Dedication

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

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HUMAN WHARTON'S JELLY DERIVED MESENCHYMAL STEM CELLS IN PANCREATIC ISLET TRANSPLANTATION

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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 –depedent diabetes mellitus
IDO	Indoleaminedeoxygenase
IEq	Islet Equivalent
IPGTT	Intra-peritonealglucose tolerance test
ITS	Insulin-transferrin-sodiumselenite
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
Von Willebrand factor	vWF
WJ	Wharton's jelly
α-SMA	Alpha- smooth muscle actin

INTRODUCTION

1.1 Pancreas anatomical and general features

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 1). On average, the pancreas measures 20 centimeters (cm) in length and weighs 90 grams (g) in men and 85 g in women [1]. 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 (Fig. 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 [2].

The exocrine portion of the pancreas, which constitutes 80-85% of the pancreas in entirety, is made up 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 [3]. 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 (Fig 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 [4]. 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.

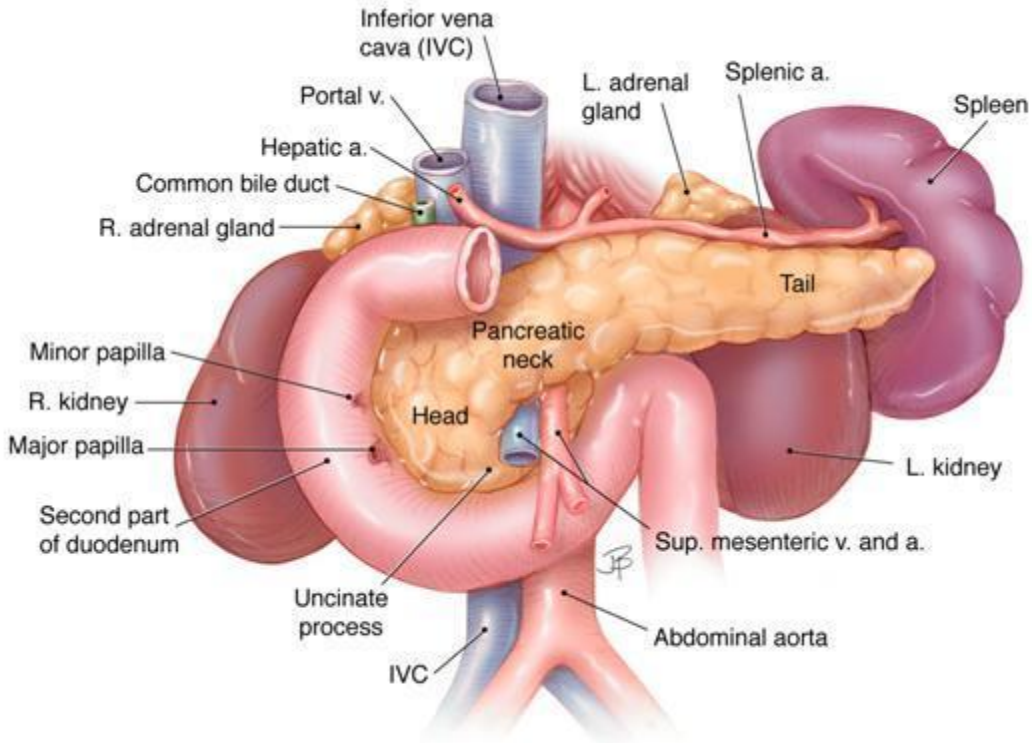


Figure 1. Pancreas anatomy (http://imgarcade.com/1/pancreas-anatomy-uncinate)

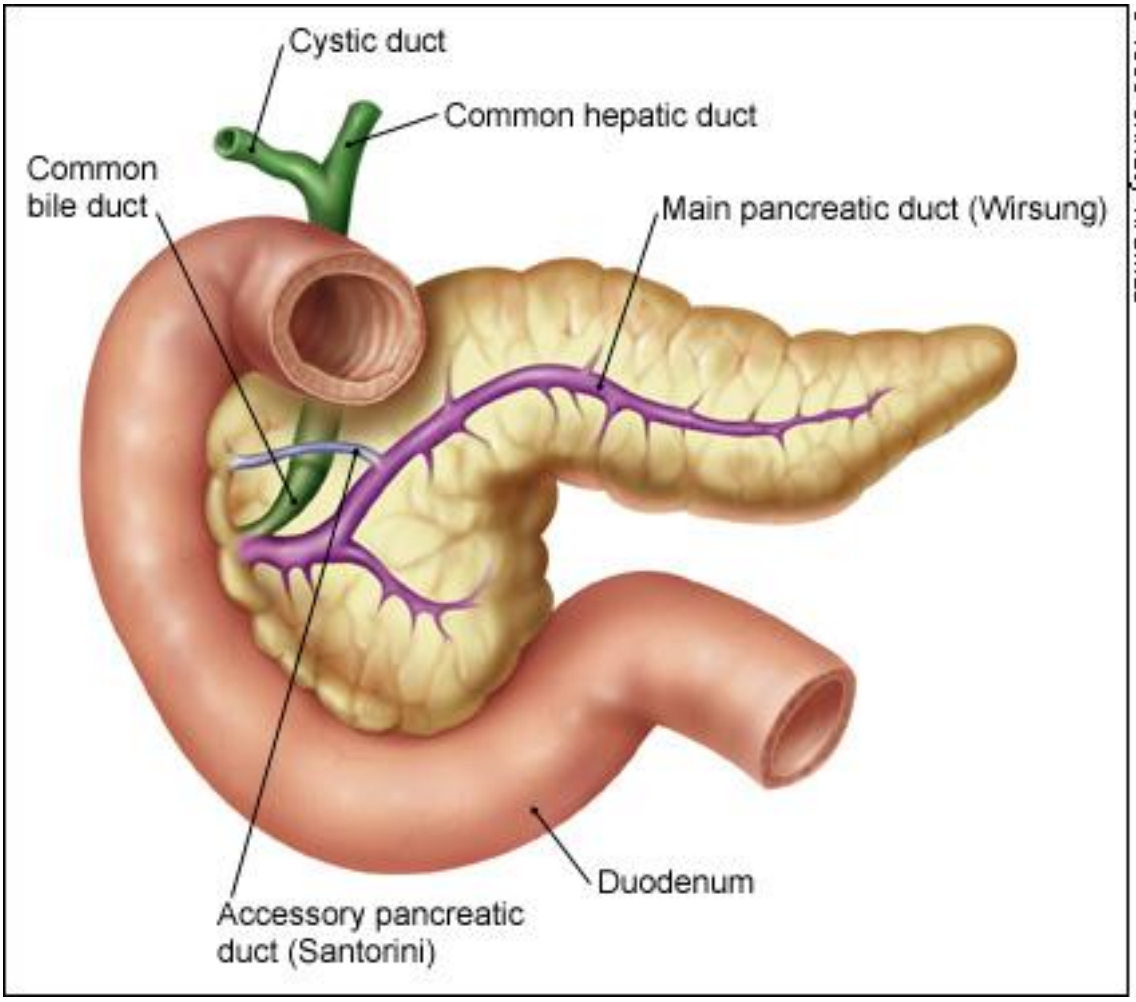


Figure 2. Pancreatic ductal anatomy (Krames C, 1999)

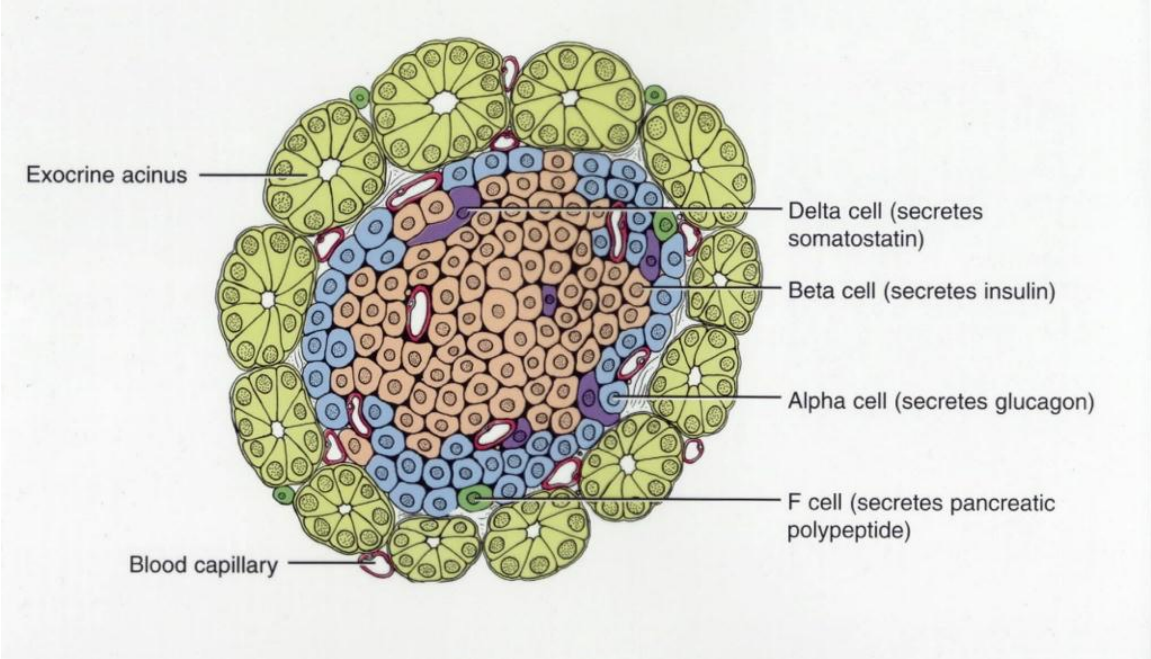


Figure 3. Islet of Langerhans (www.cram.com/flashcards/block-4-histology-3553915)

1.2 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 [5]. 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 [6-7]. 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) [8].

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 1) [3].

At CS 12-13 in humans, many important transcription factors have emerged, such as Sox-9, Hnf1-β, GATA-4 GATA-6, (Fig. 3) [10-11] and the duodenal homeobox factor 1 (PDX1), which is detected at the pancreatic endoderm level [9] (Fig. 3) [10-11]. Moreover, there has been no expression of endocrine factor 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 [12-13] 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 [12]. Neurog3 achieves high expression values by the end of the first trimester (fetal period), but then begins decreasing in the following semester [14-15]. At 10 wpc (weeks post-conception), β-cells of the islets of Langerhans become vascularized and islet clusters are observable [16].

