to maintain the endothelial differentiation and that the placenta is an independent stem cell site for regeneration before fetal colonization (Vija et al., 2009).

UMBILICAL CORD BLOOD (UCB)

During the past twenty years UCB has been exploited as a rich stem cell source and hemopoietic progenitor to determine therapeutic effects. It has been shown that, when tested *in vitro,* approximately 1% of UCB cells expresses CD34 antigen, a major cell marker for hematopoietic stem cells. UCB-MSCs are the second largest cord blood stem cell population. In addition to the typical stem cell markers (CD105, CD44 and CD73), UCB cells also expresses Oct-4, which is essential for tissue-specific gene inhibition, self-renewal maintenance, and Nanong.

Interestingly, various studies on hematopoietic cells have demonstrated neuronal

protein expression. In fact, these hematopoietic cells can differentiate into neurons and glial-like cells. Their differentiation potential is more prone toward the osteogenic lineage as compared to the adipogenic lineage. and more orientated to, when compared with MSCs from bone marrow (Vija et al., 2009).

AMNIOTIC FLUID (AF)

Recent studies have evaluated stem cell potential of MSCs isolated from AF as another alternative source of MSCs. AF cells are heterogeneous since they originate from the three germ lines. In particular, AF cells are primarily comprised of the epithelial cell lineage.

AF stem cells seem to express mesodermal and endodermal markers at high levels in the early gestation stages, while ectodermal markers have been found in the late gestational period. AF cells are positive for CD29, CD90, CD166, CD73, CD105, CD49 and CD44 antigen (HCAM-1). Recent studies have proven that AF cells possess multi-potential stem-like characteristics including expression of Oct-4, SSEA-4, and Nanong (Vija et al., 2009).

THE FEATURES OF HUMAN UMBILICAL CORD (UC)

UC is derived from the extra-embryonic mesoderm layer at twenty-six days post gestation. Umbilical cord weight is around 40 g post-partum and it stretches approximately 30-65 cm with a diameter of approximately 1.5 cm (Figure 1.2). During pregnancy, the umbilical cord is a vascular connection between mother and fetus; it protects the blood vessels that provides oxygenated blood to the fetus while also acting as a transport system for waste removal (Conconi et al., 2011; Pappa K., Anagnou, 2009). The UC originates during the first month of the fetus's life, when the morula cells (zygote development stage) will give rise to the embryo. At this time, the UC will modify creating the placenta and fetal annexes (the amniotic sac and umbilical cord). The UC has an external cubic epithelial cell layer, named the umbilical cord epithelium (UCE). It has three vessels (two arteries and one vein). Mizoguchi et al. demonstrated that the epithelium cells express not only mucous epithelium keratins, as found in the amniotic epithelium, but also stratified epithelium keratins and cornified cell envelope (CCE) associated proteins (Mizoguchi et al., 2004).

The portal vein brings oxygenated blood and nutrients from the mother to the fetus. The umbilical arteries remove carbon dioxide as waste elimination. These three vessels are embedded in a matrix made of connective tissues (fibroblastic-like cells, myofibroblast and proteoglycans), called Wharton's jelly. Wharton's jelly acts as scaffolding material for the three umbilical vessels. Capillaries and lymphatic vessels are not present in the umbilical cord (Figure 1.3).

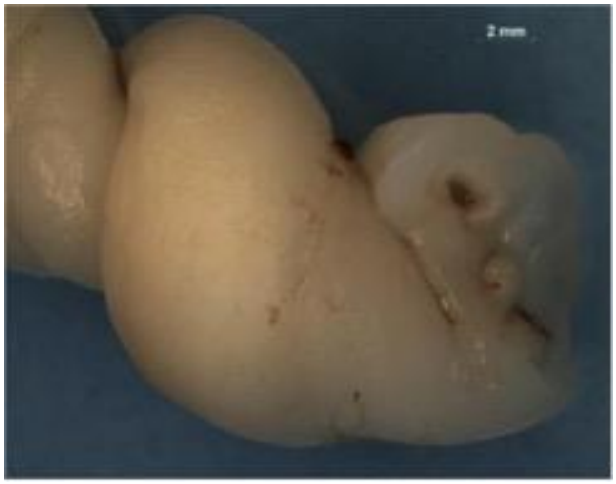

Figure 1.2: Umbilical cord (Semenov and Breymann, 2011)

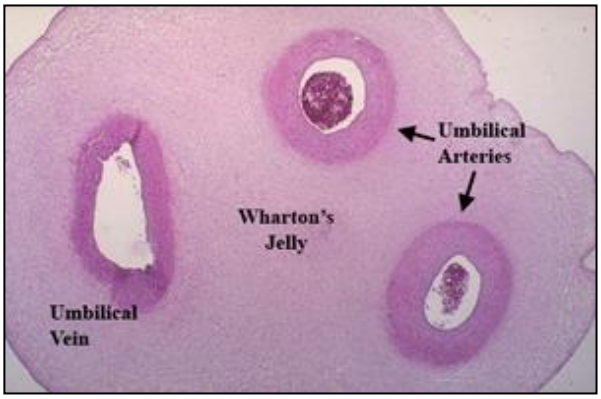

Figure 1.3: Umbilical cord H&E section

WHARTON'S JELLY: STRUCTURE AND FUNCTION

Wharton's jelly (WJ) acts as the umbilical cord's extracellular matrix, a connective mucous tissue. WJ is constituted of a glycosaminoglycan rich substance (GAGs) that contains hyaluronic acid (HA) and proteoglycans, with some collagen fibers (collagen type I and III). The role of Wharton's jelly is to prevent the umbilical vessels that provide a two-way blood flow between the maternal and fetal circulation, from compressing by torsion and/or bending (Tamura et al., 2011).

Myofibroblast and fibroblast-like cells are the two cell types existing in WJ. Myofibroblast have muscle-specific cytoskeletal filaments, and are positive for vimentin (Kasper et al., 1988), a typical fibroblastic marker, and desmin (Can and Karahuseyinoglu, 2007), a muscle cell marker. The fibroblast-like cells have similar features to fibroblasts and they produce collagen and other extracellular matrix components.

Several studies have shown that WJ cells support *ex-vivo* hematopoietic expansion (Lu et al., 2006) and *in vivo* engraftment of hematopoietic stem cells (Friedman et al., 2006). Raio demonstrated that WJ cells are a source of hyaluronic acid, which is another element of the hematopoietic stem cell niche (Raio et al., 2005).

Furthermore, WJ cells secrete cytokines, similar to bone marrow-MSCs, and they are able to synthesize granulocyte-macrophage colony stimulating factor (GM-CSF) and granulocyte colony stimulating factor (G-CSF).

WJ cells are slower to differentiate towards adipocytes than MSCs from bone marrow, but WJ cells have a shorter doubling cell line time and can be isolated with 100% success (Baksh et al., 2007).

OVERALL SIGNIFICANCE

It is well documented that MSCs are defined as immature cells with the ability to self-renew and differentiate into specialized cells belonging to different lineages. Due to their high proliferative potential MSCs can be rapidly expanded *in vitro* when growing in adhesive plastic dishes containing a classical culture medium. In addition,

they are able to differentiate into mature cell-types. Various parts of the umbilical cord (UC) have been considered a promising source of MSCs because they can be isolated in relatively high numbers when compared to other mesenchymal sources and they can be easily cultured or cryopreserved. Thus, obtaining a better knowledge of typical MSCs markers from WJ-originated cells and additional peculiar markers that are still unknown, will define the detailed markers profile and introduce a new concept in the utilization of MSCs in regenerative medicine where instead of replacing function of cells/tissue, cells could be used as adjuvant when transplanted with other cells, tissue or organs.

CHAPTER 2

MATERIALS AND METHODS

WHARTON'S JELLY MESENCHYMAL STEM CELLS ISOLATION

Isolation protocol was adopted from our previously published data (La Rocca et al., 2009). All umbilical cords were obtained after the mother's consent according to tenets of the Declaration of Helsinki and local ethical regulation. In particular, seven cords were provided by the Department of Maternal Fetal Medicine (OB-Gyn) at UTMB. After normal or caesarean delivery, following full-term birth, as described in Table 1, UCs were stored aseptically in cold saline and cellular isolation was initiated within six hours post-partum.

Maternal Age	Race/Ethnicity	Gestational Weeks	Baby Gender	Note
19	Hispanic	39	Male	Cord length between 15-30 centimeters
32	Hispanic	39	Male	
20	Hispanic	39	Female	
33	Caucasian	37	Male	
22	Caucasian	39	Female	
27	Caucasian	39	Male	
40	Asian	39	Female	

Table 1: Criteria of the collected umbilical cords.

Cords were washed in warm HBSS (Gibco), cut in small pieces (approximately 1.5 cm long) and sectioned longitudinally in order to expose the WJ under the amniotic membrane. Different incisions, without vessels removal, were made within the matrix with a sterile scalpel to increase the area exposed to the medium composed of low glucose DMEM (Sigma), supplemented with 10% fetal bovine serum (FBS, Hyclone), 1x non-essential amino acids (Sigma) and 1x antibiotics (Gibco). This isolation protocol, that does not use enzymatic processes to dissociate cells from the embedding matrix, relays on the migratory capacity of these cells that after being released from the tissue are free to attach to the culture plate. Cord pieces were maintained in culture for 15 days and the media was replaced every two days. The slow degradation of the matrix allowed secretion of growth factors and signalling molecules from the cord while preserving stem cell properties.

CELL CULTURING AND PASSAGING

After 15 days of culture, cells that were widely adherent to the plastic surface and cord fragments were removed. Routine culture was then initiated. After reaching confluence, cells were removed from the flasks with Tryple Select (Invitrogen) and were cultured for up to 15 passages in chamber slides, 96 wells and different size flasks. To validate the fibroblastic-like shape we monitored WJ cell morphology overtime under light microscopy. For immunocytochemistry analysis, cells were plated in 8-well chamber slides (BD Bioscience) and were used after reaching 90% confluence. For RNA extraction, cells were cultured either in 6-well tissue culture plates or in 25 cm^2 tissue culture flasks (Corning).

IMMUNOCYTOCHEMISTRY ANALYSIS

Immunocytochemistry detects the expression of specific antigens recognized by a primary antibody, which is then bound by a secondary antibody.

Cells were washed in PBS and then fixed and dehydrated with methanol for 20 minutes at -20°C. After rinsing with PBS, cells were treated for 3 minutes with Triton X-

100 0.1% in PBS 1X. The removal of Triton-X involved two washes with PBS followed by the addition of 0.3% hydrogen peroxide in order to inactivate endogenous peroxidases. After 20 minutes in a blocking solution (complete medium with 10% of serum in PBS 1X, in a ratio of 1:10), cells were incubated with specific primary antibodies for 1.5 hours at room temperature. Following an additional wash in PBS, cells were incubated with species-specific secondary antibodies for 10 minutes. Subsequently, streptavidin-peroxidase (Dako-Cytomation) was added, followed by 3.3'-diaminobenzidine (DAB chromogenic substrate solution, Dako). Finally, Hematoxylin (Dako) was used to counter stain the cell nuclei. Immuno-positivity was scored using a semi-quantitative approach. Three independent observers evaluated the immunocytochemistry results and semi-quantified the percentage of positive cells for each specimen. Ten high-power Fields were examined in each culture slide and the counting of the cells was performed at 40X magnification. The antibodies used in the present study, with indications of the working conditions used, are listed in Table 2.

Antigen	Host	Manufacturer
HLA-DR	Mouse	Santa Cruz
B_{7-1}	Mouse	Santa Cruz.
B_{7-2}	Mouse	Santa Cruz
HLA-ABC	Mouse	Santa Cruz
Vimentin	Mouse	Santa Cruz
v-Wf	Mouse	Santa Cruz
Desmin	Mouse	Santa Cruz
α-SMA	Mouse	Santa Cruz
Cx43	Rabbit	Santa Cruz
Ck8	Mouse	Sigma
Ck18	Mouse	Sigma
Ckmix (18-19-8)	Mouse	Sigma
Ck19	Mouse	Millipore
C-kit	Rabbit	Epitomics

Table 2: List of antibodies used in the present study

FLOW CYTOMETRY

Flow cytometry (FACS) was used to characterize cells at the 2^{nd}, 5^{th}, 10^{th} and 15^{th} passage. Cultured cells were collected for antibody staining at each time-point. The fluorescein isothiocyanate (FITC) – conjugated, phycoerythrin (PE) – conjugated, or allophycocyanin (APC) – conjugated antibodies and isotype control were used (Table 3). Isotype control antibodies were used as negative control for the measurement of the non-specific binding of the specific antibodies. Forward and side scatter gates were set to include all viable cells. Routinely, debris and doublets were excluded from the cell population data by the application of forward and side scatter selection. The two co-expression markers were analysed by the gating of the population that was positive for the first marker and the subsequent analysis of the second marker percentages. FC data were acquired with a BD FACS Aria II instrument cell sorter with two laser and seven colours. Data were analysed using three softwares: FACS Diva 6.1.2, Cell Quest (BD Biosciences) and ModFit LT (Verity Software House).

Antigen	Clone	Conjugation	Dilution	Manufacturer
Albumin	188835	Un-conjugated	01:20	R&D Systems
CD31	WM59	FITC	undiluted	Becton Dickinson
CD34	581	FITC	undiluted	Becton Dickinson
CD44	G44-26 (known as C26)	APC	undiluted	Becton Dickinson
CD45	2D1	PerCP	undiluted	Becton Dickinson
CD73	AD2	APC	01:11	Miltenyi Biotec GmbH, Bergisch Gladbach, DE
CD105	SN6	FITC	undiluted	Abcam, Cambridge, MA
CD117	YB5.B8	PE	undiluted	Becton Dickinson
CD276	FM276	APC	01:11	Miltenyi Biotec GmbH
CK18	C-04	FITC	01:20	Abcam, Cambridge, MA
CK19	RCK108	PE	01:20	Santa Cruz Biotechnology, Santa Cruz, CA
HLA ABC	W6/32	FITC	1:300	Abcam Cambridge, MA
HLA DR	L243 (G46-6)	PerCP	undiluted	Becton Dickinson
HLA-G	87G	PerCP	undiluted	eBioscience Inc., San Diego, CA
HLA-E	3D12HLA-E	APC	undiluted	eBioscience

Table 3: List of antibodies used in the present study

PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS

Western blotting was performed on whole cell lysates to detect protein expression. Cells were lysed using a modified RIPA buffer, 150 mMNaCl, 25 mM Tris (pH 7.4), 1mM EDTA, 1 mM EGTA, 2 mM Na3VO4, 10 mM NaF, 1% NP40, 10% glycerol, aprotinin (10 mg/ml) and leupeptin (10 mg/ml) for 15 minutes. Lysates was centrifuged for other 15 minutes, the supernatant was collected and quantified by a BCA protein assay (Pierce, Rockford, IL). Equal amounts of proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane, which was blocked using 5% non-fat dry milk in Tris-Buffered saline with Tween 20. The membrane was incubated overnight at 4°C with the primary antibodies as listed in table 2. After incubation, the membrane was washed 3 times with T-PBS and then rinsed and incubated for 1 h at room temperature in appropriate anti-mouse or anti-rabbit IRDye 680-800 secondary antibodies. The membrane was rinsed, developed with Odyssey Imaging Systems Li-Cor and specific protein bands were detected with Image Studio Software Version 4.0.21 Li-Cor. GAPDH served as loading control.

OSTEOGENIC DIFFERENTIATION

Differentiation of cells was performed using protocols reported in literature (Zuk et al., 2002) with minor modifications. Briefly, the cells were cultured in osteogenic medium for three weeks (DMEM, 10% FCS, supplemented with dexamethasone 0.1μM, 50μM ascorbate-2-phosphate, 10 mM β-glycerophosphate, Antibiotic / Antifungal, L-glutamine 2mM). The formation of cell clusters resembling intramembranous ossification was monitored by phase-contrast microscopy during culture. WJ-MSCs cultured in standard growth medium (not supplemented) for 3 weeks, were included as controls. At the end of the differentiation period, the cells were exposed to the Alizarin Red stain which stains specifically the deposits of extracellular calcium (La Rocca et al., 2009).

ADIPOGENIC DIFFERENTIATION

Differentiation of cells was performed by culturing WJ-MSCs at different passages in the adipogenic differentiation medium (DMEM, 10% FCS, 0.5 mM isobutyl-methylxanthine, 1 micron dexamethasone, insulin 10μM, 200μM indomethacin, Antibiotic / Antifungal, 2mM L-Glutamine) for 3 weeks. Controls included WJ-MSCs cultured in standard growth medium. At the end of the period of differentiation, the differentiated cells and control cells were subjected to staining with Oil-Red O, which stains the lipid deposits vacuoles neutral, as reported previously (La Rocca et al., 2009).

CHONDROGENIC DIFFERENTIATION

Differentiation of cells was performed by seeding WJ-MSCs into alginate beads, using slight modifications of previously published protocols (Petit et al., 1996; Anzalone et al., 2013). Briefly, WJ-MSCs were suspended in sodium alginate (Sigma-Aldrich) (4 x 10^6 cells/ml at a final concentration of 1,2 % sodium alginate in sterile physiologic solution). Beads were formed by slowly dispensing droplets of the alginate cell suspension from a 22-gauge needle syringe into a 100 mM $CaCl_2$ solution. After washing with 0.15 M NaCl, the beads were rinsed with DMEM. Beads were then cultured either in standard growth medium (controls) or chondrogenic medium, prepared following published protocols (Can and Karahuseyinoglu, 2007) with slight modifications (DMEM supplemented with 1% FBS, 6.25 g/ml insulin, 10 ng/ml TGF-β1, 50nM ascorbate-2-phosphate, 1% antibiotic/antimycotic, 1x NEAA). Beads were maintained in culture for three weeks, with medium changes every second day. For fixation and paraffin embedding, beads were processed as previously described (Ishikane et al., 2010; Ende et al. 2004). The beads were fixed in 4% paraformaldehyde, 0.1 M cacodylate buffer, pH 7.4, with 10 mM $CaCl_2$ for 4 hours at 20°C and then washed over-night at 4°C in 0.1 M cacodylate buffer (pH 7.4) containing 50 mM $BaCl_2$. The beads were standard dehydrated through alcohols and xylene and embedded in paraffin. Sections (6μm) were processed for histology (Alcian Blue and nuclear fast red staining) and IHC.