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 1. Human embryonic development stages (Jennings RE et al, 2015)

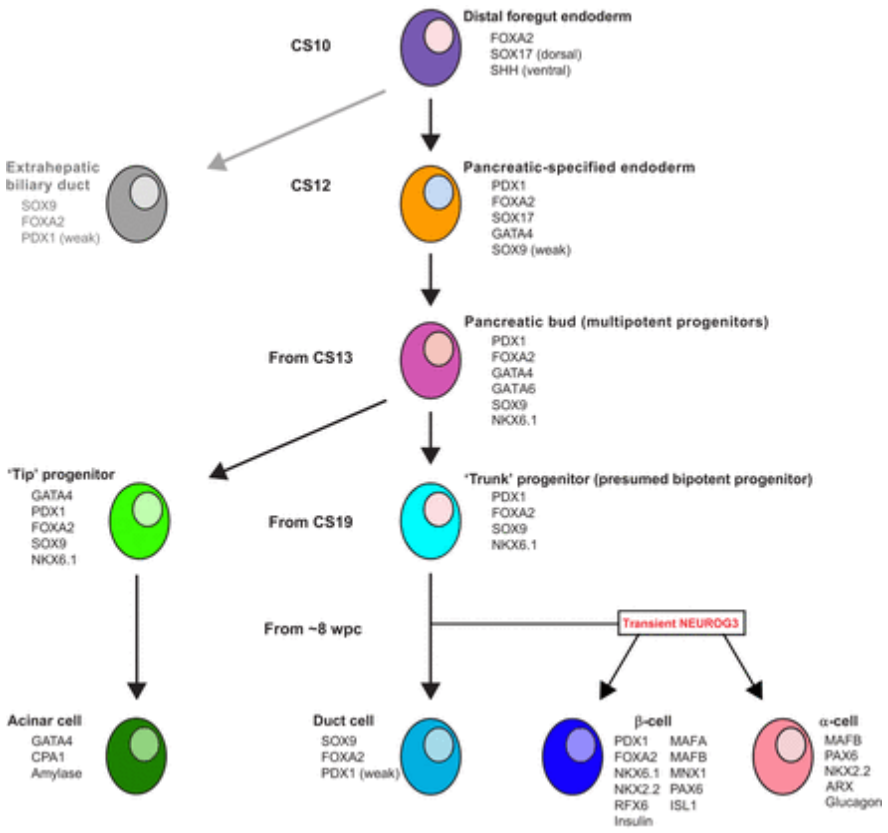


Figure 4. Transcription factor network of human pancreas development (Jennings RE et al, 2015)

1.3 Diabetes disease and therapeutic applications

Diabetes is a common disease. 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 [17]. 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 [18]. 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 [19]. 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. 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 [20-22].

1.4 Therapeutic applications for IDDM and transplant

Patients with IDDM require daily insulin injections. Unfortunately, this is not a long-term therapy, and it can generate serious side effects (severe hypoglycemic episodes). There are numerous recombinant insulin types, both human and porcine, however they are not currently intended for daily use because of the immune reactions they can generate. 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 seem to be the best option to treat patients with diabetes. However, even if 80% of patients achieve insulin independence following-surgery in their first year, the whole organ transplantation is associated with a high mortality rate. In fact, common complications include: vascular reconstruction as a complex limit, high thrombosis risk, and the ever-present immunosuppression effect. In 1966, first whole pancreas transplant was performed on an old woman by William Kelly and Richard Lillehei [23], 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 these reasons, researchers' and clinicians' interests were focused on the transplantation of the endocrine portion of the pancreas. In 1999, James Shapiro performed the first pancreatic islet transplantation on seven patients with type I diabetes using an immunosuppressive regimen without glucocorticoids. A year after the transplant, these people were insulin-independent [24]. Islet transplantation has important advantages over the whole pancreas. Islet transplantation is a less invasive technique and only requires local anesthesia.

1.5 The limitations of islet transplantation

The pancreatic islet transplant procedure presents various limitations, in particular, the lack of donors. Every year, approximately 3,000 pancreases are available for transplant in the US, but about 35,000 patients are affected by IDDM every year.

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 attachment 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 [25].

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 [26].

2. Stem cell features

Recently, stem cells have caught researchers' attention because of their promising cell features in regenerative medical therapies. The term stem cell was adopted at the end of the nineteenth century as a theoretical postulate to describe their self-regenerative capacity. Normal tissues, in which resident cells have a limited life time, need other cells in order to support the renewal of functional cell types for the organism's lifespan. There are two main criteria classically defining stem cells: 1) self-renewal: capacity to go through numerous cycles of cell division while maintaining the undifferentiated state, 2) potency: ability to differentiate into specialized cell types. In vivo, stem cell proliferation is strictly regulated by feedback mechanisms and their excessive production may result in pathological conditions such as cancer [27].

In 1978, Schofield explained for the first time the concept of "niche", a specialized microenvironment that helps maintaining stem cells features. [28]. According to the differentiation ability (Fig. 4), stem cells can be classified as:

- Totipotent stem cells: can differentiate in any mature cell type. Example: zygote and the first blastomers;
- Pluripotent stem cells: can differentiate into cell types derived from the three germ layers (but not form extra-embryonic tissues and adnexa). Example: the blastocyst inner mass cells or as many authors suggest, umbilical cord stem cells;
- Multipotent stem cells, can give rise to multiple cell types and are present in the adult (hematopoietic stem cells, adult nervous system cells);
- Unipotent stem cells, can give rise to 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.

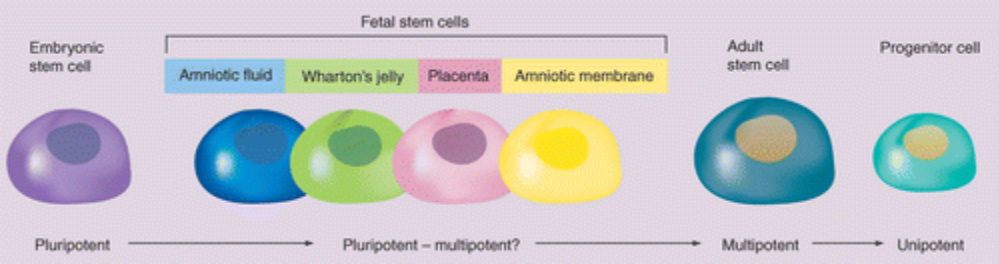


Fig. 5. Stem cells differentiation ability: potency decreases from embryonic to adult periods of life (Pappa K., Anagnou N.P. *Novel sources of fetal stem cells: where do they* fit on the developmental continuum?. Regen Med 2009;4(3),423-433)

2.1 Embryonic stem cells

Embryonic stem cells (ESCs) are derived from a limited embryo cellular population at the 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 the inner cell mass (ICM) formed by 30 cells, defined as embryonic stem cells, located at the embryonic node. Generally ESCs are permanently diploid, immortal, able to maintain an undifferentiated state, and can propagate and differentiate into cellular types derived from three embryonic germ layers [29]. Therefore, ESCs were considered as an alternative cell source for several diseases treatments. However, ESCs have some limitations: embryo cells need to be removed within 14 days of fertilization, the embryo graft can induce teratomas, and there is risk of immune and other adverse reactions in the host recipient [30].

2.2 Adult stem cells

Adult stem cells are responsible for the tissue structure functionality and supporting the cell repair process. Adult stem cells can be isolated from tissue samples, can self-renew, proliferate to elevated cell passage numbers, and do not induce teratoma formation. Recently, it has been shown that adult stem cells can even generate mature cells belonging to different lineages. For example, bone marrow hematopoietic stem cells can

give rise to muscle cells after transplant [31]; bone marrow cells can also repopulate the liver after transplantation or can be differentiated into cells that express neuronal markers [32-33]. This cell ability is known as plasticity and was also described in cases of cell fusion events [34].

2.3 Mesenchymal Stem Cells (MSCs)

MSCs constitute various cell populations. They were first described at the bone marrow stromal level thirty-five years ago [35]. Later, Pittenger and colleagues demonstrated MSC isolation and multi-potency from human bone marrow [36]. BM-MSCs were considered the primary source to obtain stem cells due to their numerous well-documented features.

Despite their huge success, bone marrow cells have some disadvantages:

1) Only a very small fraction (0.05-0.001%) of the entire bone marrow cell is made up of MSCs;
2) Their collection requires a painful and invasive procedure. For this reason, scientists continue to search alternative mesenchymal cell sources [37].

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 belonging to different lineages. They have a fibroblastic-like morphology with only a few cytoplasm and mitochondria, and an less developed Golgi apparatus. 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 almost three mature cell types: osteocytes, chondrocytes and adipocytes. The stromal localization is a typical MSC feature: known sources of MSCs are represented by (but not limited to) adipose tissue, skeletal muscle, amniotic fluid and the umbilical cord matrix.

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 easily cultured and even cryopreserved [38].
Many other properties make these cells a comparatively more efficacious therapeutic agent compared to bone marrow MSCs. For instance, UCs are usually discarded after birth and obtaining cells does not involve ethical restrictions. UC MSCs have low immunogenicity and can also modulate immune functions by producing several cytokines and growth factors. In addition, the lack of type II MHC and co-stimulatory molecules implies that these cells evade allogeneic immune responses [39]. Human UC cells share many of the surface phenotype markers with BM-MSCs (see below). Under suitable stimulation, UC MSCs can differentiate in vitro into adipocytes, osteoblasts, chondrocytes, hepatocytes, cardiac cells and neurons [40-41].

2.4 Comparison between UC-MSCs and BM-MSCs

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 the donor age [42].
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, (described below in the paragraph 3.1), are quite similar to BM-MSCs phenotypically, even if there are differences between these 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 show higher levels of HLA-I expression than BM-MSC's. After the endothelial induction, stem cells express typical endothelial markers, such as von Willebrand factor and the VE-cadherin [43].

2.5 Other sources of stem cell

2.5.1 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 cell which belong to the chorion and trophoblast cells 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 [46].

PDSCs have immune-modulatory properties. They have also Nanog and Oct3-4 inducible expression. This justifies the typical wide range of differentiation capacity, which includes neuronal and adipogenic differentiation as well as heart valve generation when stem cells are implanted into biodegradable scaffolds. Recently experimental data have 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 [44].

2.5.2 Umbilical Cord Blood (UCB)

During the last twenty years, umbilical cord blood (UCB), has been exploited as a rich stem cell source of haemopoietic progenitors. It has shown that approximately 1% of UCB cells expresses CD34 antigen, the major cell marker for hematopoietic stem cells. Even if present in very low numbers, UCB-MSCs are the second largest cord blood stem cell population. In addition to the typical MSC markers (CD105, CD44 and CD73), UCB-MSCs also expresses Oct-4, which is essential for tissue-specific gene inhibition and self-renewal maintenance, as well as Nanog.

Interestingly, various studies on hematopoietic cells have demonstrated neuronal protein expression. In fact, these hematopoietic cells can differentiate into neurons and glial-like

cells [46]. They also possess a less marked differentiation potential towards the adipogenic line and more orientated to the osteogenic type, when compared with BM-MSCs [44].

2.5.3 Amniotic Fluid (AF)

Recent studies have evaluated stem cell potential of cells isolated from amniotic fluid (AF). AF cells are heterogeneous, originating from the three germ lines.

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 [44].

3. The features of Human Umbilical Cord (UC)

Human umbilical cord (UC) is a vascular connection between mother and fetus; it protects the blood vessels that provide oxygenated blood to the fetus while also acting as a transport system for waste removal [45-46]. Umbilical cord weight is around 40 g post-partum and it stretches approximately 30 - 65 cm with a diameter of 1.5 cm (Fig. 6). The umbilical cord is mainly constituted by a specialized tissue named extra-embryonic mesoderm, which originates from the epiblast proliferation during second week of development, giving rise to a connecting stalk, which lacks vascularization. The proper UC originates during the fourth week of development, with the embryo folding that gives the external layer of amniotic epithelium. This cubic epithelial cell layer, properly named the umbilical cord epithelium (UCE), delimits the organ stroma, which contains three vessels at maturity (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 [47].

The umbilical vein brings oxygenated blood and nutrients from the placenta to the fetus, while the umbilical arteries carry blood flowing from the fetus to placenta. These three vessels are embedded in a matrix, made by a specialized connective tissue called Wharton's jelly (WJ). Wharton’s jelly is a mature mucous tissue, made up by fibroblastic-like cells and myofibroblasts (according to the classical view) embedded in a complex extracellular matrix enriched in proteoglycans. WJ acts as scaffolding material for the three umbilical vessels, impeding vascular constrictions during pregnancy. Capillaries and lymphatic vessels, as well as nervous branching, are not present in the umbilical cord (Fig 7).

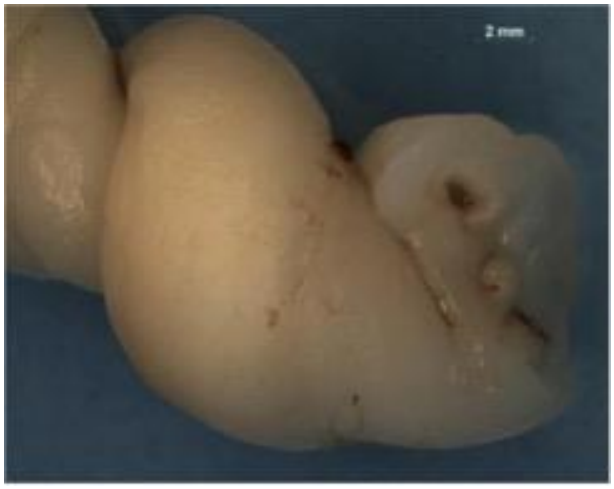


Fig. 6 Macroscopic view of human umbilical cord at term

(Semenov OV, Breymann C. *Mesenchymal Stem Cells Derived from Wharton's Jelly and their Potential for Cardio-Vascular Tissue Engineering.* Open Tissue Eng Regen Med J, 2011, 4:64-71)

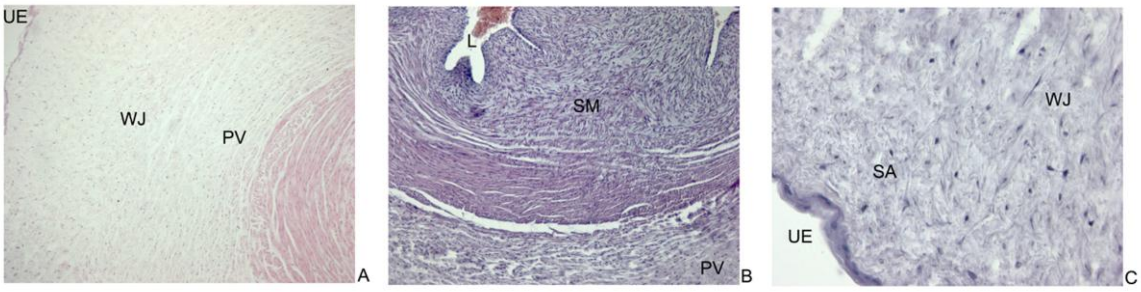


Fig. 7 Umbilical cord H&E section

3.1 Wharton's jelly: structure and function

Wharton's jelly is made up of a glycosaminoglycan (GAGs) rich substance that contains hyaluronic acid (HA) and proteoglycans, with some collagen fibers (mainly 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 [48].

Myofibroblast and fibroblast-like cells are the two cell types existing in WJ. Myofibroblast have muscle-specific cytoskeletal filaments. They are positive for vimentin [49], a typical fibroblastic marker, and desmin [50], a muscle cell marker. The fibroblast-like cells have similar features to fibroblasts and they produce collagen and other extracellular matrix components. The extracellular matrix, including WJ, the subamniotic stroma and the adventia vessels, figure 7, demonstrated immunoreactivity also for collagen type IV, heparin sulphate proteoglycan and lamin [50]. The distributions of the different collagen types over the UC, has been suggested to be responsible for the mechanical properties of the UC [51]. Extracellular matrix components can be considered as growth factors storage and support stromal cells [52]. ECM proteins showed an increasing number of growth factors such as IGFs (insulin-like growth factors), FGFs (fibroblast growth factors) and TGF-β (transforming growth factor- beta). These growth factors, in turn, control cell proliferation, differentiation and ECM remodeling.

Several studies have shown that Wharton's jelly cells (WJCs) support ex-vivo hematopoietic expansion [53] and in vivo engraftment of hematopoietic stem cells [54]. Raio demonstrated that WJC are a source of hyaluronic acid, which is another element of the hemapoietic stem cell niche [55]. Furthermore, WJC secrete cytokines similar to bone marrow-MSCs and are able to synthesize granulocyte-macrophage colony stimulating factor (GM-CSF) and granulocyte colony stimulating factor (G-CSF).

WJCs are slower to differentiate towards adipocytes than BM-MSC, but WJCs have a shorter cell doubling time and can be isolated with 100% success from typical specimens

[56]. The multiplicity of similarities with BM-MSCs, led to consider WJCs as part of the growing MSC family.

3.2 Wharton's jelly as mesenchymal stem cells: markers expression

Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs) are multipotent stem cells that have many advantages as a potential source of MSCs, since the tissue is readily available, cells are easy to grow in culture, can be cryopreserved, and have great expansion capacity in vitro. Over the years, our research group has gained reasonable experience both in isolation and the primary cell culture of WJ-MSCs [57]. Moreover, WJ-MSCs have faster and better ex vivo expansion abilities than 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 [57]. Previously, only GATA-4 expression had been reported in BM-MSCs [58].

La Rocca et al demonstrated that WJ-MSCs express connexin-43 [57], 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 with their undifferentiated state. In fact, it is known that Nanog and Oct3/4A expression in MSC 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 [59], while CK-7 is not detected [60]. Immunocytochemistry analyses have highlighted that WJ-MSCs lack expression of CD14, CD31, CD33, CD34, and CD45 [61]. Moreover, both BM- derived and WJ-MSCs do not express HLA-DR [62-63]. WJ-MSCs express: CD73, CD90, CD105, HLA class I [64] CD10, CD13, CD29, CD44, CD49e, CD166 and CD117 a receptor for the stem cell factor [65-67].

WJ-MSCs are also positive for nestin [57], an intermediate filament of the neuro-ectodermal lineage as a neurofilament precursor, which is also present in the pancreatic progenitors of β cells [68]. Furthermore, the glial fibrillar acidic protein (GFAP) and neuron-specific enolase (NSE), have been described in the literature [69-70].

Umbilical cord derived cells can also differentiate toward cells specific of endoderm-derived tissues: hepatocyte nuclear factor 4α (HNF-4α) expression by WJ-MSCs suggests a possible role in hepatocytes and pancreatic endocrine cell differentiation [57].

3.3 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). This would open new frontiers in regenerative medicine. These three differentiation cell lineages have been considered part of the minimal MSC criteria stated in 2006 [71].

The standard protocol to obtain MSC osteogenic differentiation can be confirmed by specific histological stains for extracellular calcium (Alizarin Red S and Von Kossa) [57 – 72]. Moreover, differentiated cells should express specific proteins such as osteonectin, osteocalcin, periostin and runx2 [73].

After adipogenic differentiation, mature adipocytes are confirmed by a specific histological stain such as Oil Red O [74]. In addition, the mature 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 [75]. The chondrocyte phenotype can be confirmed by the expression of collagen type II, cartilage oligomeric matrix protein (COMP) and aggrecan [76].

Neurogenic differentiation. WJC cultured in medium supplemented with basic fibroblast growth factor (bFGF), butylatedhydroxyanisole, dimethylsulfoxide (DMSO), and low

serum percentages, have been successfully induced to differentiate into glial cells and neurons by Mitchell group [70]. 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 [77].

Myocardiocyte differentiation. Myocardial repair via heterologous stem cells is an amazing area of stem cells research. Recent experiments suggest that WJ-derived cells can play a role in myocardial regeneration. The first case of myocardial cells derived by WJCs was reported by Wang et al in 2004, who described that WJCs started to express typical myocardial markers (cardiac troponin I, connexin-43 and desmin) and exhibited myocardiocyte morphology at 3 weeks post treatment with 5-azacytidine [78]. Later, Wu et al, 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 b-FGF and platelet-derived growth factor (PDGF). Wu showed that the myocardial-differentiated cells, after being applied in an acute myocardial infarction animal model, had been incorporated into the vasculature and occasionally were positive for cardiac troponin T (cTnT) [79]. Other works have claimed that MSCs may act as supportive populations through inflammation suppression in an acute myocardial infarct model, as well as paracrine effects on the repairing myocardium [80 – 81].