ANIMALS

C57/BL10 male mice (9-12 weeks old) were used as pancreatic islet donors and recipients (syngeneic islet transplant model). Animals were purchased from Harlan, housed in a standard animal facility and provided ad libitum with rodent chow and tap water. All animals were cared for according to the international guidelines on Animal Care. The study protocol was approved by the Institutional Animal Care and Use Committee (IACUC) and performed under standard regulatory guidelines for research involving animals.

DIABETES INDUCTION

Diabetes was induced 4-5 days prior to ITX by a single streptozotocin injection (intra peritoneal (IP), 220 mg/Kg; Sigma) into the recipient animals. Animals with a blood glucose level (BGL) of > 300 mg/dl for three consecutive days became transplant candidates.

ISLET ISOLATION

Donor animals were anesthetized by isoflurane (Terrell ^{TM} Isoflurane, USP, Novaplus, Piramal Healthcare) and the pancreas was harvested and stored in cold Hank solution (Gibco), until digestion. In brief, following surgical preparation, a midline abdominal incision was performed. After clamping of the duodenal ampulla, the pancreatic duct was cannulated and collagenase solution (0.8 mg/ml; Sigma) was injected. Following adequate distention, the pancreas was harvested and stored on ice. Pancreata were digested and islets were purified using Ficoll (Cellgro- Corning) density gradients (1.108, 1.096, and 1.037). Isolated islets were counted by islet Equivalent (IEq: islet of an average diameter of 150 μm).

ISLET TRANSPLANT

Diabetic animals (BGL>300 mg/dl) were transplanted with either 600 IEq or 200 IEq. Full mass (600 IEq) is expected to reverse diabetes in 100% of the cases (group 1), while marginal mass (200 IEq) is expected to not reverse diabetes (group 2). As shown on Table 4, in the study group (group 3), a marginal mass of islets was co-transplanted with $3x10^6$ W-MSCs. Briefly, following anesthesia and surgical preparation the animals were positioned exposing their left flank. An incision of approximately 1 cm was performed exposing the left kidney. After puncturing of the kidney capsule a P50 tubing connected to a Hamilton syringe was inserted and islets (or islets + WJ-MSCs) were transplanted under the kidney capsule. The capsule opening was then cauterized, the kidney repositioned and the fascia and skin sutured.

	Number of islets	**WJ-MSCs**	**n =**
Group 1	600	-	2
Group 2	200	-	3
Group 3	200	$3x10^6$	3

Table 4: Groups of animals transplanted

BLOOD GLUCOSE LEVEL (BGL) AND INTRA-PERITONEAL GLUCOSE TOLERANCE TEST (IPGTT): ENGRAFTMENT AND FUNCTIONALITY ASSESSMENT

Animals were monitored daily for BGL and body weight for the first 2 weeks and then twice weekly thereafter. Blood glucose concentrations were determined using a blood glucose meter (Accu-Chek; Roche Diagnostics, Indianapolis, UN, USA) after tail vein puncture. Transplanted islets were considered to be engrafted when a BGL of <150

mg/dl was attained and maintained. Recipients that experienced reversal of diabetes (i.e., normal BGL) within 7 days post-transplantation, were considered successful.

In animals that had reversed diabetes, islet functionality was assessed by IP glucose tolerance tests (IPGTTs). IPGTTs were performed at long-term follow-ups (day 60–120). IPGTT from naive (non transplanted) animals were included as controls. Briefly, animals were fasted, and following the detection of baseline BGL, 2 mg/kg body weight of 50% dextrose (Hospira, Inc., Lake Forest, IL, USA) in 0.5 ml was injected IP. BGL was then measured at 15, 30, 45, 90, and 120 min after injection.

In all animals that reversed diabetes within 7 days and concluded the study, the graft-bearing kidney was removed to perform a histological examination (H&E and insulin). Animals were humanely sacrificed, and the pancreas was harvested for histological analysis to confirm absence of insulin producing cells.

STATISTICS

Data was plotted using MS Excel software and statistical analyses were performed using GraphPad Prism 4 software (GraphPad Software, San Diego, USA).

The statistical methods used were nonparametric analyses. Values were considered significant for $p < 0.05$.

CHAPTER 3

WJ-MSCs: ISOLATION, PHENOTYPICAL CHARACTERIZATION AND POTENCY

INTRODUCTION AND BACKGROUND

Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs) are multipotent stem cells that have many advantages as a source of MSCs. WJ-MSCs are a readily available source (cord is usually discarded after birth), are easy to growth in culture, can be cryo-preserved and have great expansion capacity *in vitro*. Over the years, our research group has gained reasonable experience both in isolation and the primary culture of WJ-MSCs (La Rocca et al., 2009). Moreover, WJ-MSCs have better *ex vivo* expansion abilities when compared to BM-MSCs due to their lasting telomerase expression and activity. Several studies have shown that WJ-MSCs express GATA-4, GATA-5, and GATA-6 transcription factors that are involved in different development pathways of the mesoderm and endoderm – derived organs (La Rocca et al., 2009). Previously, only GATA-4 expression had been reported in BM-MSCs (Bayes et al., 2005).

La Rocca et al. demonstrated that WJ-MSCs express connexin-43 (La Rocca et al., 2009), a molecule typically present in embryonic and myocardial cells that is responsible for intercellular gap junction formations. Matsuyama and Kawara established that Connexin-43 expression increases in a stage-related trend along the myocardial differentiation pathway and that it is linked to proliferation arrest as well as mature phenotype acquisition.

WJ-MSCs are able to regenerate, a key mesenchymal cell feature, while maintaining their replicative potential and undifferentiated state. In fact, it is known that Nanog and Oct3/4A expression in MSCs are some of the factors responsible for maintaining long-term self-renewal status.

WJ-MSCs, isolated with no enzymatic methods, express various cytokeratin types (CK), such as CK-8, CK-18, and CK-19 (Moll et al., 2008), while CK-7 is not detected (Xie et al., 2009). Immunocytochemistry analyses have highlighted that WJ-MSCs lack

expression of CD14, CD31, CD33, CD34, and CD45 (Battula et al., 2009). Moreover, both BM-derived and WJ-MSCs do not express HLA-DR (Turnocova et al., 2009; Zummo et al., 2007). WJ-MSCs express: CD73, CD90, CD105, HLA class I (Troyer and Weiss, 2007), CD10, CD13, CD29, CD44, CD49e, CD166 and CD117 a receptor for the stem cell factor (Hung et al., 2002; Weiss et al., 2006; Bakhshi et al., 2008).

WJ-MSCs are also positive for nestin (La Rocca et al., 2009), an intermediate filament of the neuro-ectodermal lineage as a neurofilament precursor, which is also present in the pancreatic progenitors of β cells (Zulewski et al., 2001). Furthermore, the glial fibrillar acidic protein (GFAP) and neuron-specific enolase (NSE) have been described in the literature as other neuronal markers (Karaoz et al., 2009; Mitchell et al., 2003).

Mesenchymal stem cells derived from umbilical cord can differentiate toward endoderm-derived organs (e.g. hepatocyte). In fact, the hepatocyte nuclear factor 4α (HNF-4α) has been expressed by WJ-MSCs, suggesting a possible role in hepatocytes and pancreatic endocrine cell regeneration (Conconi et al., 2011).

Bone marrow mesenchymal stem cells (BM-MSCs) have been extensively studied for regenerative medicine applications. They have a fibroblastic-like morphology and can differentiate into adipocytes, osteoblasts, and chondrocytes. Although bone marrow represents the main stem cells source in the clinical setting, the cell number obtained after isolation is low and decreases with donor age (Rao and Mattson, 2001).

While there are several disadvantages in using BM-MSCs for experimental and therapeutic use, they still remain the 'gold standard' in regards to the mesenchymal stem cell concept when compared to other sources.

MSCs derived from Wharton's jelly, the whitish jelly present inside the umbilical cord, (widely described in chapter 1), are similar to BM-MSCs phenotypically, with some differences between the two cell populations. For instance, BM-MSCs appear to be more directed in the osteogenic differentiation and can express genes as biglycan, vitronectin, or CD44. In contrast, UC-MSCs exclusively express high levels of genes related to angiogenesis, such as IL-8 and IL-1 receptor ligand. BM-MSCs are also positively associated with nestin and collagen type I and II by immunocytochemistry, while CD106

is only in the bone marrow.

Recent studies have shown that UC-MSCs produce chemokines as well as cytokines that can induce hematopoietic stem cell proliferation. They also contain higher levels of HLA-I expression as compared to BM-MSC's. After the endothelial induction, stem cells express high endothelial markers levels, such as von Willebrand factor and the VE-cadherin (Di Nicola et al., 2002).

UC AND WHARTON'S JELLY MESENCHYMAL STEM CELL (WJ-MSC) DIFFERENTIATION ABILITY

WJ-MSCs are multipotent cells, able to generate different mature cytotypes. There are numerous studies that demonstrate WJ-MSCs differentiate toward connective tissue phenotypes (osteoblasts, chondrocytes and adipocytes). These three differentiation cell lineages have been considered part of the minimal MSC criteria stated in 2006 (Dominici et al., 2006). Their potency would open new frontiers in regenerative medicine.

The standard protocol to obtain MSC osteogenic differentiation was confirmed by specific histological stains for extracellular calcium, Alizarin Red S and Von Kossa (Zuk et al., 2002). Moreover, the same MSC differentiation should express specific proteins such as osteonectin, osteocalcin, periostin and runx2 (Ciavarella et al., 2009).

After adipogenic differentiation, the adipocytes differentiated are confirmed by lipid- specific histological stain such as Oil Red O (Janderova et al., 2003). In addition, the new cells should express specific proteins such as adiponectin, leptin and peroxisome proliferator-activated receptor gamma (PPAR-γ).

Regarding the chondrogenic lineage, differentiated MSCs are confirmed by Alcian blue or Safranin O-Fast Green stains (Steck et al., 2009). The chondrocyte phenotype can be confirmed by the expression of collagen type II, cartilage oligomeric matrix protein (COMP) and aggrecan (Dvorakova et al., 2008).

Neurogenic differentiation. WJC cultured in medium supplemented with basic fibroblast growth factor (bFGF), butylated hydroxyanisole, dimethyl sulfoxide (DMSO), and low serum percentages, have been successfully induced to differentiate into glial

cells and neurons by Mitchell and his group (Mitchell et al., 2003). Their group described the neural markers expression (NSE and GFAP) by undifferentiated cells, while the differentiated neurons and glia cells over-expressed these molecules and showed catecholaminergic neuron markers. Yan and colleagues were able to differentiate UC-MSCs into neuronal lineage and then transplant them in a rhesus monkey model as a potential therapeutic application for Parkinson's disease (Yan et al., 2013).

Myocardiocyte differentiation. Myocardial repair via heterologous stem cells is a promising area of research. Recent experiments suggest that WJ-derived cells can play a role in myocardial regeneration. The first case of myocardial derived WJ was explained by Wang et al in 2004. In this study, WJC started to express typical myocardial markers (cardiac troponin I, connexin-43 and desmin) and exhibited myocardial morphology at 3 weeks post treatment with 5-azacytidine (Wang et al., 2004). Later, Wu et all, demonstrated a new myocardial differentiation protocol with WJ-MSC, where 5-azacytidine use (24 hours) was followed by 4 weeks of culture in medium supplemented with β-FGF and platelet-derived growth factor (PDGF). Wu showed that the myocardial-differentiated cells, after being injected into a heart experiencing acute myocardial infarction, had been incorporated into the vasculature and occasionally were positive for cardiac troponin T (cTnT) (Wu et al., 2009). Other works have claimed MSC supportive populations through inflammation suppression in an acute myocardial heart attack model, as well as paracrine effects on the myocardium repairing (Gnecchi et al., 2006; Henning et al., 2008).

Hepatogenic differentiation. In the literature, there are various protocols in support of the hepatocyte–derived MSCs lineage in an *in vitro* model. This model uses many of the factors in culture medium as hepatocyte growth factor (HGF), fibroblast growth factors (FGFs) for the first inductive phase, and oncostatin M (OSM), involved in the final differentiation phase. Furthermore, insulin-transferrin-sodium selenite (ITS), dexamethasone and epidermal growth factor (EGF) have been used. These factors should be applied in a monolayer culture, in 3D scaffolds (Baharvand et al., 2006), or in a co-culture system with fetal or adult hepatocytes (Quiao et al., 2007). Most experiments have been performed using low (1%) serum culture media, PAS, and indocyanine green

stains, which have been tested to confirm the hepatocytes metabolic activity and viability (Zheng et al., 2008). Numerous researchers have demonstrated that UC-MSCs can be successfully differentiated in an *in vitro* model as well as an *in vivo* model.

METHODS

Detailed methods for WJ cell isolation, primary culture, characterization and differentation protocols were described in chapter 2.

RESULTS

Morphological features of Wharton's jelly Mesenchymal stem cells (WJ-MSCs)

Briefly, isolation of WJ cells from the umbilical cord matrix has been performed using standard methods: leaving cord fragments in culture medium allowed cells to exit and attached to the culture vessel according to their mesenchymal migratory capacity, without any enzymatic treatment. WJ-MSCs were grown on culture surfaces and were easily expanded *in vitro* under standard conditions (Figure 3.1 D - F). According to our original hypothesis, the slow matrix degradation allows growth factors and signalling molecules to be released by the cord and maintain the stem cell potency.

Standard histochemical staining (H&E) was performed on paired paraffin tissue sections of embedded umbilical cords. As visible in figure 3.1 (panels A – C), the specimens showed the expected substructures of the umbilical epithelium, vessels, and intervascular stroma known as Wharton's jelly.

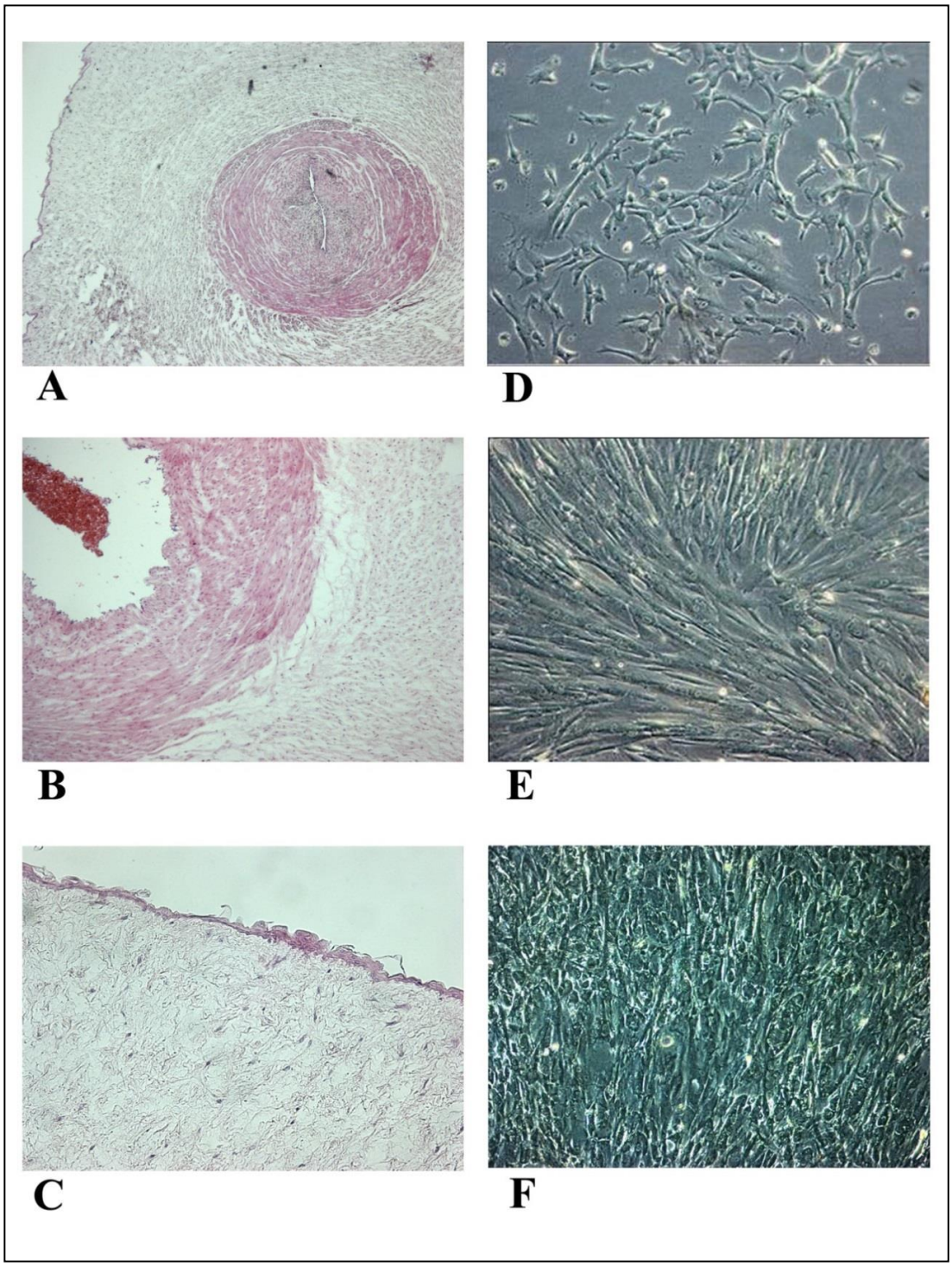

Figure 3.1 Demonstration of umbilical cord and WJC morphology:
Umbilical cord appears lined by a continuous amniotic epithelium, with vessels embedded in a ECM-rich mucous tissue (A); (B), detailed view of the perivascular area; (C), detailed view of the sub-amniotic area of the stroma. Cells obtained from the same cord specimen are depicted in panels D (passage 1), E (passage 6), F (passage 8),

showing the expected fibroblastic-like morphology. Magnification: (A, 5x), (B, D, E, F, 10x), (C, 20x).