Hepatogenic differentiation. In the literature, there are various data supporting the concept of WJ-MScs differentiation towards hepatocytes. In vitro protocols are based mainly on the administration to cultured cells of factors such 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 [82], or in a co-culture system with fetal or adult hepatocytes [83]. 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 vitality [84 – 85]. Numerous researchers

have demonstrated that UC-MSCs can be successfully differentiated in an in vitro model as well as an in vivo model.

4. MSCs and immune system interactions: immunological properties

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 factors secretion [43]. An immune function of MSCs is to inhibit T-cell proliferation and dendritic cell (DC) differentiation [44]. MSCs are able to do this because they have low expression of co-stimulatory molecules and the absence of HLA-II molecules [43-57]. Recent studies have shown that soluble factor secretion is a consequence of MSCs and T-lymphocytes cross talk, and not a constitutive process [86].

HLA molecules have been involved in both NK cell self-tolerance induction as well as maternal immune system tolerance toward the embryo [87-88]. Several publications pointed out that the existence of HLA non-classical type I in the mesenchymal stem cells. Specifically HLA-G and its soluble form, HLA-G5 [44- 89].

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 [43].

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 [90]. MSCs express indoleaminedeoxygenase (IDO) and nitric oxide (NO), molecules which are involved in the immune response in differen models [91].

4.1 MSCs and inflammatory targeting

After systemic infusion, MSCs are able to migrate to the tissue injury site and start to accumulate there [92]. Migrating resident phagocytes find MSCs around the damaged tissue area. MSC treatments have positive effects on neurological disorders due to their anti-inflammatory and oxidative stress stem cell properties.

Kemper and et al. described the superoxide dismutase 3 (SOD3) secretion by MSCs as a neuro-protective agent, decreasing the inflammation and tissue damage [93].

Other in vivo experiments have confirmed MSCs power to reduce inflammation in obstructive apnea [54], renal failure [55], liver fibrosis [56], asthma [57] and acute myocarditis [58].

In addition, UCB stem cells have normalized blood glucose level in NOD diabetic mice and reduced insulitis [59]. 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 in acute and chronic inflammatory diseases.

5. Aims

The aim of this project was to investigate the potential of WJ-MSCs to be used in cell therapy of TID as a co-transplanted, supportive population for the engraftment of pancreatic islets.

To pursue this objective, he project has been divided into two main tasks:

1) In vitro generated data: WJ-MSCs have been isolated from umbilical cord, cultured according to the standard methods described in the literature and developed in our laboratory [57]. Then we characterized WJ cells lines through different analyses (immunocytochemistry, immunohistochemistry, flow cytometry, western blot, RT-PCR) in order to define the immune-modulatory and / or anti-inflammatory molecules expression profile. In addition, we tested the expression of further molecules belonging to several mature lineages. We also investigated different neuronal markers (NSE, Nestin, GFAP, NF68 and GDNF). Some of them, as GDNF, have been specifically linked to the nervous regulation of the exocrine and endocrine pancreas development. These experiments have been carried out on naive cells, in order to deeply characterize their features prior to transplantation.

2) In vivo generated data: We have developed a TID in vivo model to test the anti-inflammatory effect of WJ-MSCs, namely the streptozotocin (STZ)-induced diabetes in a mouse (anti-inflammatory syngeneic study) model. STZ evokes an inflammatory effect on beta cells therefore inducing hyperglycemia. WJ-MSCs were administered to animals receiving sub-optimal (marginal mass) doses of islets, in order to stress test the capacity of WJ-MSCs to improve the viability of the islets and their engraftment, leading to diabetes reversal.

MATERIAL AND METHODS

6.1 Wharton's Jelly Mesenchymal Stem Cells Isolation

Isolation protocol was adopted from our previously published data [57]. All umbilical cords were obtained after the mother's agreement according to tenets of the Declaration of Helsinki and local ethical regulation. After normal vaginal or caesarean delivery, following full-term birth, umbilical cords were stored aseptically in cold saline and cellular isolation was started within six hours post-partum. Cords were washed in warm HBSS (Gibco), and then were cut in small pieces about 1.5 cm long, sectioned longitudinally so that to expose the Wharton's Jelly under amniotic membrane. Different incisions, without vessels removal, were made within the matrix with a sterile scalpel to increase the area exposed to the contact with medium composed of low glucose DMEM (Sigma), supplemented with 10% FBS (fetal bovine serum, Hyclone), 1x NEAA (non-essential amino acids, Sigma), 1x antibiotics-antimycotics (GIBCO), and 2mM L-glutamine (Sigma). For this isolation protocol we did not use enzymatic processes to dissociate cells from the embedding matrix, leaving cells free to attach to the culture vessel based on their migratory ability. Cord pieces were left for 15 days with medium changed every two days. Therefore, the slow degradation of the matrix allowed secretion of growth factors and signalling molecules from the cord, maintaining stem cell potency and providing a positive stimulation to the cultured cells.

After 15 days of culture, cells widely adhered to the plastic surface, cord fragments were removed, and routine culture was performed.

6.2 Cell culturing and passaging

After reaching confluence, cells were removed from the flasks with Tryple Select (Invitrogen) and were cultured for up to 15 passages (corresponding to about 60 population doublings). 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 25cm^2 tissue culture flasks (Corning).

6.3 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 with PBS and then fixed and dehydrated with methanol for 20 minutes at -20C°. After rinse 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 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. After another wash with PBS, cells were incubated with species-specific secondary antibodies for 10 minutes. Subsequently, streptavidin- peroxidase (Dako-Cytomation) was added, followed by 3.3'-diaminobenzidine (DAB chromogenic substrate solution, Dako). Finally, Hematoxylin (Dako) was used to counter stain the cell nuclei.

The antibodies used in the present study, with indications of the working conditions used, are listed in table n.2

Antigen	Host	Manufacturer	Dilution
HLA-DR	Mouse monoclonal	Santa Cruz	1:50
$B_{7\text{-}1}$	Mouse monoclonal	Santa Cruz	1:50
$B_{7\text{-}2}$	Mouse monoclonal	Santa Cruz	1:200
HLA-ABC	Mouse monoclonal	Santa Cruz	1:50
Vimentin	Mouse monoclonal	Santa Cruz	1:100
v-Wf	Mouse monoclonal	Santa Cruz	1:50
Desmin	Mouse monoclonal	Santa Cruz	1:100
α-SMA	Mouse monoclonal	Santa Cruz	1:100
Cx43	Rabbit polyclonal	Santa Cruz	1:100
Ck8	Mouse monoclonal	Sigma	1:200
Ck18	Mouse monoclonal	Sigma	1:800
Ck mix(8-18-19)	Mouse monoclonal	Sigma	1:50
Ck19	Mouse monoclonal	Millipore	1:100
C-kit	Rabbit monoclonal	Epitomics	1:50

Table 2: List of antibodies used in the present study

6.4 Total RNA extraction

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

The cells were lysed by adding 350µl of Buffer and 3.5 µl of β-mercaptoethanol, and were mixed vigorously. The obtained lysate was filtered through RNAspin Mini Column and was centrifuged for 1 minute to 10000 rpm. After centrifugation, the mini filter was discarded and the filtrate was transferred to a new 1.5 ml tube where we added 350µl of 70% ethanol. After mixing, the lysate was pipetted 2-3 times and was transferred to a RNAspin Mini Column, placed in a 2ml tube and centrifuged at 8500 rpm for 30 seconds. After centrifugation, the column was placed in a new tube, where 350 µl of MDB (Membrane Desalting Buffer) was added, and centrifugation was performed for 1 minute at 10000 rpm to dry the membrane. Each sample was incubated for 15 minutes with 95 µl of a mixture containing DNase to avoid a possible DNA contamination. The following washes were carried out with specific buffers supplied with the kit and then the column was transferred into a 1.5 ml tube (nuclease-free). The RNA was eluted from the filter in 100 µl of RNase-free H_2O (by centrifugation at 10000 rpm for 1 minute). The RNA extract was stored at -20 ° C until use. The concentration of RNA extracted was determined by spectrophotometer with a wavelength of 260 nm. Only samples with A260/A280 ratio over 1-6 were considered usable for the following analyses.

6.5 RT-PCR (Reverse Transcription Polymerase Chain Reaction)

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

After treatment with DNase, 2µg of RNA were processed:. The reaction comprised a reverse transcription step of 50 minutes at 42 C° and an inactivation phase of 5 minutes at 92 C°.

Next, 2 µl of cDNA were added to 10pM of specific primers, which was then followed by 4 µl of 5x PhusionBuffer, 0,4µl 10mM dNTP, 0,6µl DMSO, 0,2 µl of Phusion DNA Polymerase and then water added until a final volume of 20 µl. The amplification reaction

was performed according to five steps. The initial denaturation of 30 seconds at 98C°, followed by another denaturation step of 10 second at 98 C°, the annealing phases at 30 seconds each at specific-primers temperatures, the extension step of 30 seconds at 72C°, and the final extension for 10 minutes at 72 C°.

6.6 Agarose gel electrophoresis

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

6.7 Flow cytometry

Flow cytometry (FACS) was used to characterize cells at 2^{nd}, 5^{th}, 10^{th} and 15^{th} passage. Hundred thousand cells were used for antibody staining. The fluorescein isothiocyanate (FITC) – conjugated, phycoerythrin (PE) – conjugated, or allophycocyanin (APC) – conjugated antibodies and isotype control are summarized in Table 3. Isotype control antibodies were used as negative control for the measurement of the no 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 was 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, a cell sorter with two laser and seven colours and were analysed with three softwares: FACS Diva 6.1.2, Cell Quest (BD Biosciences) and ModFit LT (Verity Software House).

Antigen	Clone	Conjugated	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

6.8 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, 150mMNaCl, 25mMTris (pH 7.4), 1mM EDTA, 1 mM EGTA, 2 mM Na3VO4, 10 mMNaF, 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 were 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, listed in table 2, and other ones. 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.

6.9 Osteogenic differentiation

Differentiation of cells was performed using protocols in the literature with minor modifications [100]. Briefly, the cells were cultured in osteogenic mediumfor 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 along culturing. 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 subjected to the Alizarin Red stain, which stains specifically the deposits of extracellular calcium [57].

6.10 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 for 3 weeks to monitor the spontaneous formation of lipid vacuoles. 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 [57].

6.11 Chondrogenic differentiation

Differentiation of cells was performed by seeding WJ-MSCs into alginate beads, using slight modifications of previously published protocols [101 - 102]. Briefly, WJ-MSCs were resuspended in sodium alginate (Sigma-Aldrich) (4×10^6cells/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 washes with 0.15 M NaCl, the beads were rinsed with DMEM. Then, beads were cultured either in standard growth medium (controls) or chondrogenic medium, prepared using published protocols with slight modifications (DMEM supplemented with 1% FBS, 6.25 g/ml insulin, 10 ng/ml TGF1, 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 [98, 99]. 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 (6 μm) were processed for histology (Alcian Blue and nuclear fast red staining) and IHC.