Phenotypical characterization of Wharton's jelly Mesenchymal stem cells (WJ-MSCs) for the expression of classical MSC markers and standard differentiation.

To confirm that the isolated cells adhered to the classical definition of mesenchymal stem cells, multiple analyses were made in accordance with the minimal criteria stated by the ISSCR. Flow cytometry (figure 3.2) showed that WJ-MSCs were amply positive to classical MSCs markers (such as CD29, CD44, CD73, CD90, CD105), while lacking CD34 and CD45, therefore adhering to the classical pattern of markers expression expected by MSCs. Standard differentiation experiments (figure 3.3) demonstrated the ability of these cells to differentiate towards the osteogenic, adipogenic and chondrogenic lineages.

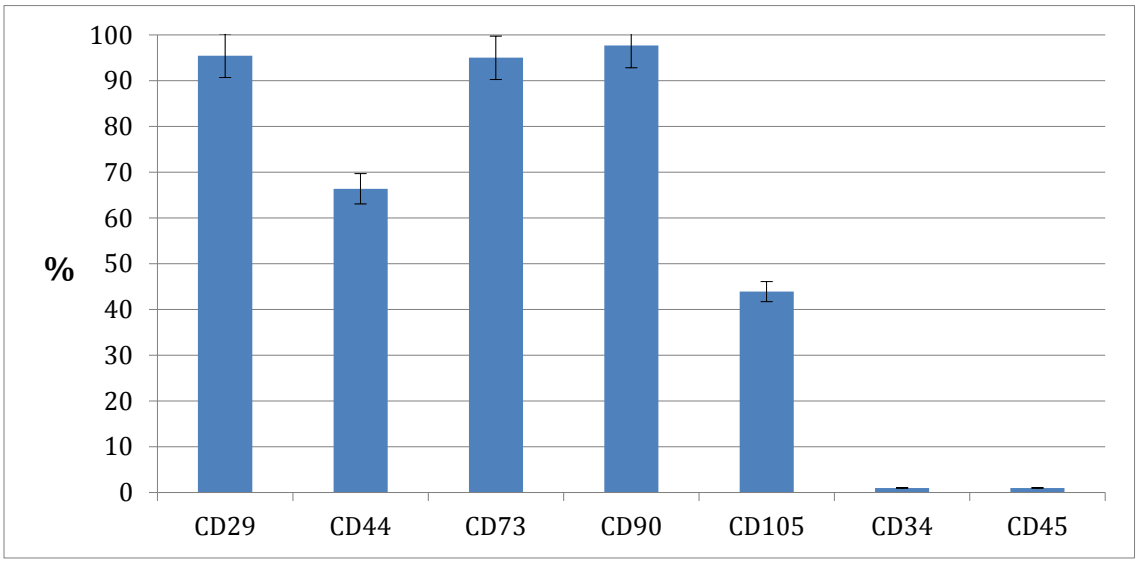

Figure 3.2: Flow cytometry analysis of standard MSC markers expression in WJ-MSCs. Cells were positive for the expected markers of MSC populations, namely CD29, CD44, CD73, CD90 and CD105, while being almost negative for the hematopoietic markers CD34 and CD45.

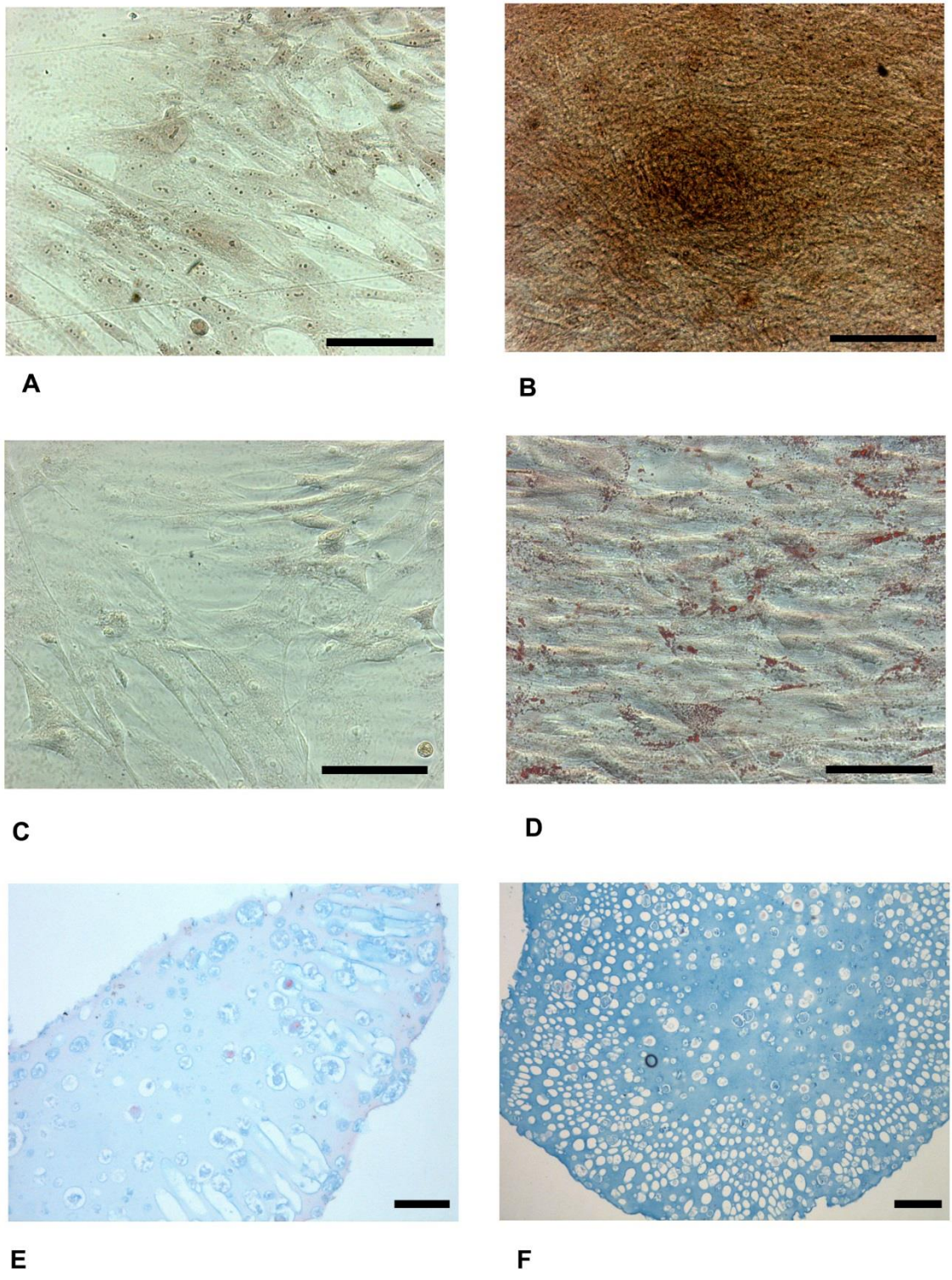

Figure 3.3: Demonstration of the tri-lineage differentiation by WJ-MSCs. Cells were subjected to differentiation as previously described (Methods) and stained with specific histological stains. Osteogenic-differentiated cells showed large extracellular calcium

deposits as confirmed by Alizarin Red (B), as compared to undifferentiated cells (A). Adipogenic differentiation was confirmed by Oil Red O positive stain (D) as compared to undifferentiated cells (C). Chondrogenic differentiation of alginate hydrogel-embedded cells was confirmed by Alcian Blue (F) as compared to negative in controls (E). Magnification: 20x (A-D), 10x (E, F).

Phenotypical characterization of Wharton's jelly Mesenchymal stem cells: additional markers.

In order to provide a more detailed characterization of WJ-MSCs, we investigated their expression in the umbilical cord tissue and cultured cells. As shown in figure 3.4 (A, B), α-smooth muscle actin (α-SMA) is expressed at the vascular muscle component and Wharton's jelly cells level. Vimentin (C, D) and desmin (E, F) are other two intermediate filaments expressed in WJ and UE zones. Vimentin is a typical intermediate filament of mesenchymal lineage cells. Desmin expression plays an important role since it is usually associated with the muscle cell phenotype. As shown in figure 3.4 (F) vessel wall stains positive for desmin, and it is also expressed in Wharton's jelly (Figure 10 E, F) as well as umbilical epithelium. The von Willebrand factor (vWF), typically restricted to mature endothelial cells, was detected only in vascular endothelium as expected, Figure 3.4, (G, H). The same results observed in tissue for (α-SMA, vimentin, desmin and vWF) have been confirmed subsequently in cell culture by immunocytochemistry (ICC), as shown in Figure 3.5 (A, B, C, D). This is of central importance for the characterization of the phenotypical features of WJCs. In fact, even if their environment changed with the *ex-vivo* culture, the expression of multiple molecules remains unaltered. This will be of further importance for the maintenance of features leading to the immune privilege of those cells.

Moreover we have evaluated the cytokeratin (CK) expression, intermediate filaments at the human body epithelial level. Previous studies have described the cytokeratin family presence in the umbilical cord blood (Petit et al., 1996). We demonstrated that cytokeratins like CK-8, CK-18 and CK-19, are expressed in the umbilical epithelium and Wharton's jelly, Figure 3.6 (A, B). Our group have shown that WJ-MSCs expressed c-Kit antigen, the stem cell factor receptor, differently from BM-MSCs. c-Kit is localized at the perivascular region by IHC analysis, and less expressed in WJ, Figure 3.6 (C, D).

Connexin-43 (Cx-43) is another interesting molecule, belonging to the stem population, expressed mostly in the embryonic and mesenchymal stem cells. From a cell

therapy perspective, Cx-43, together with others, may help engraftment in the host pancreatic parenchyma, by allowing the establishment of physical interactions between cells. It has also been demonstrated that Cx-36 and Cx-43 have opposite effects on beta cell mass and insulin production levels. The IHC analysis has allowed us to highlight that Cx-43 is widely expressed at the UE and Wharton's jelly level, Figure 3.6 (E, F). ICC method has been used to evaluate the expression of the same molecules (cytokeratins, c-Kit, and Cx-43) in the cell culture. As visible in Figure 3.7 (A, B and C), WJ-MSCs showed expression of CK-8, CK-18 and CK-19, thus confirming the IHC data. WJ cells have been found to both express c-Kit and connexin 43, Figure 3.7 (D and E) molecules also *in vitro*.

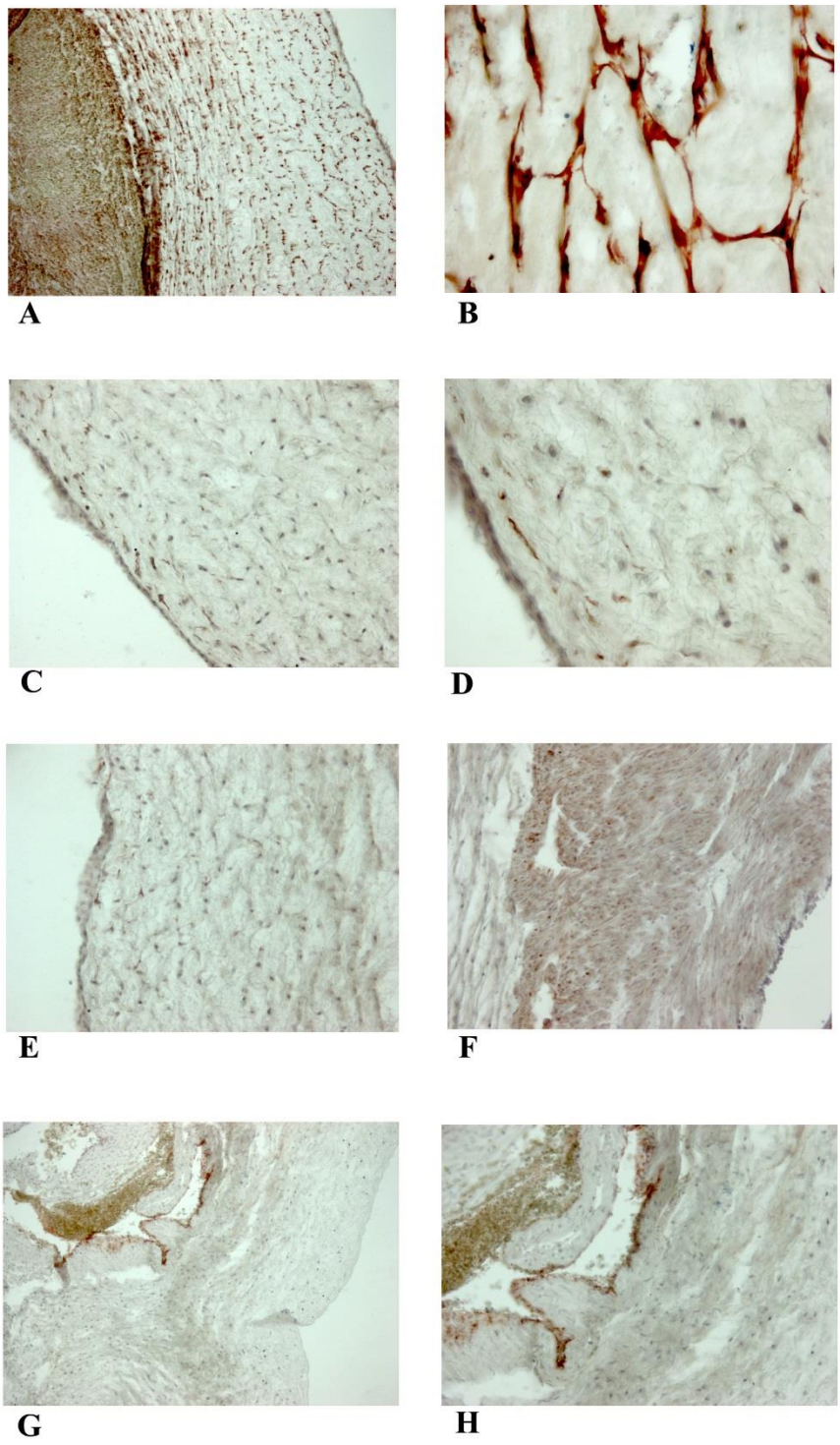

Figure 3.4. Representative panels of immunohistochemical detection of various markers in umbilical cord specimens: α-SMA (A and B), Vimentin (C and D), Desmin (E and F), vWF (G and H). Magnification: A and G 10x, B-F, H, 20x.

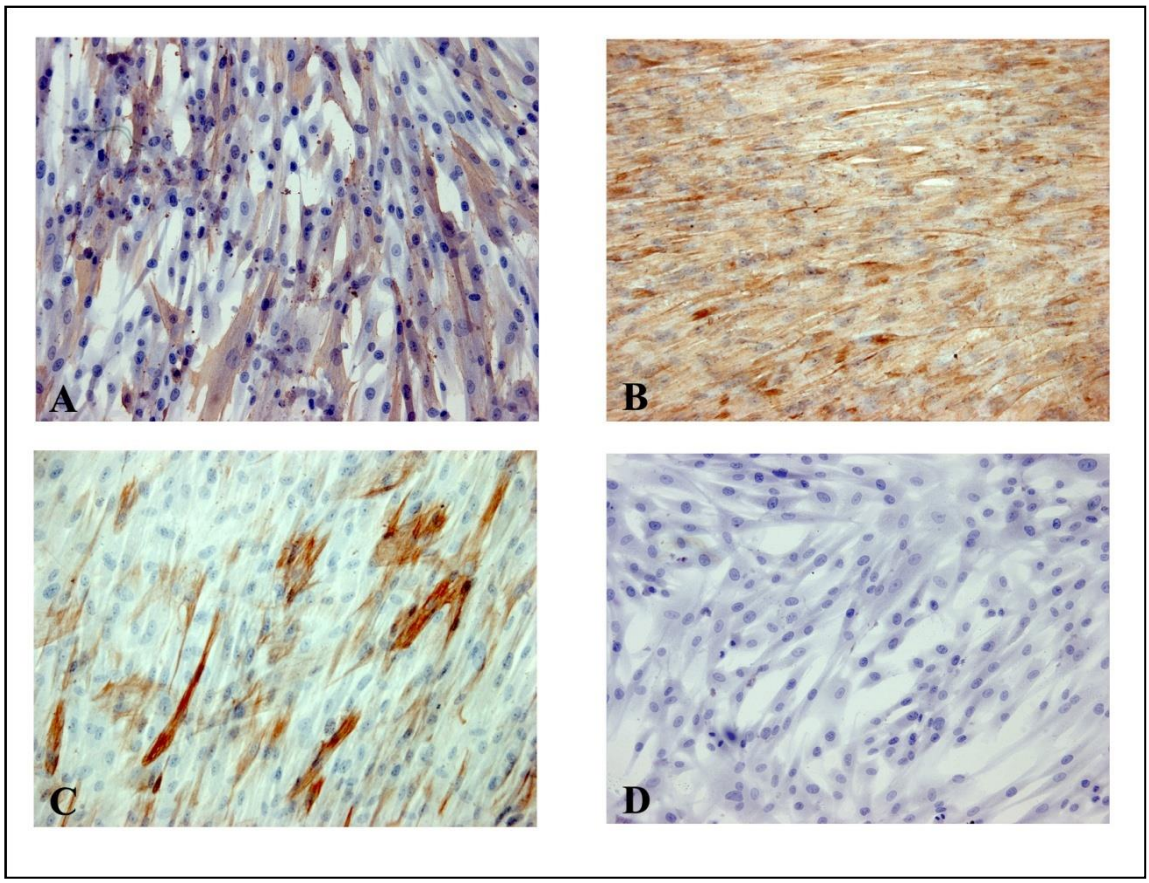

Figure 3.5: Representative panels of immunocytochemical detection of markers in umbilical cord specimens: α-SMA (A), Vimentin (B), Desmin (C), vWF (D). Magnification: 20x.

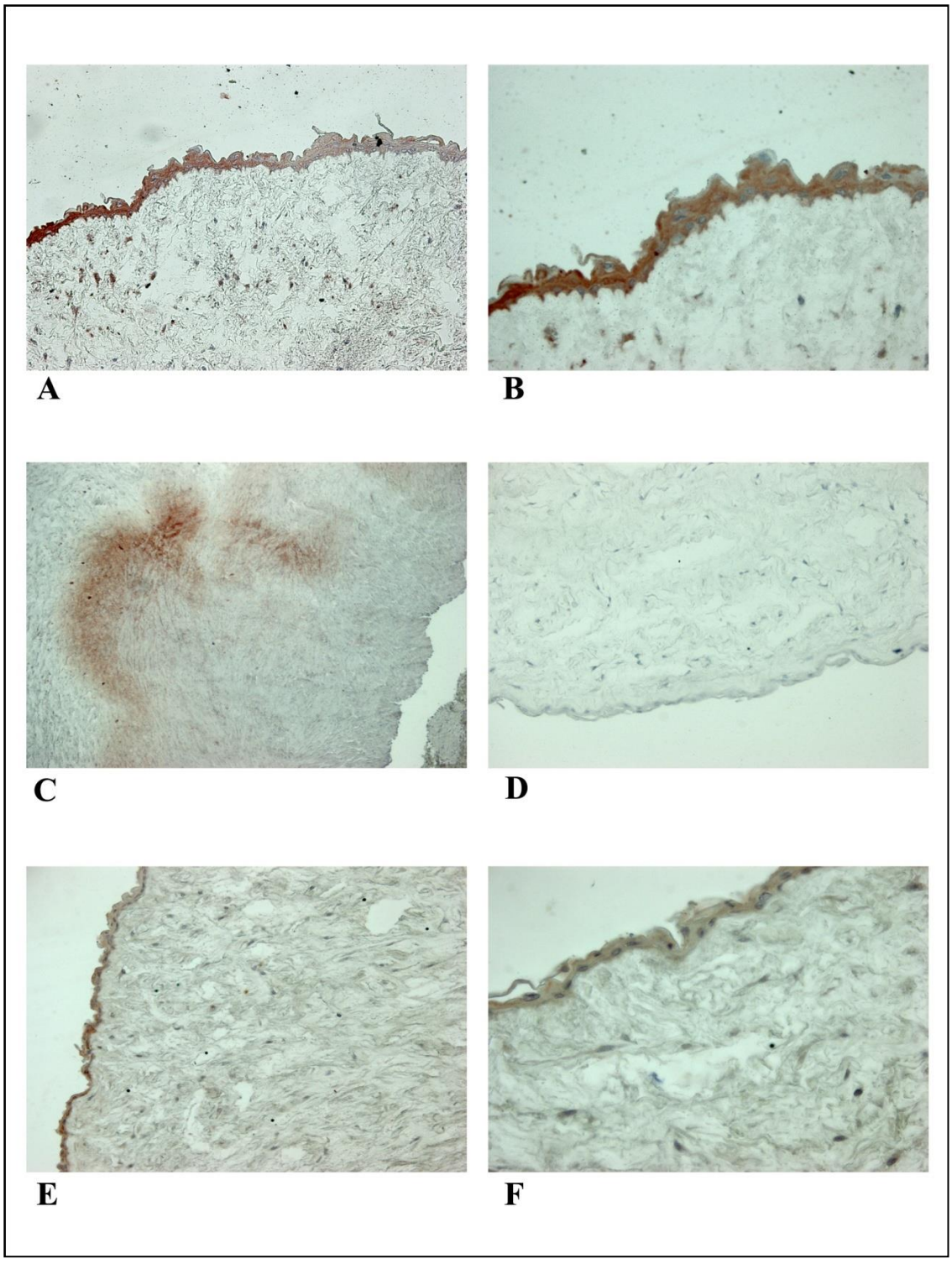

Figure 3.6: Representative panels of immunohistochemical detection of markers in umbilical cord specimens: Ck-mix (A and B), C-kit (C and D), Cx-43 (E and F). Magnification: (A, D, E, 20x) , (B, F, 40x) and C 10x.

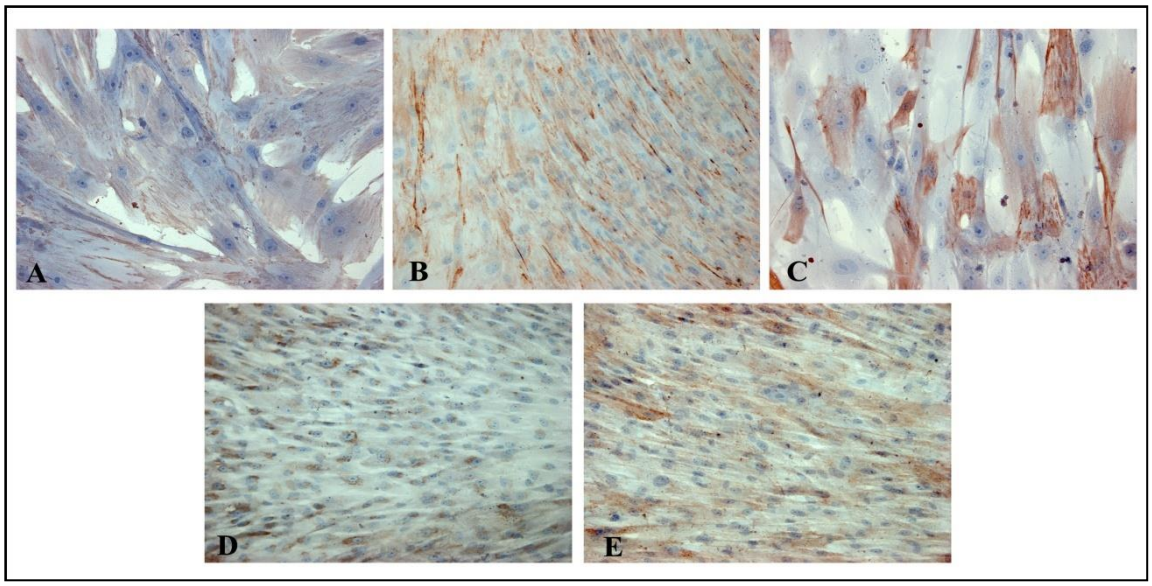

Figure 3.7: Representative panels of immunocytochemical detection of markers in umbilical cord specimens for Ck-8 (A), Ck-18 (B), Ck-19(C), C-kit (D), Cx43 (E). Magnification: 20x.

Neuronal markers and glial factor expression of Wharton's jelly Mesenchymal stem cells

Another interesting observation, that emerge from the phenotype analysis of the un-differentiated WJ-MSCs, is their ability to express molecules belonging to several mature lineages, such as neuro-ectodermal. Due to the importance of neural cells and molecules in the early differentiation process of pancreas tissues, both exocrine and endocrine, and the possible establishment of a cross-talk between infused stem cells and islets populations via diffusible factors, we aimed to determine if our undifferentiated populations, to be used in subsequent cell transplantation experiments, would be able to express such molecules.

Figure 3.8 shows the results of immunocytochemical analyses on cultured WJ-MSCs: cells were positive for the expression of Nestin, Figure 3.8 (B), which is an intermediate filament expressed in neuronal precursors but found also in pancreatic stem cells. This is normally replaced by the mature intermediate filaments along the standard differentiation of the neural lineages, so it is not surprising to find a very low number of NF68-expressing cells, Figure 3.8 (D) scattered between the Nestin-positive cells. WJ-MSCs express also other markers of the neural lineage, as shown for neuron-specific enolase (NSE) , Figure 3.8 (A) and the glial fibrillar acidic protein, GFAP, Figure 3.8 (C), which shows the classical filamentous cytoplasmatic staining.

These data show that WJ-MSc are able to express several molecules specifically associated with neuro-ectodermal differentiation pathways. On these premises, we wanted to determine whether the GDNF (glial derived neurotrophic factor) expression could be detected in these cells. In fact, GDNF represents a secreted molecule associated with the differentiation of islet cells in pancreas, as observed in cat development (Lucini et al., 2008). Further studies of overexpression of GDNF in pancreatic glia showed that the factor was associated with an increased beta cell mass and improved glucose tolerance (Mwangi et al., 2008).

Figure 3.9 shows the results of a western blotting analysis of GDNF expression in WJ-MSCs as well as in cerebral cortex (used as a positive control) or pancreases of mice

either diabetic or normoglycemic. GDNF was expressed in all samples as a mature form with a band of 15 kDa. The glycosylated form, with a reported molecular weight of 25kDa (Garcia et al., 2010) was detected only in mouse tissues. The most abundant bands referred to the higher supra-molecular complexes that GDNF forms with heparin around 37 kDa, which range from 33 to 45 kDa. These data constitute the first observation of GDNF expression in Wharton's jelly cells, and due to its importance in pancreas development and beta cell proliferation and glucose tolerance, this factor may constitute an important player in the subsequent experiments aiming to evaluate the contribution of WJ-MSCs in islets transplantation.

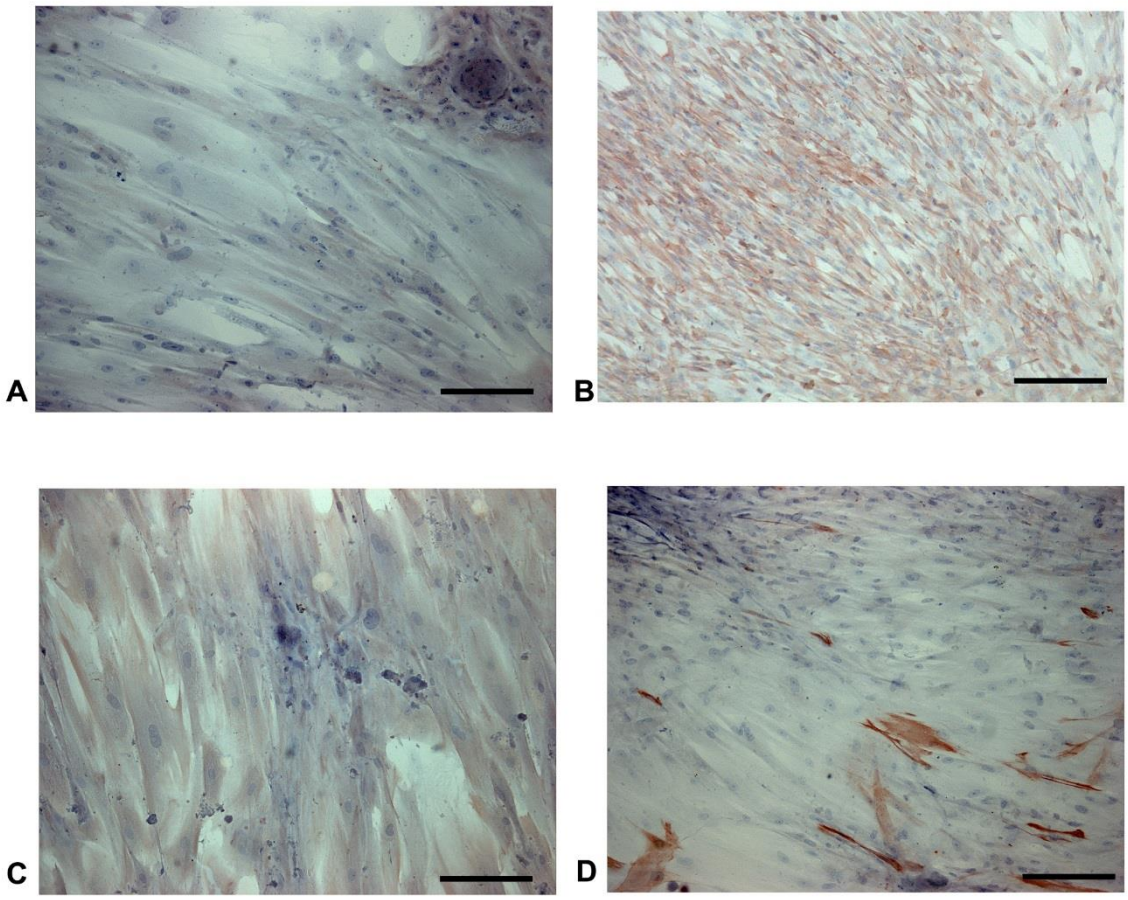

Figure 3.8: Representative panels of immunocytochemical detection of neural markers in WJ-MSCs. Cells were amply positive for the expression of NSE (A), Nestin (B) and GFAP (C). Scattered cells were also positive for the expression of the mature intermediate filament NF68 (D). Magnification: 10x.

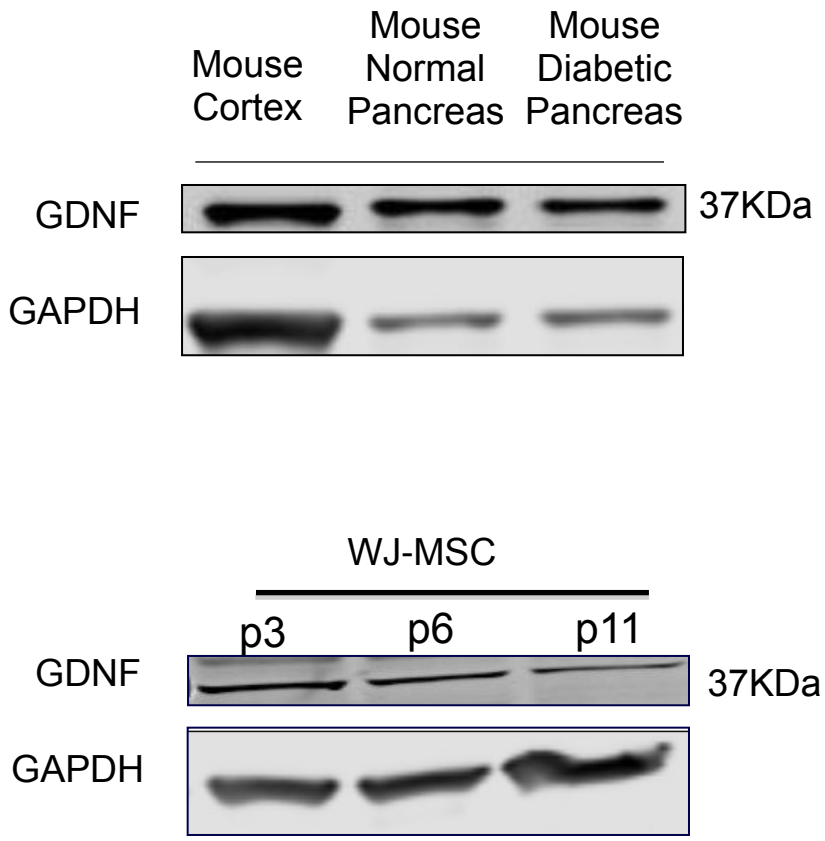

Figure 3.9: Western blotting analysis of GDNF expression in mouse tissues and WJ-MSCs.
GDNF was detectable as the mature form, which weighs 16kDa in all the considered specimens, the free glycosylated isoform was detected in mouse tissues but not in WJ-MSCs (not shown here). In all samples, the predominant forms were the numerous supra-molecular complexes with heparin, which range in MW around 37 kDa. GAPDH was used as control for protein expression.

DISCUSSION

The first objective of this project was to obtain a better characterization of the basic biology features of WJ-MSCs, which were first demonstrated to possess the standard expected features in term of classical markers expression as well as the standard differentiation abilities. However, these are only part of the data that have been generated during the project: in fact, we pursued a detailed characterization of the WJ-MSCs phenotype, not only in the cultured cells, but in their *in organ* counterpart, by analyzing the expression of the same molecules also in the umbilical cord tissue. This allowed to detect markers belonging to the ectodermal, endodermal and mesodermal-derived tissues in these cells, which were present also in the cells *in vivo*. Such a study, which has limitedly exploited elsewhere, strongly suggested that the cells maintain even in culture conditions, the same phenotype, which they feature in the umbilical cord and warrants their properties *in vivo*. Another feature of the WJ-MSCs is the expression of various molecules of the neuro-ectodermal lineage. We characterized in this project the expression of markers such as NSE and GFAP, but also nestin positivity and the appearance of rare NF68-positive cells tend to confirm that WJ-MSCs may have the possibility to differentiate towards neural lineages. However, the most important finding, to this regard, was the characterization of GDNF expression in WJ-MSCs. This factor has been implied with many processes related to beta cells, from the developmental patterning of islets populations, to the effect of its overexpression in pancreatic glia on beta cells numbers and glucose tolerance improvement. The discovery that WJ-MSCs express this secreted molecule may pose a new basis on the way in which these cells may favor islets engraftment and survival in the host. Further research is necessary to better detail the role of GDNF in pancreas, but the data showed for the first time in this project constitute a solid basis to proceed further.

CHAPTER 4

IMMUNO-MODULATORY AND IMMUNE RELATED MOLECULES EXPRESSION IN WJ-MSCs

ABSTRACT

We have previously shown that WJ-MSCs express the core-markers of mesenchymal-like stem cells and their potency (ability of WJ cells to differentiate according to three differentiated mature cell lineages). Furthermore, WJ-MSCs possess a unique ability to cross lineage border, they have immunomodulatory and anti-inflammatory properties and these characteristics make them promising for therapeutic approaches.