6. 12 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.

6.13 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.

6.14 Islet Isolation

Donor animals were anesthetized by isoflurane and the pancreas was harvested and stored in cold Hank's solution (Gibco), until digestion. In brief, 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. Pancreases were digested and islets were purified using Ficoll (Cellgro-Corning) density gradients (1.108, 1.096, and 1.037). After purification, islets were separated by size in three different groups using stainless steel mesh (Bellco Glass, Inc Tissue Sieve) filtrations by gravity (150 μm and 300 μm pore size, respectively). Following filtration, islet size was confirmed through observation at light microscopy. Aliquots from each islet size batch were harvested and stained with dithizone (Sigma) for sizing and counting. Isolated islets were transplanted as 600 (full mass) and

200 IEq (marginal mass). In each islet-sized group, the number of islets transplanted was adjusted to match the same islet mass. From each isolation, islet functionality was confirmed in vivo by diabetes reversal in control animals receiving 600 IEq.

6. 15 Blood Glucose level (BGL) and Intra-peritoneal glucose tolerance test (IPGTT): engraftment and functionality assessment

Animals were monitored daily for BGL and body weight changes for the first 2 weeks and then twice weekly thereafter. Blood glucose concentrations were determined using a blood glucose meter and strips (Accu-Chek; Roche Diagnostics) 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 test (IPGTT). IPGTT was performed at long-term (day 90) follow-up. 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. Animals were humanely sacrificed, and the pancreas was harvested for histological analysis.

RESULTS

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

Isolation of WJ-MSCs from the umbilical cord matrix has been performed using standardised methods developed in our laboratory: leaving cord fragments in culture medium allowed cells to exit and attach to the culture vessel according to their mesenchymal migratory capacity, without any enzymatic treatment. WJ-MSCs have grown robustly on culture surfaces and have been easily expanded in vitro under standard conditions (Fig.8 D - F). According to our original hypothesis, the slow matrix degradation allows growth factors and signalling molecules to exit from cord and maintain the stem cell potency.

Standard histochemical staining (H&E) was performed on paired tissue sections of paraffin-embedded umbilical cords. As visible in figure 8 (panels A – C), the specimens showed the expected substructures such as umbilical epithelium, vessels, and the intervascular stroma known as Wharton's jelly.

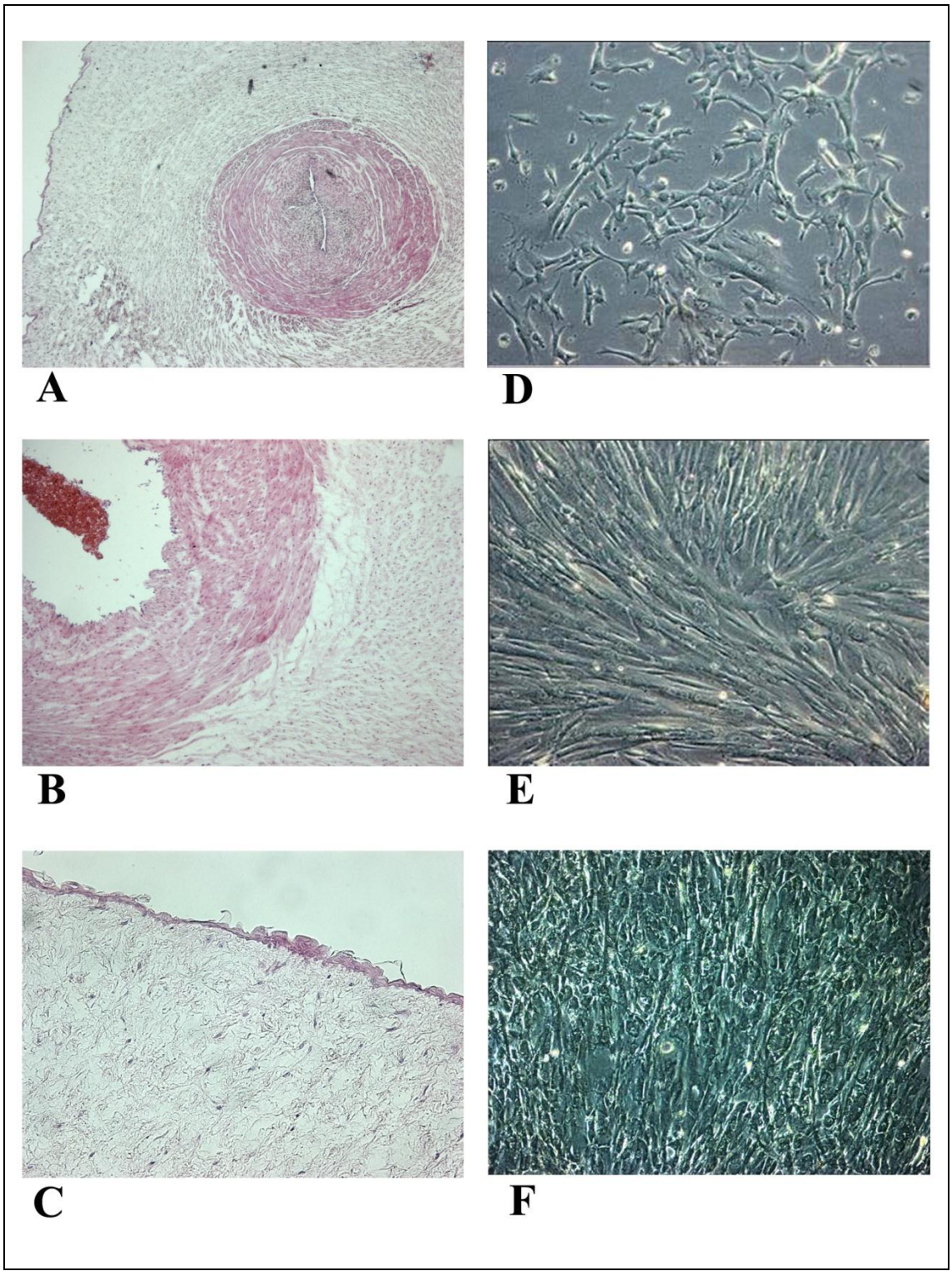


Fig.8 Demonstration of umbilical cord and WJC morphology:
Umbilical cord appears lined by a continuous amniotic epithelium, with vessels embedded in a GAG-rich mucous tissue (A); B shows a more detailed view of the perivascular area; C detailed view of the sub-amniotic area of the stroma. Cells obtained 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).

7.2 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 9) 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 expected pattern of markers expression of MSCs. Standard differentiation experiments (figure 10) demonstrated the ability of these cells to differentiate towards the osteogenic, adipogenic and chondrogenic lineages according to tissue specific stainings.

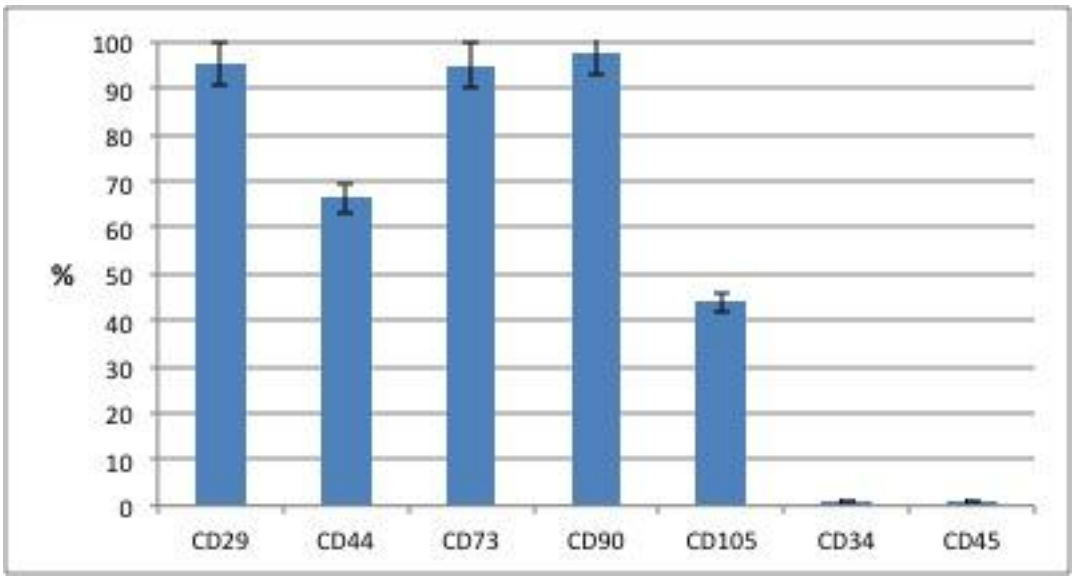


Figure 9: Flow cytometry analysis of standard MSC markers expression in WJ-MSCs. Cells were positive for the expected markers of MSC populations, namely CD29, CD44, CD73, CD90 and CD105, while being almost negative for the hematopoietic markers CD34 and CD45.