Literature data have demonstrated the expression of immunomodulatory markers in UC tissue as well as in primary culture and more importantly the maintenance of the immune modulating features in cell types differentiated from MSCs.

The present chapter underlines another important ability of WJ-MSCs, the immune modulation of WJ cells in *in vitro* model, defining their potential utilization for the future cellular therapy.

INTRODUCTION

In recent years, it is known that the MSCs have an ability to interact with the adaptive and innate immune system by cell contact and soluble factor secretion (Di Nicola et al., 2002). An immune function of MSCs is to inhibit T-cell proliferation and dendritic cell (DC) differentiation (Vija et al., 2009). MSCs are able to do this because they have low expression of co-stimulatory molecules and the absence of HLA-II molecules secretion (Di Nicola et al., 2002; Conconi et al., 2011). Recent studies have shown that soluble factor secretion is a consequence of MSCs and T-lymphocytes cross talk, but not as a total constitutive process (Pappa and Anagnou, 2009).

In particular, HLA molecules have been involved in both NK cell self-tolerance induction as well as maternal immune system tolerance toward the embryo (Tamura et al., 2011; Kasper et al., 1988). Several publications pointed out that the espression of HLA non-classical type I in the mesenchymal stem cells. Specifically HLA-G and its soluble form, HLA-G5 (Vija et al., 2009).

Di Nicola and colleagues suggested that transforming growth factor beta (TGF-β) and hepatocyte growth factor (HGF) are two possible mediators for T-cell suppression in a mixed lymphocytes reaction (Di Nicola et al., 2002).

Ren and colleagues have observed that the adhesion molecules ICAM-1 (inflammatory cytokine-induced intercellular adhesion molecule-1) and VCAM-1 (vascular cell adhesion molecule-1) are critical for MSCs immunosuppression on T-cells, and are inducible by IFN-γ, IL-1 and TNF-α presence (Can, 2007). MSCs express indoleamine deoxygenase (IDO) and nitric oxide (NO), two molecules involved in the immune response (Takechi et al., 1993).

After systemic infusion, MSCs are able to migrate to the tissue injury site and start to accumulate there (Sobolewski et al., 2005). Migrating resident phagocytes find MSCs around the damaged tissue area. MSC treatments have positive-protection effects on neurological disorders due to their anti-inflammatory and oxidative stress stem cell properties.

Kemper et all. described the superoxide dismutase 3 (SOD3) secretion by MSCs

as a neuro-protective agent, decreasing the inflammation and tissue damage (Lu et al., 2006).

Other *in vivo* experiments have confirmed MSCs power to reduce inflammation in obstructive apnea (Friedman et al., 2006), renal failure (Raio et al., 2005), liver fibrosis (Baksh et al., 2007), asthma (La Rocca et al., 2009) and acute myocarditis (Bayes-Genis et al., 2005).

In addition, UCB stem cells have normalized blood glucose level in NOD diabetic mice and reduced insulitis (Moll et al., 2008). Another key question remains, however, as to how inflammatory cytokines play a part in the MSC regulation potential and the related *in vivo* interaction pathways.

Evidence from *in vivo* model suggests that MSCs are able to attenuate inflammation, by secreting certain mediators like cytokines and growth factors in acute and chronic inflammatory diseases.

METHODS

Methods for the immune modulatory features of WJ-MSCs were described before in chapter 2.

RESULTS

Immunomodulatory and immune related molecules expression in WJ-MSCs by immunohistochemistry, immunocytochemistry and flow cytometry.

An important goal of this project was to understand the pathways and interactions between the mesenchymal stem cells and recipient immune system, to better detail the mechanisms to prevent the acute post-rejection, either after organ transplant or after cell therapy. The major histocompatibility complex (MHC), class I and II, are between the main molecules involved in the immune response. By IHC, we have demonstrated that class I MHC (HLA-ABC) is widely expressed at the umbilical epithelium level as well as

WJ cells, Figure 4.1, (A) in the cord tissue. In contrast, no positivity has been found for the HLA-DR, typical MHC molecule type II, Figure 4.1 (C).

Attention has been recently given to non-classical MHC class I complexes, effectors of the immunologic tolerance, modulating lymphocyte proliferation and NK cells. Previously, our lab has underlined the HLA-G expression in the WJ-MSCs (La Rocca et al., 2009). Our interest has then been focused on the HLA-E molecule. For the first time we have shown HLA-E expression at the protein level, Figure 4.1 (E) in both umbilical epithelium and WJ cells. Immunocytochemistry confirmed these data, with WJ-MScs in culture being positive for HLA-ABC, negative for HLA-DR, and, as demonstrated for the first time here, positive for HLA-E at the protein level, Figure 4.1 (B, D, F).

Flow cytometry confirmed the data of ICC experiments, as visible figure 4.2: WJ-MSCs were positive for class I MHC (HLA-ABC), negative for the expression of class II MHC (HLA-DR) and positive for the expression of non-classical class I MHC (HLA-E).

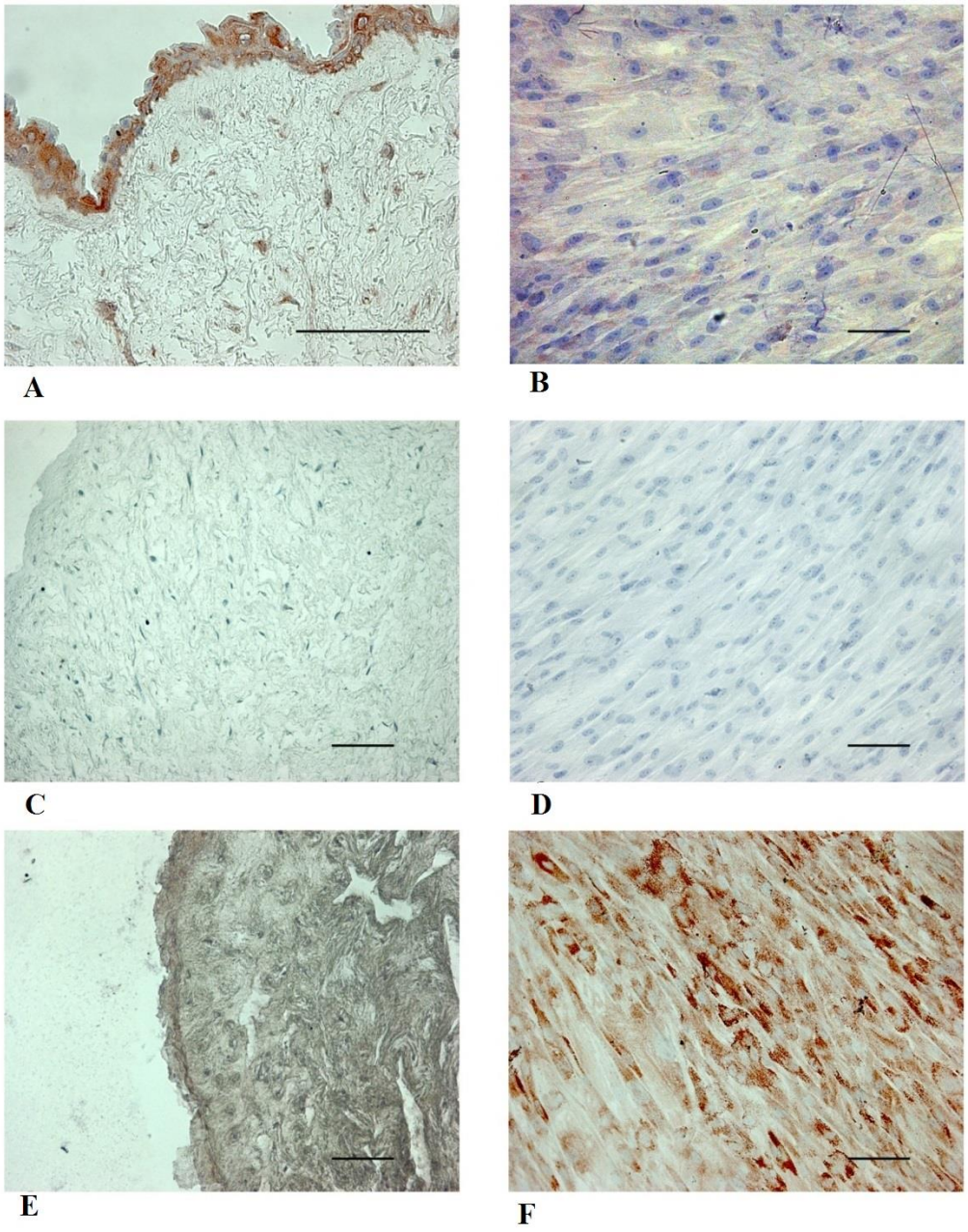

Figure 4.1: Representative panels of immunohistochemical/immunocytochemical detection of immune-related molecules in umbilical cord specimens and paired cultured cells. Both UC tissue and WJCs were positive for HLA-ABC (A, B) and HLA-E (E, F), while lacking detectable expression of HLA-DR (C, D). Magnification: 40x (A); 20x (B-F).

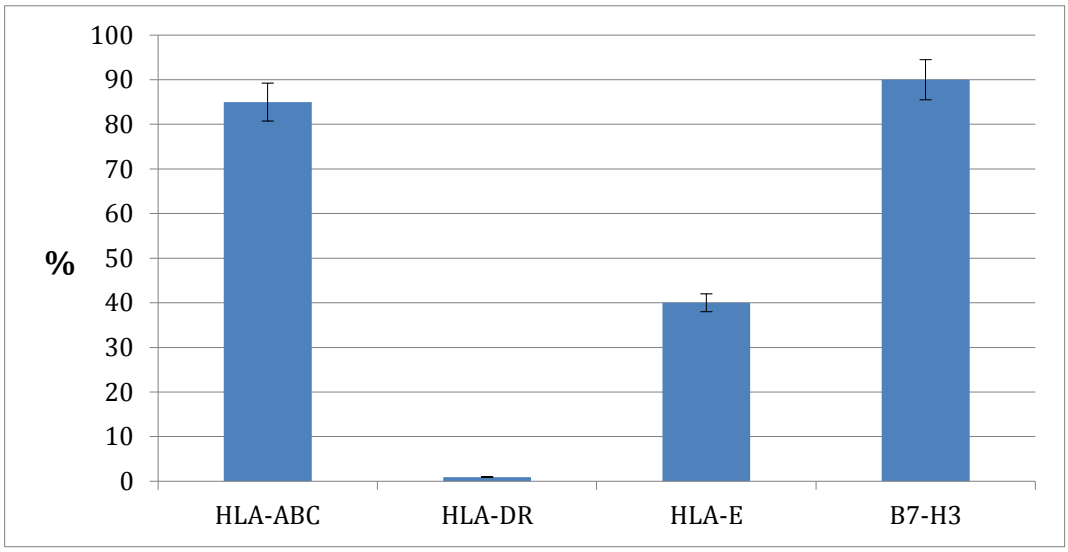

Figure 4.2: Flow cytometry analysis of the levels of expression of immune-related and immunomodulatory molecules in WJ-MSCs. As expected and confirming the results of ICC analyses, cells were positive for class I HLAs (HLA-ABC), negative for class II (HLA-DR), and showed the expression of both HLA-E and CD276 (B7-H3).

B7 co-stimulators are a growing family of molecules, which are implicated in the regulation of the immune response by interacting with specific receptors expressed by lymphocytes. The classical B7 co-stimulators are B7-1 (CD-80) and B7-2 (CD-86). As show in figure 4.3, the two co-stimulatory molecules are undetectable in the cord tissues. *In vitro* data (not shown) confirmed the lack of these molecules also in cultured WJ-MSCs.

Further members of the B7 families have been discovered in recent years, and for some of these, an inhibitory role has been proposed. B7-H3 is one of these molecules, whose expression has been demonstrated in WJ-MSCs, at the RNA level unlike B7-H1 and B7-H4. WJ-MSCs were positive for B7-H3 in cord tissue, Figure 4.4 (A) as well as in the cultured cells, Figure 4.4 (B). We evaluated additional immune-modulatory molecules such as indoleamine 2,3 dioxigenase (IDO), an enzyme implicated in the tryptophan amino acid metabolism, that is necessary for the lymphocyte proliferation. IDO was positively expressed in both umbilical cord and cultured cells, Figure 4.5 (A - B). In addition, the early pregnancy factor (EPF) was also detected in the cord tissue and cultured cells, Figure 4.5 (C - D). EPF belongs to the chaperonin family, and is required for the viability and survival of embryo, due to its immunosuppression and growth-promoting features. This is the first report describing EPF expression in WJ-MSCs and cord tissue.

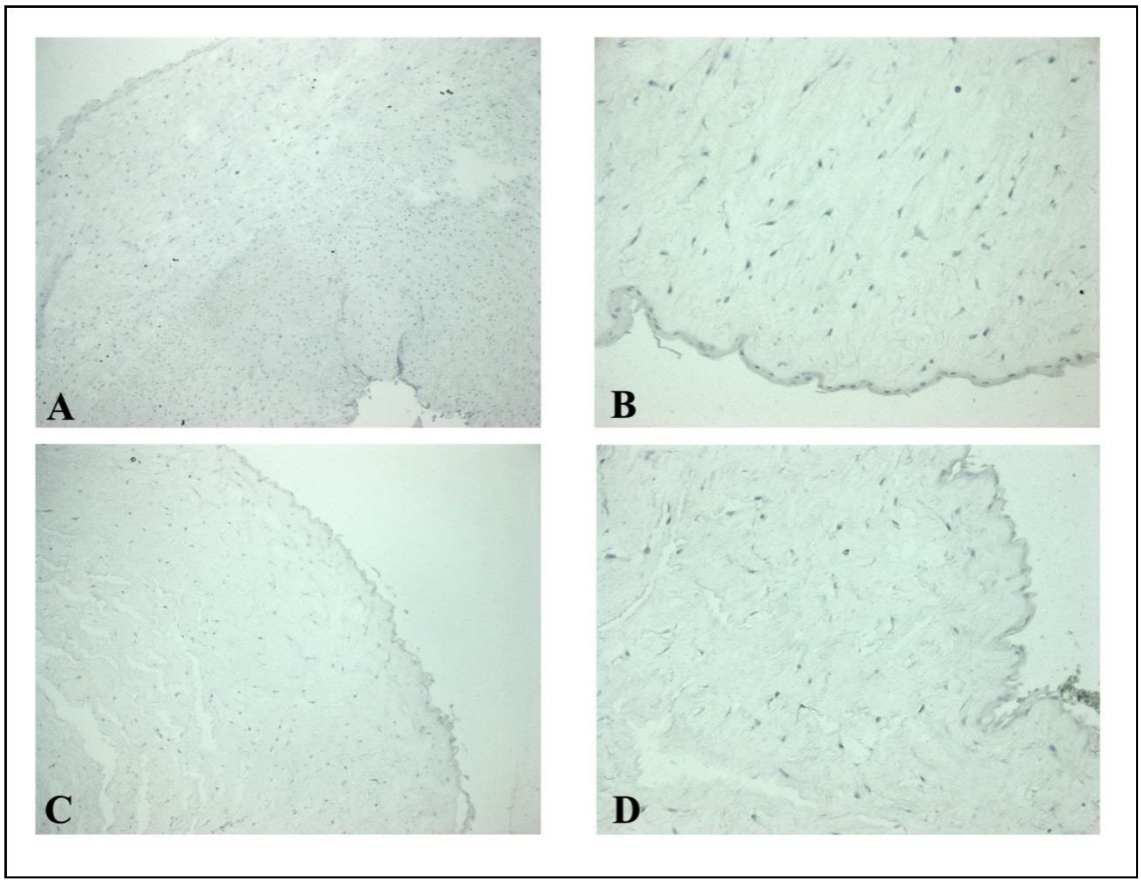

Figure 4.3: Representative panels of immunohistochemical detection of immune-related molecules in umbilical cord specimens immunohistochemistry for B7-1 (A and B), B7-2 (C and D). Magnification: (A and C 10x), (B and D 20x).

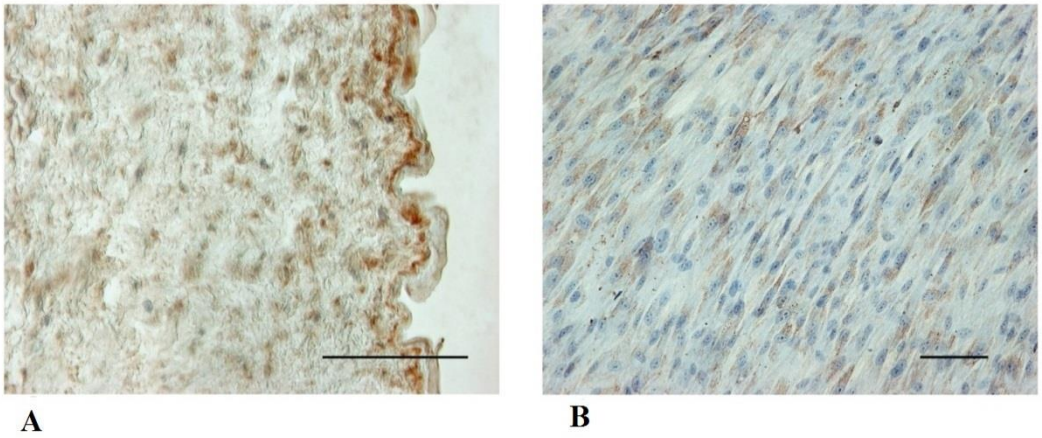

Figure 4.4: Representative panel of immunohistochemical/immunocytochemical detection of B7-H3 in umbilical cord specimens and paired cultured cells. Both UC tissue (A) and WJ cells (B) were positive for B7-H3. Magnification 40x and 20x

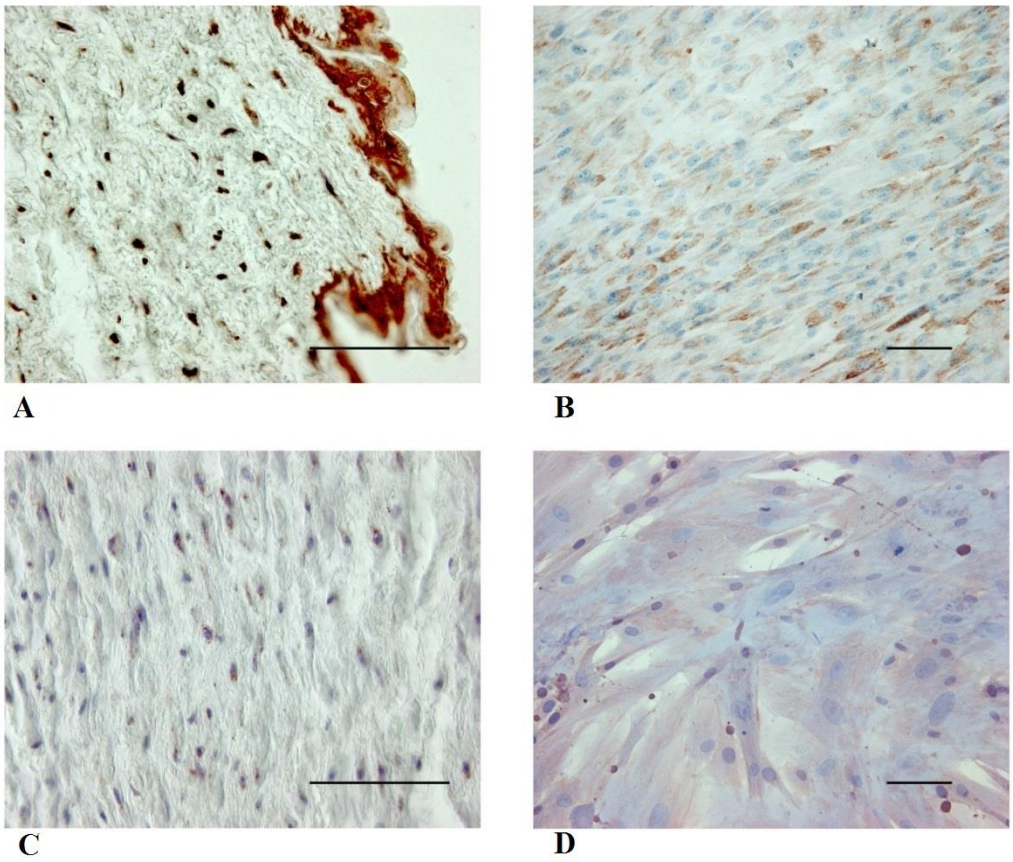

Figure 4.5: Representative panels of immunohistochemical/immunocytochemical detection of immune-related molecules in umbilical cord specimens and paired cultured cells. Both UC tissue (A, C) and WJ cells (B, D) were positive for IDO (A, B) and EPF (C, D). Magnification 20x and 10x.

Immuno-modulatory molecules expression of Wharton's jelly Mesenchymal stem cells (WJ-MSCs) by western blot analyses

We analyzed the previous markers by western blot analyses, in order to confirm the previous protein expressions. Total proteins extracted from different WJ-MSCs at 3rd, 6th, and 11th culture passages were loaded on polyacrylamide gels under non-reducing conditions, in order to preserve supra-molecular complexes, which are not influenced by SDS presence. Figure 4.6 (A) shows that HLA-E is markedly present in all the passages considered, therefore confirming the results obtained with previous experiments. Interestingly, in addition to the canonical 40 kDa band which refers to the expected MW of the un-glycosylated protein chain, a 50 kDa band (due to the glycosylation of the molecule) and a higher molecular weight complex (around 100 kDa) were also detectable. Figure 4.6 (B) shows that B7-H3 is represented by a smaller band around 55 kDa, which may refer to the unglycosylated form, and a main band around 100 kDa, which refers to the mature, glycosylated form found on cell membrane.

Another molecule, which has been associated to the immuno-modulatory phenotype of MSCs is Galectin-1. WB analyses, Figure 4.6 (C) showed that the molecule is expressed in its mature form (15 kDa band) in all the passages analyzed. At earlier passages a higher MW band (about 30 kDa) is also observable.

LIF (Leukemia inhibitory factor) has been also linked to the immunosuppressive properties of MSCs. WB analyses (figure 19D) has shown that the 22 kDa precursor band is expressed in all the cell passages, while at passage 3 a 40 kDa band (the mature glycosylated form) and a higher molecular weight band (around 160kDa) can be observed.

Overall these data confirm the expression of crucial immuno-modulatory molecules in WJ-MSCs and open new scenarios on the definition of the molecular partners in higher molecular weight complexes, even considering that the receptor for B7-H3 is still unknown.

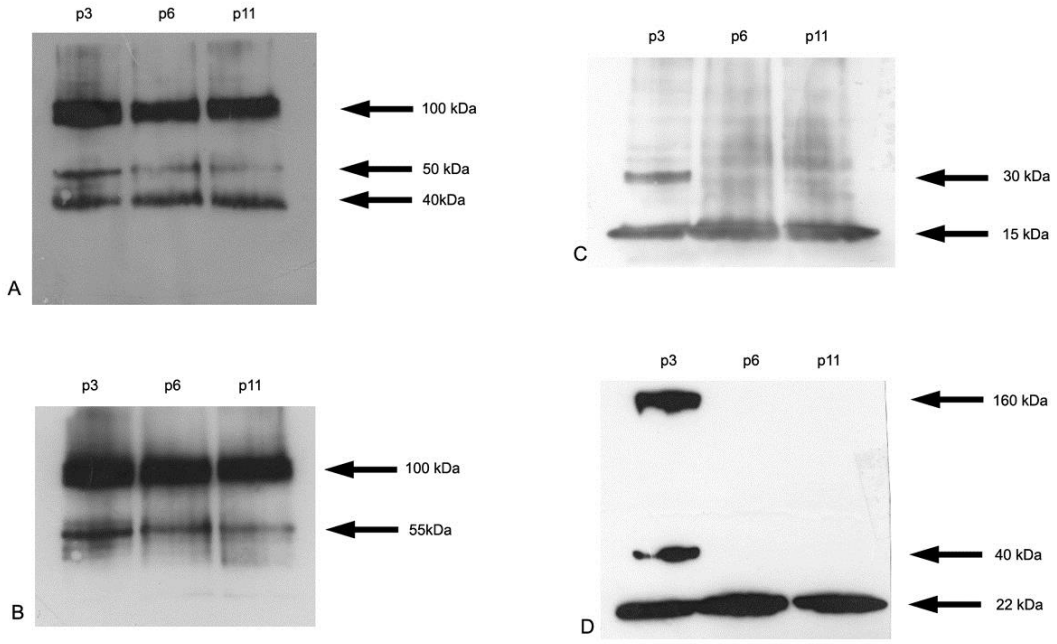

Figure 4.6: Panel of Western Blotting analyses for the expression of immunomodulatory molecules in WJ-MSCs. A: HLA-E; B: B7-H3; C: Galectin-1; D: LIF.

DISCUSSION

In this study we wanted to characterize the expression of immune modulatory/anti-inflammatory molecules both in cultured cells and in the umbilical cord tissue. Surprisingly, molecules which are thought to be expressed at the feto-maternal interface, such as HLA-E and B7-H3, are actually present in UC tissue cells, which uncovers possibly new roles for these cells in the processes related to pregnancy and fetus development. More importantly, we characterized new molecules expressed by the isolated cells, with relevance to our islet co-transplant *in vivo* study, which may justify part of the properties these cells have *in vivo* when transplanted in immune competent hosts. Of particular importance are the data on B7-H3, which is a molecule associated physiologically to the inhibition of the T cell response, but which receptor is still unknown.

Another objective of this project was to analyze the expression of other molecules, which may warrant a better engraftment of the islets in host tissues, when expressed by a co-transplanted cell type. Interesting data came from the Cx-43 immuno-localization, since this molecule, expressed at high levels by WJ-MSCs, is also known to be expressed by cell types residing at the periphery of the islet (for example, but not limited to, alpha cells). Therefore, the possibility to physically establish relations with the islet populations via the formation of gap junctions, constitutes an intriguing possibility which may be explored in further experiments stemming from this research.

CHAPTER 5

WJ-MSCs IN PANCREATIC ISLETS TRANSPLANTATION

ABSTRACT

Type I diabetes (TID), also known as insulin-dependent diabetes mellitus (IDDM), is an immune disease characterized by T-lymphocytes-mediated pancreatic beta cells destruction. TID is usually diagnosed in children and young adults and presents itself with a rapid and aggressive onset. Currently the causes leading to the immune system activation targeting the beta cells are not fully understood. Different hypothesis, including genetic and viral, have been postulated and are under investigation. Patients affected by TID require daily insulin injections and are at risk of short- and long-term complications associated to the disease, including blindness, renal and heart failure. While insulin is instrumental for diabetic patient survival, pancreas and pancreatic islet transplantation are two potential treatments for the disease. Pancreatic islet transplantation consists in transplanting only the insulin–producing cells and this is accomplished through a minimally invasive procedure. Although this approach is known to be feasible and to treat diabetes, more research is needed to improve the outcome so that it can be offered to a larger population of diabetics. In this study we have investigated the effect of Wharton's jelly derived mesenchymal stem cells (WJ-MSCs) as immune-modulatory and anti-inflammatory when co-transplanted with pancreatic islets in animals. The overall aim of the study was to demonstrate that by mediating inflammation and immunity islet engraftment and functionality could be improved. Based on our pilot experiment, improvement could be observed.

INTRODUCTION

The pancreas is an abdominal, lobulated gland with distinct exocrine and endocrine components. The adult pancreas is a transversely oriented retroperitoneal organ extending from the "C" loop of the duodenum to the hilum of the spleen (Figure 5.1). On average, the pancreas measures 20 centimeters (cm) in length and weighs 90 grams (g) in men and 85 g in women (Solcia et al., 1997). Although the pancreas does not have well-defined anatomic subdivisions, the adjacent vasculature can be used to separate the pancreas into three parts: the head, neck, body, and tail.

The pancreatic duct system is variable. The main pancreatic duct, also known as the duct of Wirsung, drains mainly into the duodenum at the papilla of Vater. Whereas, the accessory pancreatic duct, also known as the duct of Santorini, drains into the duodenum through a separate minor papilla (Figure 5.2). In adults, the main pancreatic duct merges with the common bile duct proximal to the papilla of Vater, thus creating the ampulla of Vater, a channel for biliary and pancreatic drainage. Due to developmental variability, however, this ductal architecture can differ between individuals (Kumar et al., 2004).

The exocrine portion of the pancreas, which constitutes 80-85% of the pancreas in its entirety, is made of acinar cells. Acinar cells surround the series of ducts that convey the digestive enzymes they produce to the duodenum. Acinar cells are pyramidally-shaped epithelial cells that are radially oriented around a central lumen. The basal portion of acinar cells is basophilic and contains abundant endoplasmic reticulum. Additionally, acinar cells contain a well-developed supra-nuclear Golgi complex that is part of an apically oriented secretory pathway that forms membrane-bound zymogen granules, which contain the digestive enzymes. Every day, the pancreas secretes 2 to 2.5 liters of bicarbonate-rich fluid containing digestive enzymes and pro-enzymes.

The endocrine portion of the pancreas is composed of about 1 million highly vascularized cell clusters, known as the islets of Langerhans. These islet cell

clusters are comprised of multiple, distinct cell types: α-cells, β-cells, δ-cells, ε-cells, and γ or PP-cells. Each cell type secretes distinct hormones: α-cells-glucagon, β-cells-insulin, δ-cells-somatostatin, ε-cells-ghrelin, and γ or PP-cells-pancreatic polypeptide (Jennings et al., 2015). These cell types have a precise organization. For example, β-cells are mainly located at the center of the islet, with all the other cell types comprising the perimeter of the islet (Figure 5.3). The β-cell's role is the secretion of insulin in response to increased nutrient availability in blood circulation. For example, glucose has usually a physiological concentration of 5 mM, but in response to higher blood levels of glucose, fatty acids, and amino acids at a concentration of 7-10 mM, it will stimulate a response from the β-cells (Zulewski et al., 2001). Furthermore, insulin has many other functions: 1) promotes the synthesis of fatty acids and triglycerides in the liver and adipose tissue, 2) stimulates the synthesis of glycogen in the liver and skeletal muscle, 3) allows amino acid permeability, 4) promotes protein synthesis in most tissues, 5) inhibits the depletion of energy reserves in all tissues, and 6) suppresses hepatic gluconeogenesis.

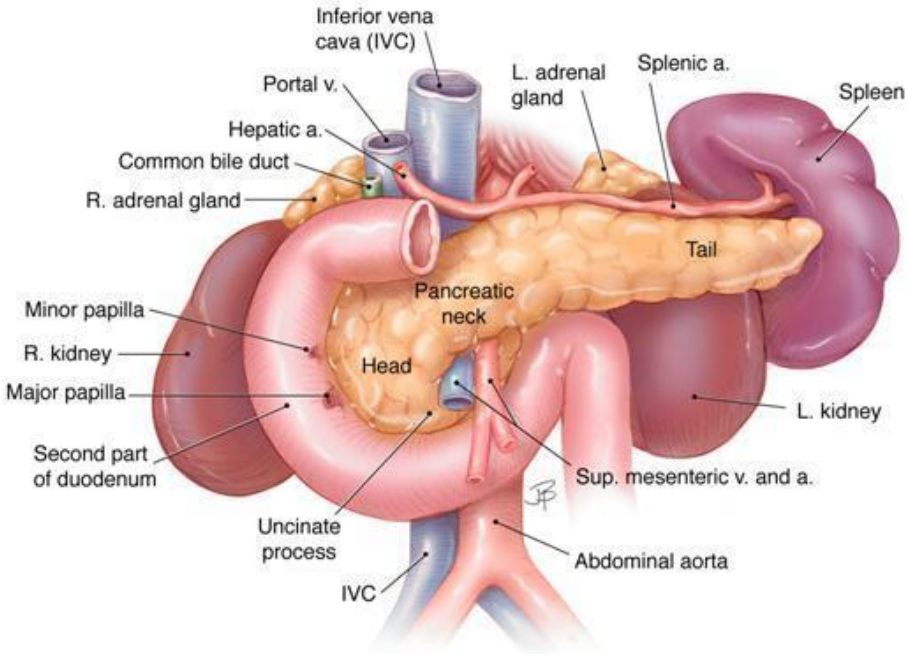

Figure 5.1. Pancreas anatomy (http://imgarcade.com/1/pancreas-anatomy-uncinate)

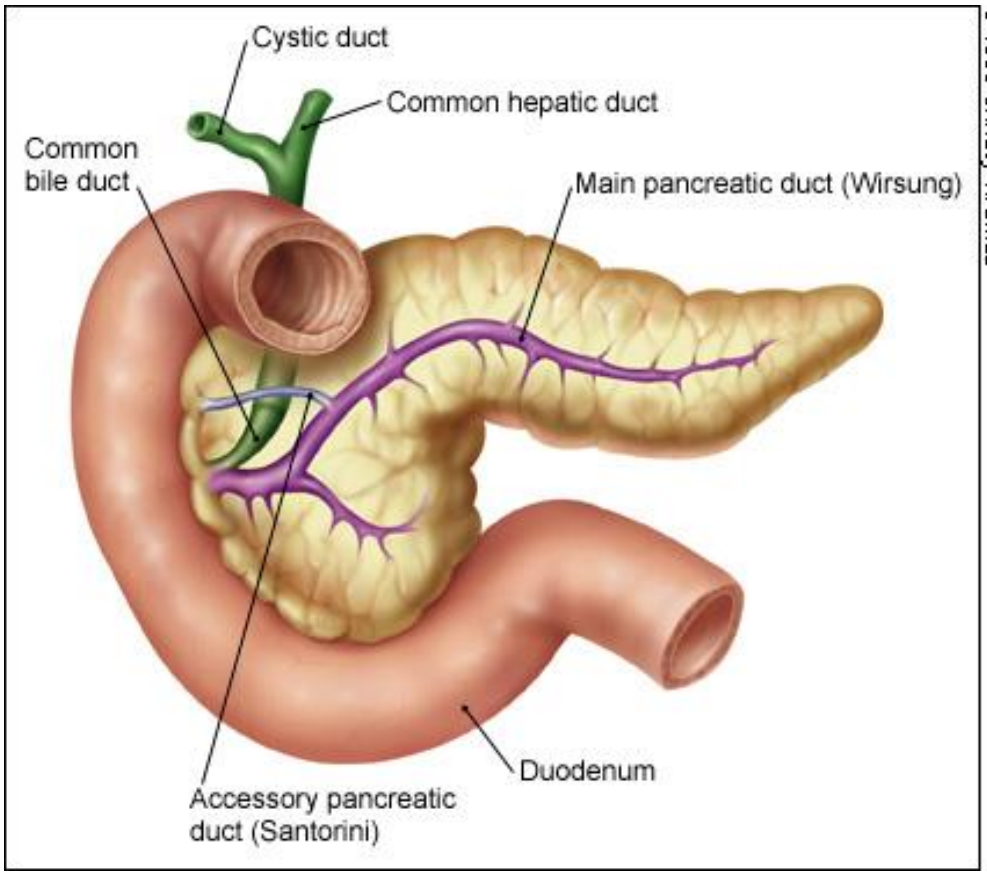

Figure 5.2. Pancreatic ductal anatomy (Krames C, 1999)