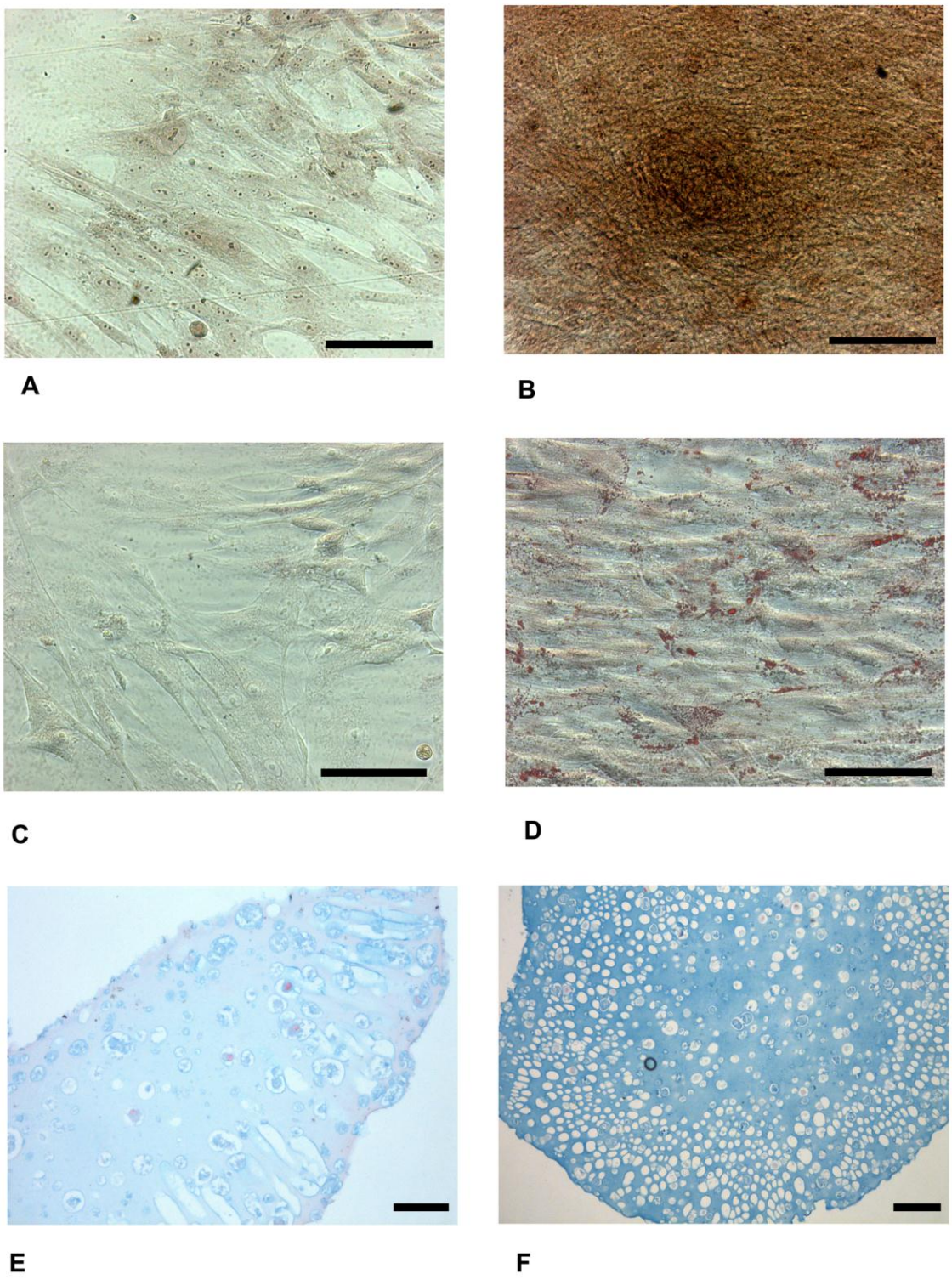


Figure 10: Demonstration of the tri-lineage differentiation by WJ-MSCs. Cells were subjected to differentiation as reported in methods and stained with specific histological stains. Osteogenic-differentiated cells showed large extracellular calcium deposits as

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

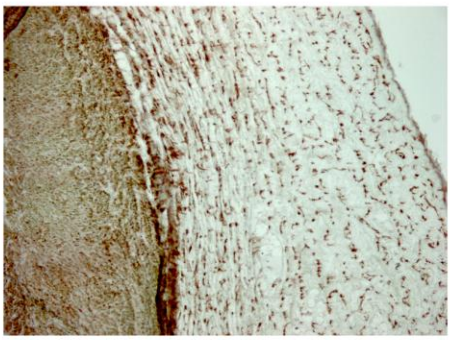
7.3 Phenotypical characterization of Wharton's jelly Mesenchymal stem cells (WJ-MSCs): further markers.

In order to provide a more detailed characterization of WJ-MSCs, we wanted to investigate their expression in parallel between the umbilical cord tissue and cultured cells. As shown in figure 11 (A, B), the α-smooth muscle actin (α-SMA) is strongly expressed at the vascular tunica media 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. In fact, figure 11 F shows that the vessel wall is amply positive to desmin, but interestingly we demonstrated its expression also in Wharton's jelly (Figure 11 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 11 G, H). The results of IHC have been confirmed subsequently in the cell culture by immunocytochemistry (ICC) for the α-SMA, vimentin, desmin and vWF, as shown in Figure 12 (A, B, C, D). This datum 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 remained unaltered. This will be of further importance for the maintenance of features leading to the immune privilege of such cells.

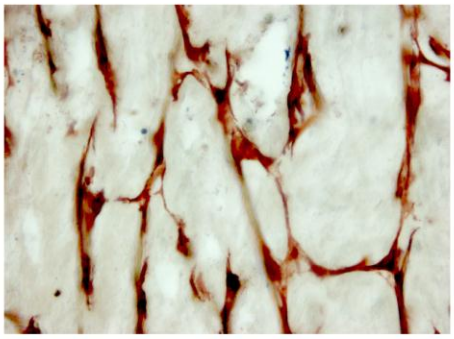
Moreover we have evaluated the cytokeratin (CK) expression, intermediate filaments at the human body epithelia level. Previous literature data have described the cytokeratin family presence in the umbilical cord blood [103]. We demonstrated that particular cytokeratins, as CK-8, CK-18 and CK-19, are expressed in the umbilical epithelium and Wharton's jelly (figure 13 A, B). Our group showed 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 13 C, D).

Connexin-43 (Cx-43) is another interesting molecule, belonging to the stem population, and expressed mostly in the embryonic stem cells, and also in the 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

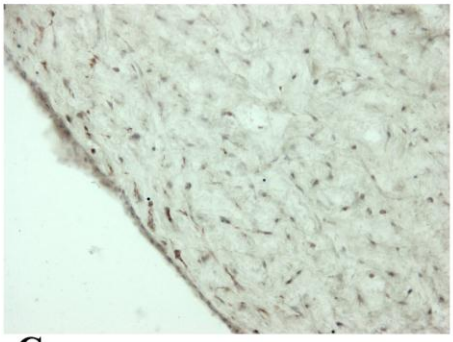
between cells. It has also been demonstrated that Cx-36 and Cx-43 have opposite effects on beta cell mass and insulin production levels. Moreover, in vivo Cx-43 positive cells localize at the periphery of islets and mark also alpha cells. The IHC analysis has allowed us to highlight that Cx-43 is widely expressed at the UE and Wharton's jelly level (Figure 13 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 14 (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 14 D and E) molecules also in vitro.



A



B



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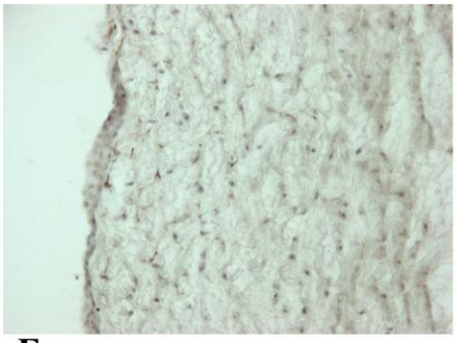

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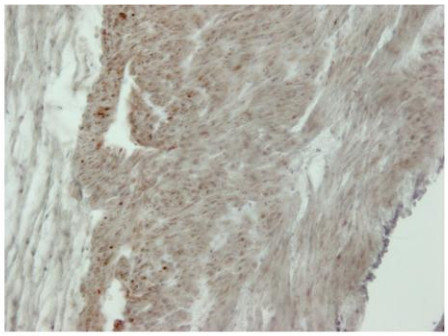

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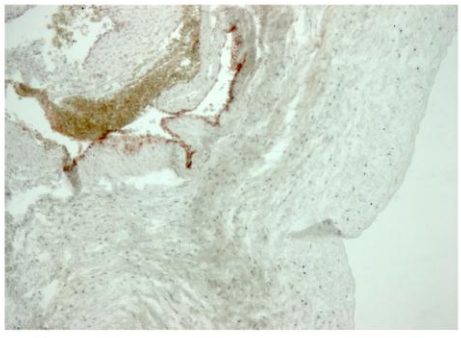

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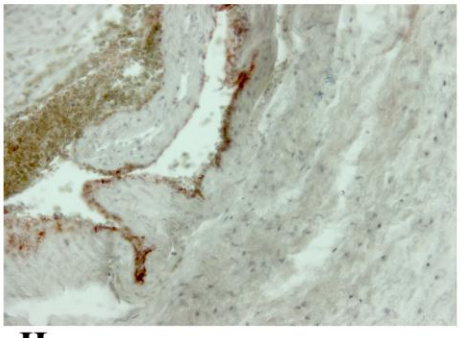

H

Fig.11: 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.

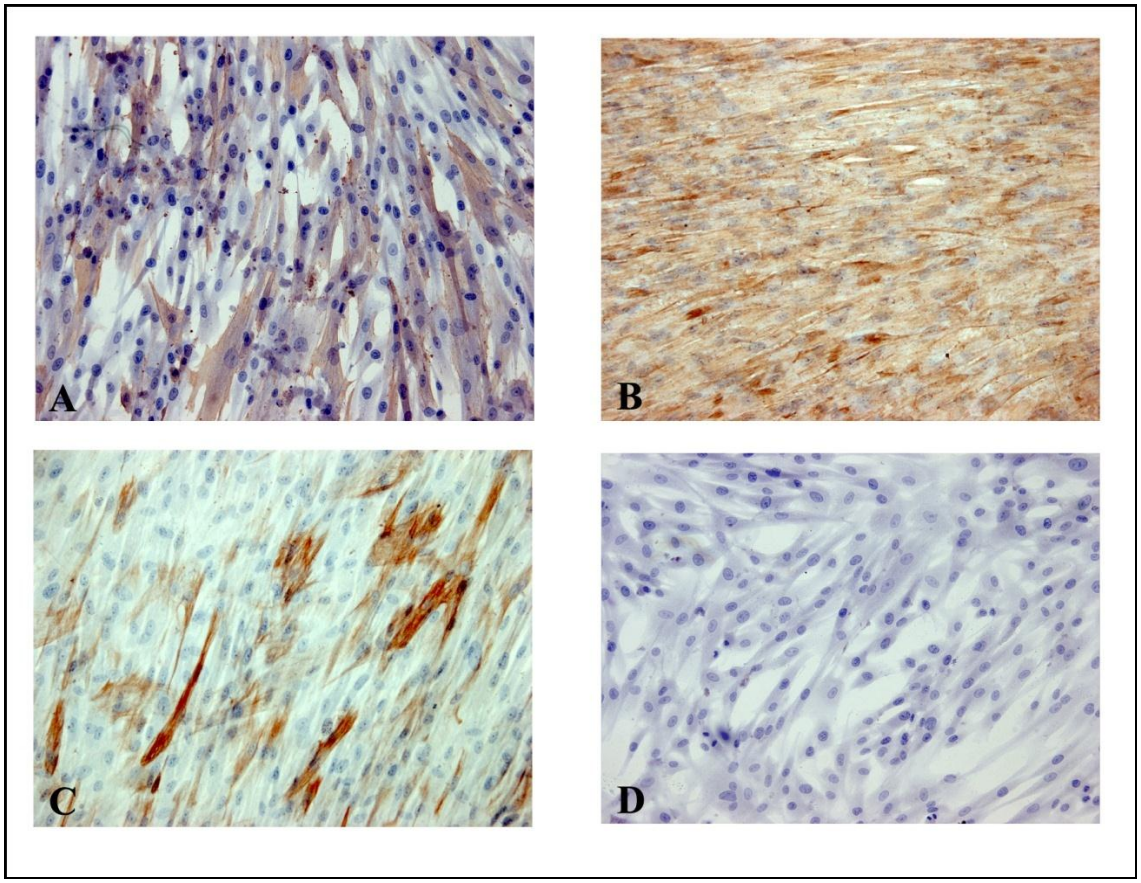

Fig.12: Representative panels of immunocytochemical detection of various markers in umbilical cord specimens: α-SMA (A), Vimentin (B), Desmin (C), vWF (D). Magnification: 20x.