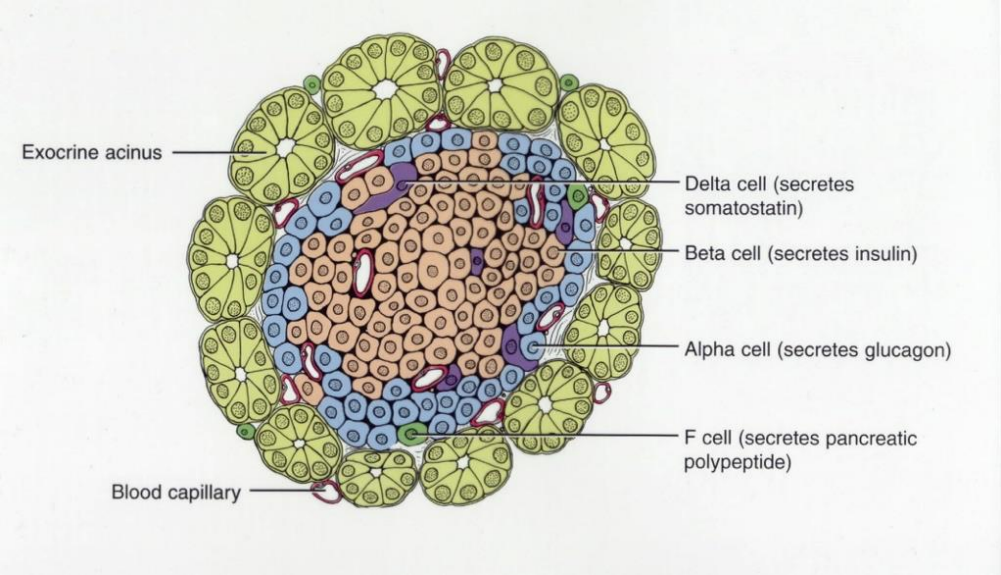

Figure 5.3. Islet of Langerhans (www.cram.com/flashcards/block-4-histology-3553915)

Pancreas development and involved factors

The pancreas develops from two distinct buds, dorsal and ventral, that arise from either side of the distal foregut endoderm. During early embryonic development, the ventral pancreatic primordia rotates and fuses with the dorsal at approximately the seventh week of gestation to form the single gland-pancreas (Oertel, 1989). The majority of the pancreas, (body, tail and superior and inferior head included), is derived from the dorsal primordium. Pancreas development is a complex process that involves differentiation of specialized cells, regionalization and morphogenesis (Avolio et al., 2013; Shin et al., 2013). In particular, pancreas organogenesis requires various sequential connections with the close mesodermal tissue as well as the inclusion of various important and stage-specific factors such as: Retinoic Acid (RA), Bone Morphogenetic Protein (BMP), Notch, and Fibroblast Growth Factor (FGF) (Bayha et al., 2009).

In 2011, O'Rahilly and Müller classified human embryonic development into 23 different Carnegie Stages (CS). The remaining development is divided into individual stages by morphology. Human embryogenesis is staged by maturity and only by time extension, as days post-conception. Human embryogenesis differs from other model species such as mouse, for the embryogenesis classifications, in term of time measured, Table III (Jennings et al., 2015).

At CS 12-13 in humans, many important transcription factors have emerged, such as Sox-9, Hnf1-β, GATA-4 GATA-6, figure 5.3 (Seymour, 2014) and the duodenal homeobox factor 1 (PDX1), which is detected at the pancreatic endoderm level, Figure 5.3. (Pan and Wright, 2011; Haumaitre et al., 2005). Moreover, there has been no expression of endocrine factors in human embryos at this time point, referred to as "primary transition". Primary transition represents the early phase of pancreas development, and all of these aforementioned factors are necessary for human pancreatic growth.

The "secondary transition" period starts from CS 15 to CS 19, and it is characterized by epithelial cell diffusion and acinar differentiation. Additionally, the pro-endocrine

transcription factor, Neurogenin3 (pro-Neurog3), begins to be expressed along the primitive pancreatic ducts (Johansoon et al., 2007; Gu et al., 2002) and will determine the specific endocrine lineage. In particular, early Neurog3^{+} (CS 20-21) will specify the human α-cells of the islets of Langerhans, while the late form of Neurog3+ will give rise to β-, δ- and PP-cells (Johansson et al., 2007**)**. Neurog3 achieves high expression values by the end of the first trimester (fetal period), but then begins decreasing in the following semester (Pinney et al., 1965; Salisbury et al., 2014). At 10 wpc (weeks post-conception), β-cells of the islets of Langerhans become vascularized and islet clusters are observable (Jennings et al., 2013).

Table 1. Stages of human embryonic development, key features and estimation of equivalent timeline of mouse development

Human embryonic stage	Approximate days post-conception (dpc)	Examples of morphological features	Key events in human embryonic pancreas development	Approximate equivalent stage of mouse development*
CS9	22-26	Up to 3 somite pairs, largely unfolded endoderm	Prior to formation of AIP	E7.5-E8
CS10	25-27	4-13 somite pairs, rostral and caudal neuropore	Folded endoderm with narrowed communication with yolk sac creating the AIP, notochord adjacent to foregut endoderm	E8-E8.5
CS11	27-29	13-20 somite pairs, rostral neuropore closing, looping of the heart tube		E8.5-E9
CS12	29-31	Lens and otic placodes, caudal neuropore closing, 1st-3rd pharyngeal arches	First detection of PDX1 in presumptive pancreatic endoderm	E9-E9.5
CS13	30-33	Early sign of upper limb bud	Clear dorsal and ventral pancreatic buds	E9.5-E10
CS14	33-35	Upper and lower limb buds clearly visible		E10-E11.5
CS15	35-37	Hand plate now visible		E11.5-E12.25
CS16	37-40	Clear retinal pigment, auricular hillocks, foot plate visible	Growth of organ and proliferation of multipotent pancreatic progenitors	E12.25-E12.75
CS17	39-42	Digital rays first visible in hand plate		E12.75-E13.25
CS18	42-45	Digital rays first visible in foot plate		E13.25-E14
CS19	45-47	Clearly notched hand plate	Distinction possible between central trunk cells and peripheral tip cells, e.g. GATA4 levels	E14-E14.5
CS20	47-50	Clearly notched foot plate, webbed fingers, scalp vascular plexus visible		E14.5-E15
CS21	49-52	Visible fingers, webbed toes, scalp vascular plexus halfway up head	Onset of detection of *NEUROG3* and first detection of insulin-positive cell (i.e. signs of endocrine commitment)	E15-E15.5
CS22	52-55	Scalp plexus two-thirds of the way up head, separated fingers	Ventral bud largely rotated around the gut and becomes opposed with the dorsal bud	E15.5-E16
CS23	53-58	Scalp vascular plexus at top of head, separated toes		E16-E16.5

Carnegie stages (CS) are shown with their estimates of corresponding days post-conception (dpc) adapted from O'Rahilly and Muller (2011) and the UNSW Human Embryo Resource (https://embryology.med.unsw.edu.au/embryology/index.php/Embryonic_Development). Human embryogenesis spans the first 8 weeks of development prior to the fetal period. Counting somite pairs by light microscopy becomes more difficult after CS11. AIP, anterior intestinal portal.
*The estimation of mouse development relates broadly to the Carnegie stage, not the key event in human pancreas development.

Table 5: Human embryonic development stages (Jennings RE et al, 2015)

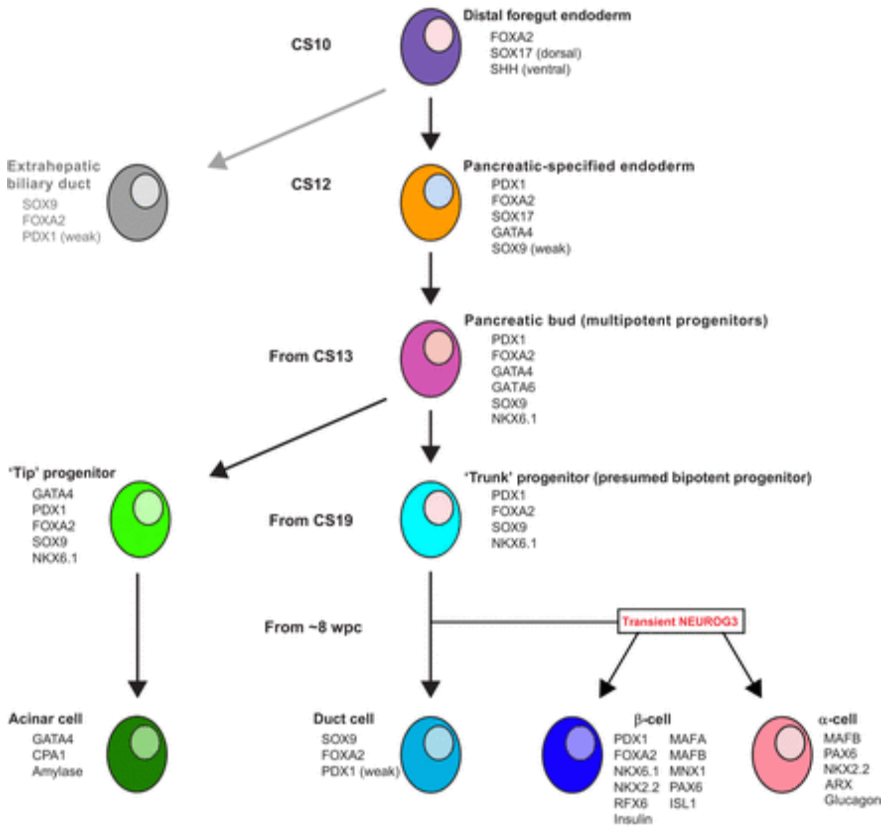

Figure 5.4. Transcription factor network of human pancreas development (Jennings RE et al, 2015)

Diabetes disease and therapeutic applications

Diabetes is a common disease. Approximately 29.1 million people or 9.3% of the U.S. population have diabetes (CDC, National Diabetes Statistics report, 2014). In the year 2000, 171 million adult cases were reported worldwide and this number is expected to reach 334 million patients globally (Vija et al., 2009). It is associated with high rates of morbidity and mortality. Patients suffer with long-term complications, such as cardiovascular disorders (myocardial infarction and coronary artery disease), kidney disease, blindness, and damage to the nervous tissue. The disease originates from a selective destruction of pancreatic β cells, which leads to a persistent hyperglycemia (Zulewski et al., 2001). Diabetes is a polygenic disease with genetic pre-conditions. Environmental factors, such as population density and climate, are also shown to contribute to the development of the disease. There are two types of diabetes: 1) Type I diabetes (TID) or insulin-dependent diabetes mellitus (IDDM), which is characterized by the destruction of insulin producing β cells, and 2) Type II diabetes, a metabolic disorder characterized by hyperglycemia due to insulin resistance.

IDDM represents 10% of cases worldwide, and its incidence is 41/100,000 people/year in Europe, and 25/100,000 people/year in North America. IDDM usually occurs in children and young adults and is characterized by pancreatic β cell failure, necessitating life-long parenteral insulin replacement (Vija et al., 2009). With 15,600 children and youth newly diagnosed annually (American Diabetes Association data, 2014), IDDM is the most commonly seen pediatric endocrine disorder and these values are still increasing. Currently, causes leading to the immune system activation are not fully understood. The pathogenesis of the disease is defined by T cell infiltration and chronic inflammation in the islets of Langerhans, with consequent β cell destruction and insulin insufficiency. The three major antigens, which interact with the immune system, are:

GAD65 - protein expressed mainly in neuroendocrine cells. 60-80% of type I diabetics have turned to GAD65 autoantibodies.

ICA512 - protein-tyrosine phosphatase trans-membrane family. It has a role in insulin secretion. 60-70% of people with diabetes have autoantibodies to ICA512.

Insulin - the antibodies are directed to the β chain and are greater in young individuals.

Insulin-dependent diabetes mellitus diagnosis can be done by searching for these autoantibodies that appear months or years before the onset of disease. However, in certain cases, it has been proved that the B-lymphocytes presence is not necessary for the disease development. The selective islet destruction can be caused by both direct and indirect mechanisms. Indirect mechanisms may comprise: CD4+ binding the MHC-II on the dendritic cells, recognition of auto-antigens, and production of pro-inflammatory cytokines, such as IL-1, IL-6, IL-10, TNF-α and IFN-γ that can recognize and attack β cells. A direct mechanism involves CD8+ T-lymphocyte cells that bind the MHC-I of β cells by activating apoptotic pathways. It has been demonstrated that animal models have limitations in the ability to reproduce the same disease event sequence.

Type II diabetes is the most common diabetic condition (90-95%) typically occurring in adults. Type II diabetes is characterized by hyperglycemia due to insulin resistance. Insulin resistance is linked to several factors: obesity, age, sedentary life style, genetic predisposition. In addition, it can be caused by altered insulin signaling, insulin receptor mutations, or glucose transporters. There is a possible relationship between the excess visceral adipose tissue and insulin resistance status. In fact, this tissue releases certain pro-inflammatory cytokines, such as TNF, that blocks the insulin receptor, and is implicated in the insulin pathogenesis resistance (Orki, 1998; Wang et al., 1997; Blomberg et al., 1998).

Therapeutic applications for IDDM

Patients with IDDM require daily insulin injections. Unfortunately, this does not prevent long-term complication, and it can generate serious side effects (severe hypoglycemic episodes). Currently, human insulin is biosynthetically produced by recombinant DNA technology.

Different insulin types, related to the time action, are available to consumers: 1) fast acting (starts to act half an hour after subcutaneous administration with a peak after 3 hours and then rapidly declines), 2) intermediate-acting (delayed with protamine or zinc) starts to work after about 2 hours and reaches its maximum effect after 4-6 hours after subcutaneous injection and then decreases; 3) prolonged action (large crystals of insulin - zinc) whose effect is maintained for about 24 hours.

Transplantation of the whole pancreas or the pancreatic islets is an alternative option for the treatment of diabetes. Despite approximately 80% of patients achieve insulin independence in their first year following-surgery, the whole organ transplantation is associated with a high mortality rate. Complications include: hemorrhage, thrombosis, enteric lick, rejection and infection. In 1966, the first whole pancreas transplant was performed on an old woman by William Kelly and Richard Lillehei (Busnardo et al., 1983), but the insulin action of the new pancreas, persisted for 6 days in the patient. The following pancreas transplantations had so many complications, such as high mortality rate and absence of long-term outcomes.

Because of this, researchers' and clinicians' interests were focused on the transplantation of the endocrine portion of the pancreas. The first islet transplant was performed in the 70's. In 1999, James Shapiro performed pancreatic islet transplantation in seven patients with type I diabetes using an innovative immunosuppressive regimen without glucocorticoids. A year after the transplant, patients were insulin-independent (Shapiro et al., 2000). Islet transplantation has advantages over the whole pancreas. Islet transplantation is a less invasive technique and requires local anesthesia.

The limitations of islet transplantation

The pancreatic islet transplant procedure presents various limitations. Limited donors availability as compared to patients waiting for a transplant is a phenomenon affecting all organs. Every year, approximately 3,000 pancreata are available for transplant in the US, but approximately 35,000 patients are affected by IDDM.

Furthermore, the islet isolation and purification methods are currently unsatisfactory and cause a high loss of endocrine tissue prior to transplantation. The islet infusion causes an increase of the portal pressure proportional to the islet mass infused. All these factors limit the total islet amount that can be implanted. Islet transplantation in the liver is associated with inflammation, instant blood-mediated thrombosis, and ischemic liver tissue associated with increased liver enzymes. During the islet engraftment stage, approximately 50-75% of the islets are lost; moreover the immune-suppressive requirement leads to a heightened risk of general infection and a toxic effect on pancreatic β-cells (Teague et al., 2007).

In addition to immune reactions, there are other factors contributing to the islet loss during the early post-transplant recovery stage such as a hypoxic state in the hepatic portal vein, as well as the balance between pro-apoptotic and anti-apoptotic mediators. Another influencing factor is the pancreatic islets size. Small diameter islets are between 50 and 150 μm, while large diameter islets are between 150 and 300 μm. During islet isolation ex vivo, the capillary structure degenerates. Immediately after the transplant, islets can receive oxygen and nutrients only through the diffusion process since revascularization starts after 7-10 days. Both *in vitro* and *in vivo* studies demonstrate an increased islet survival of the small sized islets compared to larger ones. Islet revascularization is a complex process that includes vessel wall digestion, protease action, migration, proliferation and differentiation of endothelial cells (ECs). After the vessel reconstitution, ECs produce platelet-derived growth factor (PDGF), which recruit the support cells, including mesenchymal stem cells (MSCs). MSCs contribute to the ECs migration by producing proteases, possibly up-regulating angiopoietin, VEGF synthesis by ECs, and providing immune-modulatory activity.

In 2008, Johansson and co-workers showed that the combination of islets with MSCs increases the ECs ability of covering the islet surface. In particular, ECs release prolongations at the matrix and islet level, improving the revascularization (Johansson et al., 2008).

METHODS

Methods for the animal model and co-transplantation of islets and WJ-MSCs were described in chapter 2.

RESULTS

Pilot study for the feasibility of co-transplantation of WJ-MSCs and pancreatic islets

As detailed in methods, mice of the three experimental groups were rendered diabetic by STZ injection. After three days, the study mice were transplanted with a marginal mass (200 IEq) of pancreatic islets and $3x10^6$ WJ-MSCs. Mice were then monitored for the subsequent three months for blood glucose levels and body weight. As expected, control mice, which received only 200 IEq, failed to reverse diabetes, and died within three weeks from transplantation. Of the three mice that received WJ-MSCs along with 200 IEq islets, two reversed diabetes before two weeks post-transplantation. Body weight also confirmed that mice receiving the WJ-MSCs co-transplantation were healthier. In fact they gained weight after transplant.