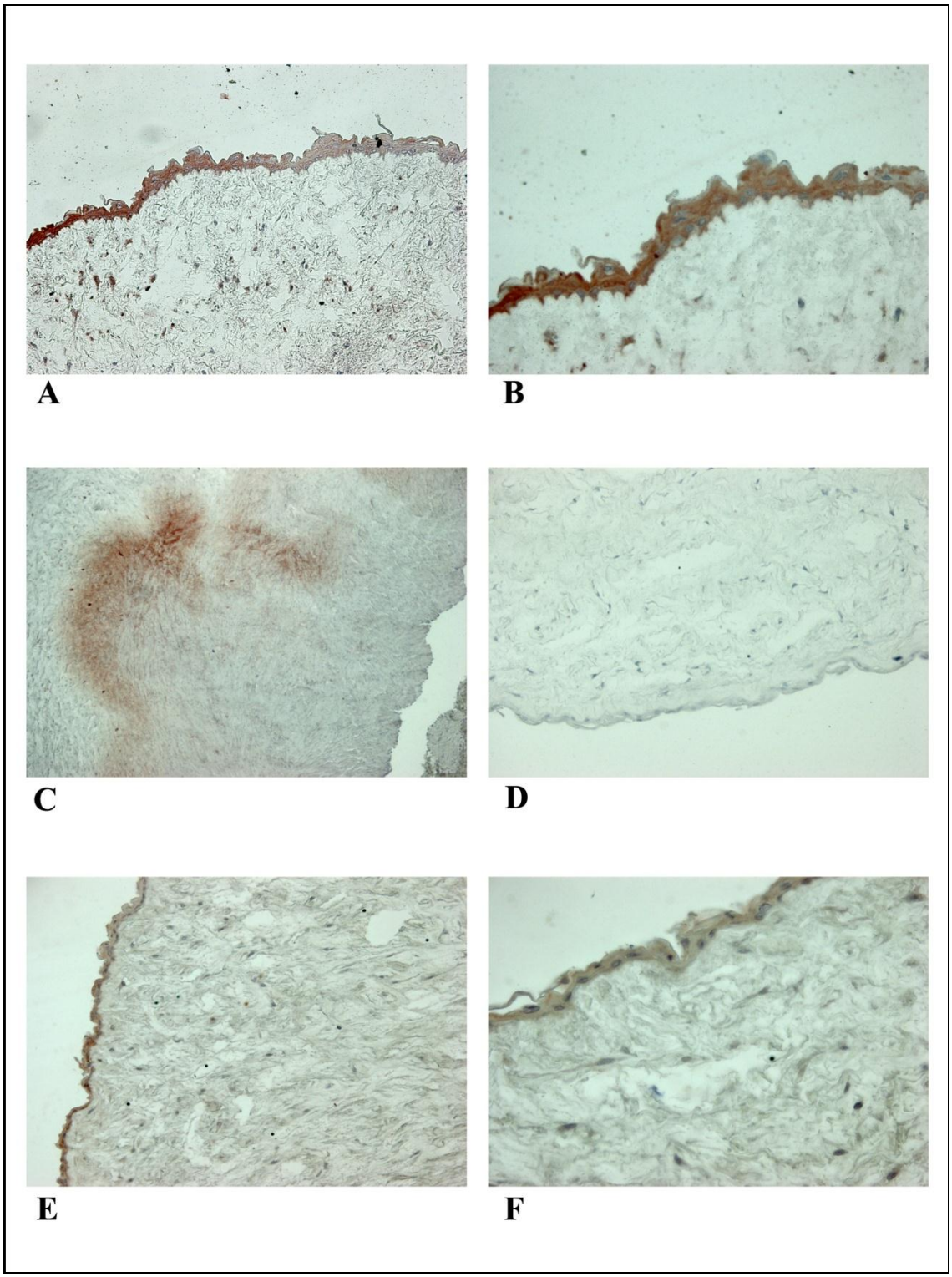

Fig.13: Representative panels of immunohistochemical detection of various markers in umbilical cord specimens: Ck-mix (A and B), C-kit (C and D), Cx-43 (E and F). Magnification: (A, D, E, 20x), (B, F, 40x) and C 10x.

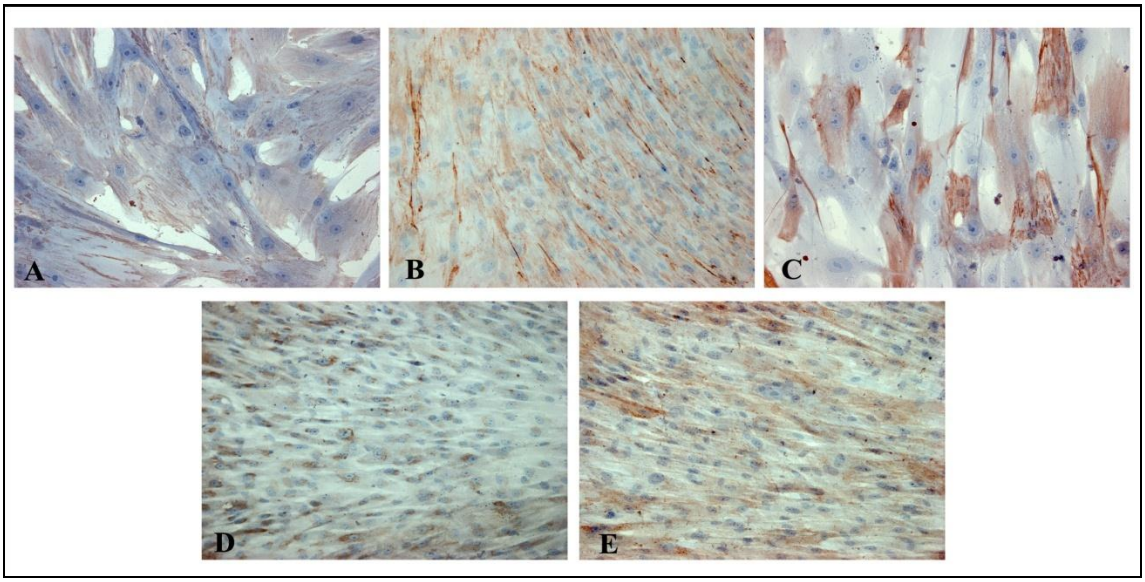

Fig.14: Representative panels of immunocytochemical detection of various markers in umbilical cord specimens for Ck-8 (A), Ck-18 (B), Ck-19(C), C-kit (D), Cx43 (E). Magnification: 20x.

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

Because of their hypo-immunogenicity and immune-modulatory features, mesenchymal stem cells have been identified as an alternative treatment for diabetes. MSCs lack co-stimulatory molecules expression and are able to modulate the immune response by releasing cytokines and growth factors. MSCs immunomodulatory features involve the suppression of T cell proliferation and the inhibition of natural killer (NK) and B-cell proliferation [104-106].

Therefore an important goal is to understand the pathways regulating the interactions between the mesenchymal stem cells and recipients' 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 15, A) in the cord tissue. In contrast, no positivity has been found for the HLA-DR, a typical type II MHC molecule (Figure 15 C).

Much attention has been recently given to non-classical MHC class I complexes, and effectors of the immunologic tolerance, modulating lymphocyte and NK cells proliferation. Previously, our lab has underlined the HLA-G expression in the WJ-MSCs [57]. Now our interest has been focused on the HLA-E molecule. For the first time we have showed HLA-E expression at protein level, (Figure 15 E) in both umbilical epithelium and WJ cells. Immunocytochemistry confirmed these data, with WJ-MSCs in culture being amply positive for HLA-ABC, negative for HLA-DR, and, as demonstrated for the first time here, positive for HLA-E at the protein level (Fig. 15 B, D, F).

Flow cytometry confirmed the data of ICC experiments, as visible figure 16: WJ-MSCs were amply 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).