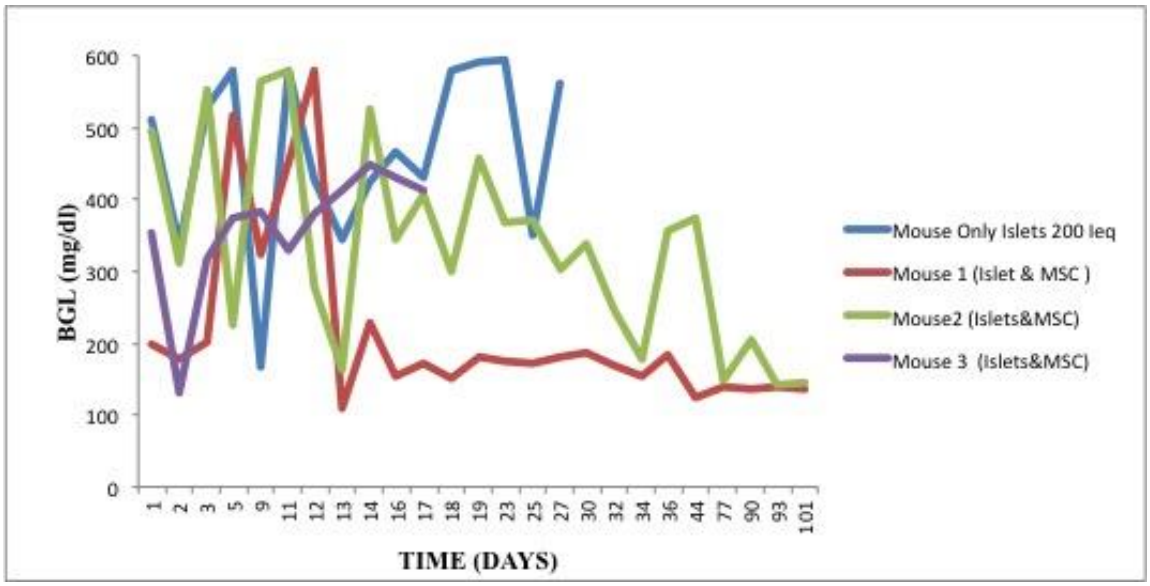

Figure 5.5: Graph depicting the blood glucose levels (BGL) of the animals following co-transplantation of pancreatic islets and WJ-MSCs.

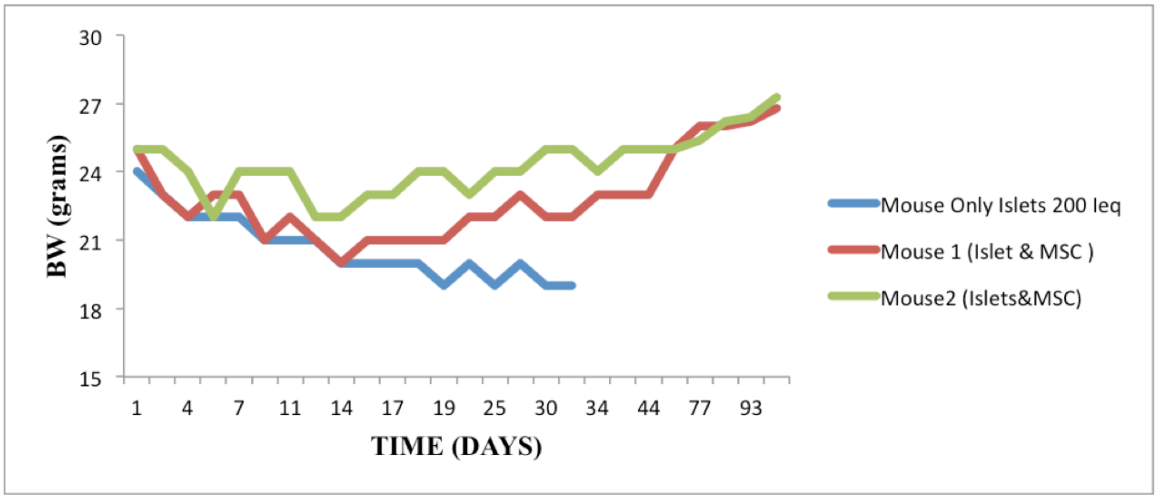

Figure 5.6: Graph reporting body weight.

IPGTT results

To confirm the diabetes reversion of the two mice, which were normoglycemic after three months, we performed an intra-peritoneal glucose tolerance test (IPGTT). Dextrose injection caused a peak in BGL at approximately 30 minutes post injection, followed by a slower decrease for the subsequent 1.5 hours. The day after, BGL was fully normalized. There are contrasting data in literature (Ayala et al., 2010) regarding the interpretation of the BGL after IPGTT, since fasting (which was the case in these animals) prior to the injection may have secondary effects, which may result in misinterpretation of the test results. The complete restoration of normal BGL attained since the day after the test confirms that the animals had functioning insulin producing cells.

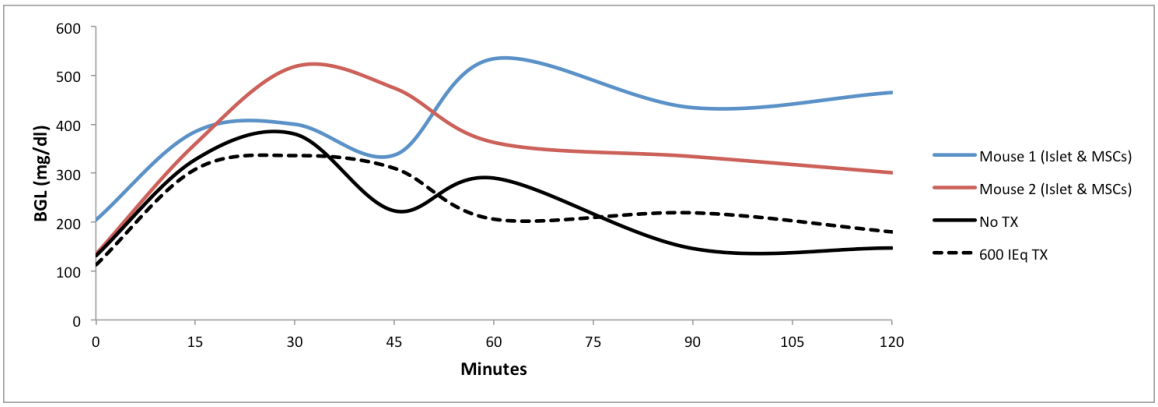

Figure 5.7: Graph depicting the results of IPGT test for the two mice of the experimental group (blue and red curves) performed after three months and compared to controls (no transplant and full mass transplant). The two mice reacted differently to the stimulation, with only one showing the characteristic peak around 30 minutes after injection. Both mice showed normal BGL values the following day.

Islets engraftment under kidney capsule (H&E and Insulin staining)

After four months, animals were sacrificed and the mouse pancreas and kidney were harvested and embedded in paraffin. As shown in figure 5.8, we investigated the presence of functional islets under the kidney capsule: H&E staining (panels A, B) confirmed formally the success of the transplant (in accordance to the observed diabetes reversal), and the islet structure preservation. Furthermore, insulin staining (figure 26 C, D) was tested in immunohistochemistry, and the largest part of the islets consisted of a preserved inner mass of insulin-positive cells, therefore testifying the successful engraftment of islets and their revascularization and resistance to the ischemic stresses suffered during the isolation/transplant procedure. Since normally the marginal mass transplantation fails to reverse we can speculate that the presence of the co-transplanted cells favored islets survival and engraftment and ultimately diabetes reversal.

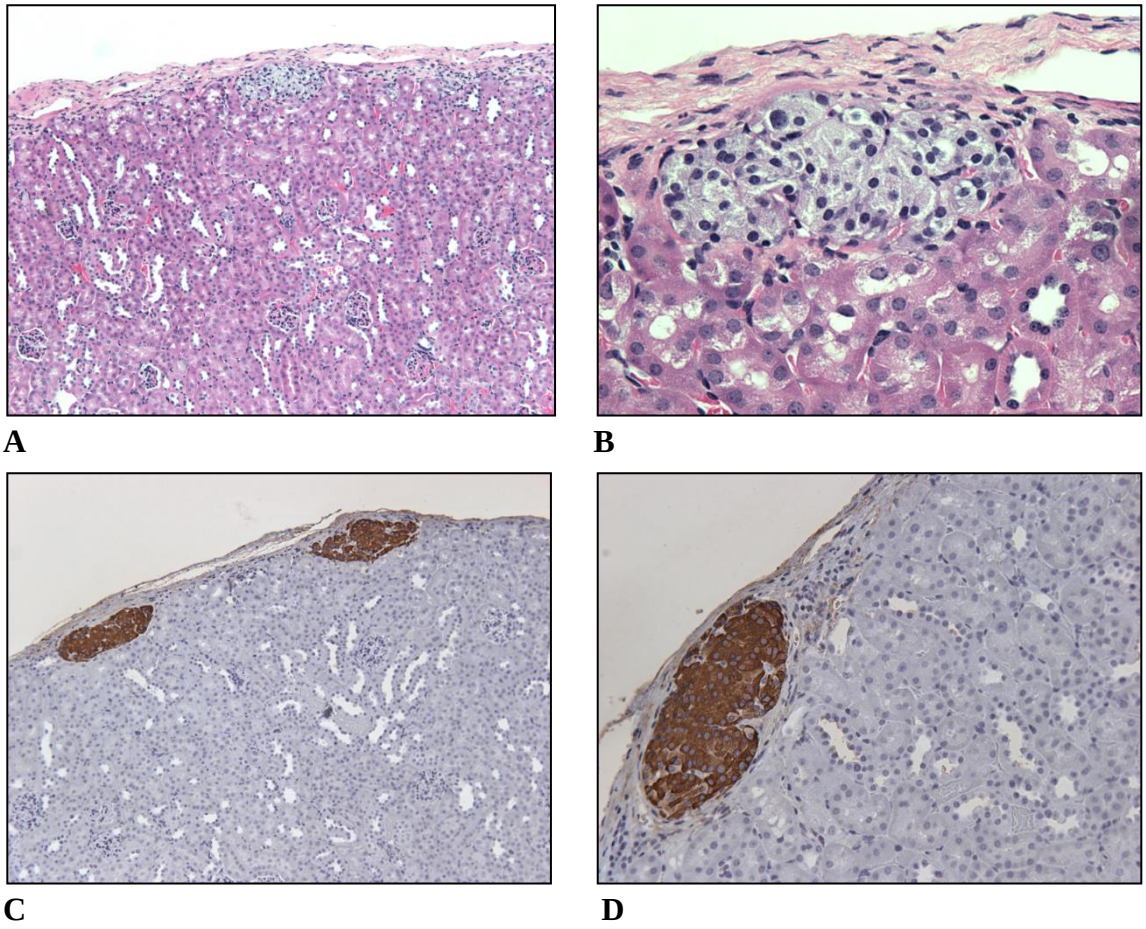

Figure 5.8: Localization of islets transplanted under the kidney capsule of mice receiving co-transplantation of WJ-MSCs. A, B: Hematoxylin/Eosin staining of islets detectable under the capsule of the transplanted kidneys. The islets show standard morphology. C, D: panels of immunohistochemical detection of insulin-positive cells under the kidney capsule: the islets were functional in that most cells belonged to the beta cells mass and were positive to insulin expression. Magnification 10x and 20x.

DISCUSSION

The aim of this part of our project was to investigate, in a pilot experiment, the efficacy of WJ-MSCs in co-transplantation with pancreatic islets in mice rendered diabetic by STZ. We explored the marginal mass (where islets are not sufficient to reverse diabetes). When the marginal mass was co-transplanted with $3x10^6$ un-differentiated WJ-MSCs we observed an adjuvant effect. The islets and cells were transplanted under the kidney capsule of recipient mice and the animals were monitored for the subsequent months. Two out of three mice survived long-term with normal BGL and body weight parameters, therefore providing a initial demonstration of the efficacy of stem cell in warranting the survival of islets. IPGT test showed that also a dextrose challenge, which resulted in a peak of BGL between 30 and 60 minutes from injection, was then metabolized by the animals, which returned to normal levels since the day after. Histological examination of the transplanted kidney showed the presence of functional insulin-positive islets, which conserved a normal morphology, therefore highlighting the role of WJ-MSCs in preserving their functions and favoring their engraftment in an immune-competent host.

Overall, the data is anticipating the potential usefulness of WJ-MSCs when co-transplanted with islets. These cells, may help islet engraftment in several ways, hampering inflammation, silencing immune responses, ameliorating vascularization and providing trophic factors to the islet cells, which may even resemble those processes which take place when the pancreatic cell populations organize themselves with other populations from different lineages and tissues (endothelia, neurons, glia) to form the multi-tissue pancreatic organ.

The researches performed during the Doctoral Course and written in this thesis is confirming WJ-MSCs as a potential source of MSCs. We have confirmed stem markers, potency and anti-inflammatory/immune modulatory properties. The overall profile of these cells and their availability may open new paths in the utilization of MSCs in regenerative medicine. Diabetes is one of the many disease that, as shown, could benefit from the utilization of WJ-MSCs and we hope our work can constitute an initial step toward the application of this therapy in many other diseases.

CHAPTER 6

CONCLUSION

Perinatal stem cells have recently emerged as promising populations, which may be reliably sourced from tissues discarded at birth. These cells, in addition to differentiate in several mature types, have hypo-immunogenicity and immune modulation features that render them promising for transplantation in immuno-competent hosts without concurrent immune suppression. In particular, WJ-MSCs have been highlighted as cells, which retain most of the immuno-modulatory features of the perinatal tissues, which are fundamental for embryo and fetus survival.

The data of this project have led a better knowledge of the WJ-MSC potential (cell characterization) as it applies to immune-modulation and anti-inflammation *in vitro*. Our goal was to define new WJ-MSCs markers like immune-modulatory and anti-inflammatory profile molecules of the cells studied, in order to introduce a new concept in the utilization of MSCs in regenerative medicine where instead of replacing function of cells/tissue, cells could be used as adjuvant in combination with other approaches like transplant.

The initial focus of this research was to isolate WJ-MSCs from umbilical cord, culture them according to the standard methods described in the literature. Then we characterized WJ cells lineage through different analyses (immunocytochemistry, immunohistochemistry, flow cytometry, western blot, RT-PCR) in order to determine the immune-modulatory and/or anti-inflammatory profile. In addition, we tested biomarkers involved in endodermal and pancreatic factors and investigated different neuronal markers (NSE, Nestin, GFAP, NF68) and the glial-derived neurotrophic factor (GDNF) involved in the exocrine and endocrine pancreas development. These experiments have been carried out on naive cells.

In addition, we have developed a TID model *in vivo* as pilot experiment and we have observed the effect of WJ-MSCs. The anti-inflammatory effect of WJ-MSCs in streptozotocin (STZ) induced diabetes in a mouse (anti-inflammatory syngeneic study)

model. STZ evokes an inflammatory effect on beta cells therefore inducing hyperglycemia. Following injection of STZ in control animals, we monitored the animals for blood glucose levels and body weight changes overtime. Blood glucose levels (BGL) have been monitored three times a week and evidence of diabetes (>350 mg/dl) has been observed approximately three days following STZ injection and preserved for approximately three weeks. To confirm the diabetes reversion, we used the Intra-peritoneal glucose tolerance test (IPGTT). Only islets (marginal and full mass), WJ-MSC-naive cells, and WJ-MSCs plus marginal islet mass have been co-transplanted in animals.

In summary, the results presented here have revealed the WJ-MSCs features and overall markers repertoire with the intent to define their mesenchymal nature and unknown markers, concept still unclear in literature. The capability to propagate these cells *in vitro* could make them a reliable source of MSCs for potential clinical applications when a high number of cells are required.

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VITA

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Publications

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Abstracts

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Summary of Dissertation

In recent years, mesenchymal stem cells (MSCs) have caught researchers' attention because of their potential application in regenerative medicine. MSCs are defined as immature cells with the ability to self-renew and differentiate into specialized cells belonging to different lineages. A potential source of MSCs is the umbilical cord and more specifically a substance here present, called Wharton's jelly (WJ). However, more research is needed in order to determine the biological features and specific markers expressed by this MSCs source. WJ-MSCs represent an easily accessible source of stem cells, with good expansion capacity *in vitro*, and mononuclear fibroblastic-like cell morphology. The overall objective of this project was to isolate WJ-MSCs and perform a fine characterization of their properties, with particular attention to the expression of specific markers. We demonstrated that WJ-MSCs possess the standard mesenchymal

features and are positive for the classical MSC markers**,** clusters of differentiation (CD), like CD29, CD44, CD73 and CD90, while they resulted negative for specific hematopoietic markers (CD34 and CD45). Furthermore, WJ cells confirmed their potency and their ability to differentiate according to the osteogenic, adipogenic and chondrogenic phenotypes in an *in vitro* model. In our study we have characterized WJ cells lineage through different analyses (immunocytochemistry, immunohistochemistry, flow cytometry, western blot, RT-PCR), determining their immune-modulatory and anti-inflammatory profile. Furthermore, we tested different neuronal markers (NSE, Nestin, GFAP, NF68) and trophic factor GDNF in order to further define the profile of WJ-MSCs. WJ naive cells were co-transplanted in an *in vivo* model of pancreatic islets transplantation. The specific aim of this part of the project was to investigate the role of WJ-MSCs as adjuvant in the engraftment of pancreatic islets in an animal model potentially through anti-inflammatory and/or immune-modulatory features. Based on the results of this research we provided a better understanding of the overall characteristics of WJ-MSCs introducing new opportunities for their utilization in regenerative medicine.

This dissertation was typed by TIZIANA CORSELLO.