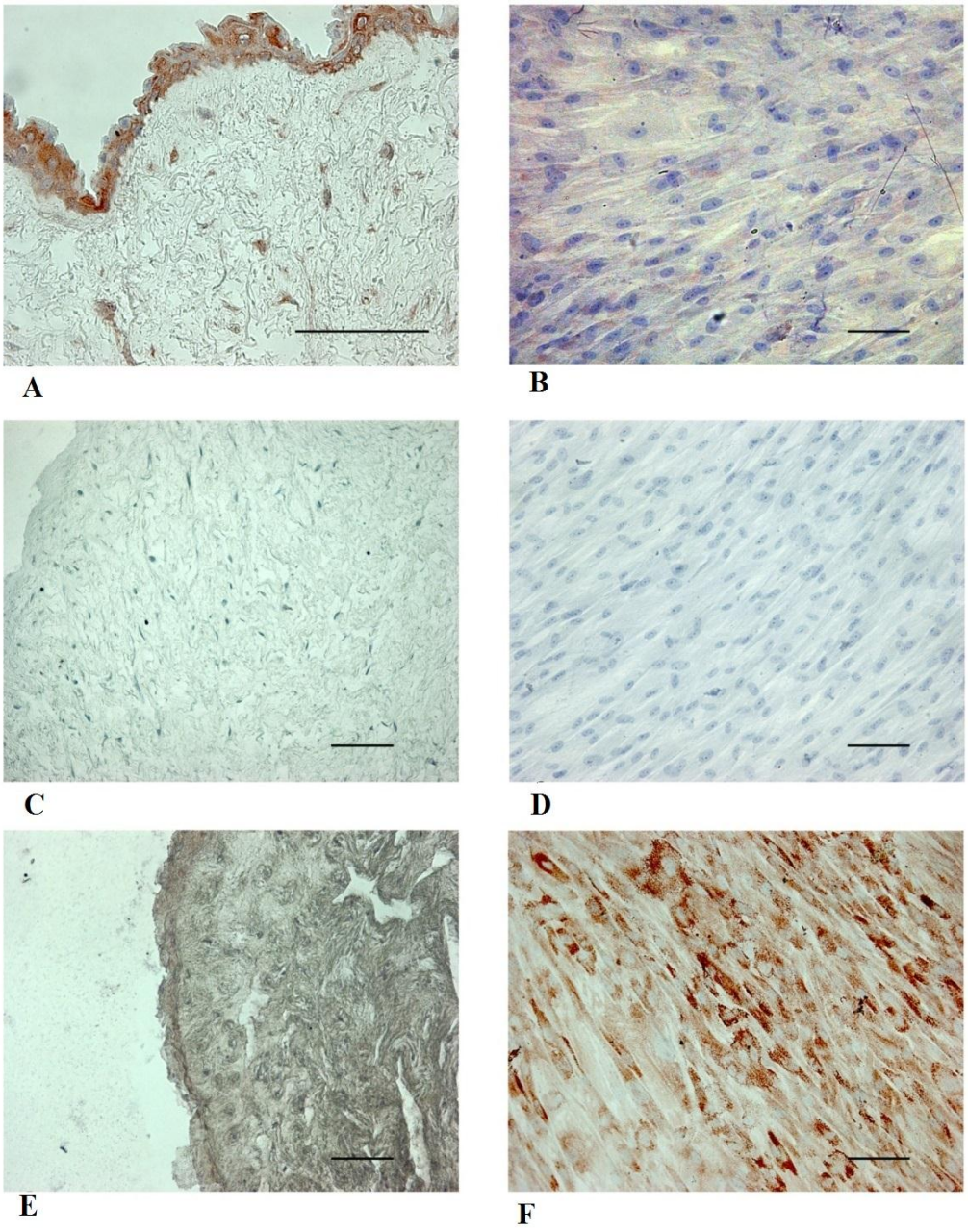

Fig.15 Representative panels of immunohistochemical / immunocytochemical detection of immune-related molecules in umbilical cord specimens and paired cultured cells. Both UC tissue and WJCs were positive for HLA-ABC (A, B) and HLA-E (E, F), while lacking detectable expression of HLA-DR (C, D). Magnification: 40x (A); 20x (B-F).

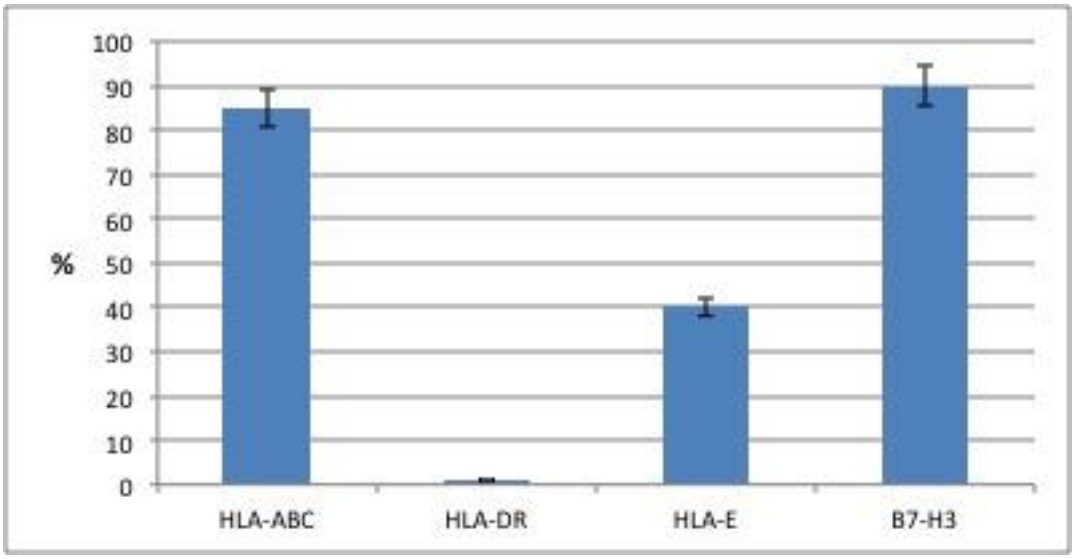

Figure 16: Flow cytometry analysis of the levels of expression of immune-related and immunomodulatory molecules in WJ-MSCs. As expected and confirming the results of ICC analyses, cells were amply positive for class I HLAs (HLA-ABC), negative for class II (HLA-DR), and showed the expression of both HLA-E 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 16, 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 both cord tissue (Figure 18 A) and cultured cells (Figure 18 B). We evaluated further 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, Fig. 19 (A and B). In addition, the early pregnancy factor (EPF) was also detected in the cord tissue and cultured cells (Fig.19 C and 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.

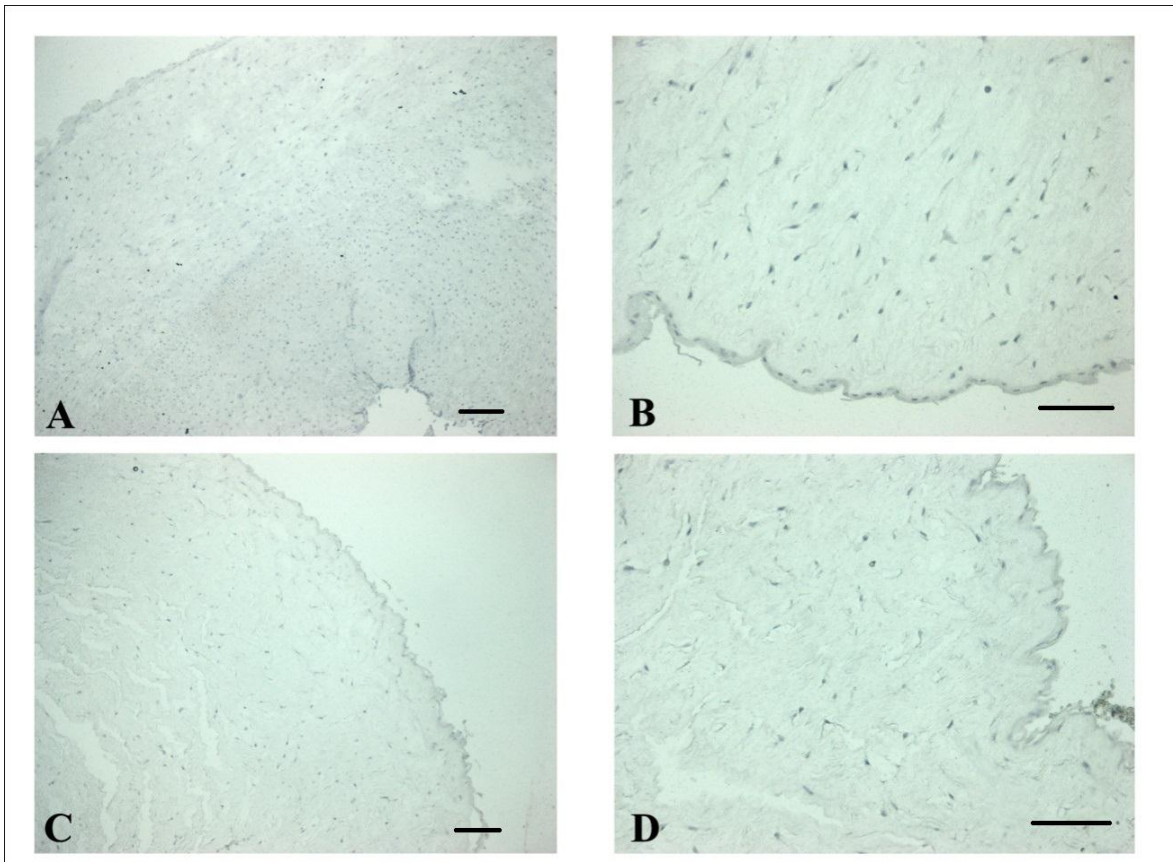

Fig.17: Representative panels of immunohistochemical /immunocytochemical detection of immune-related molecules in umbilical cord specimens Immunohistochemistry for B7-1 (A and B), B7-2 (C and D). Magnification: (A and C 10x), (B and D 20x).

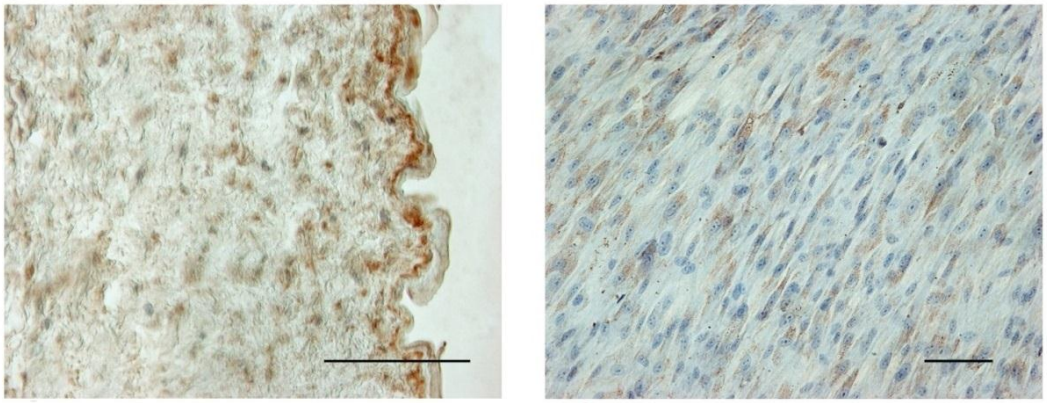

Fig.18: Representative panel of immunohistochemical / immunocytochemical detection of B7-H3 in umbilical cord specimens and paired cultured cells. Both UC tissue (A) and WJCs (B) were positive for B7-H3. Magnification 40x and 20x.

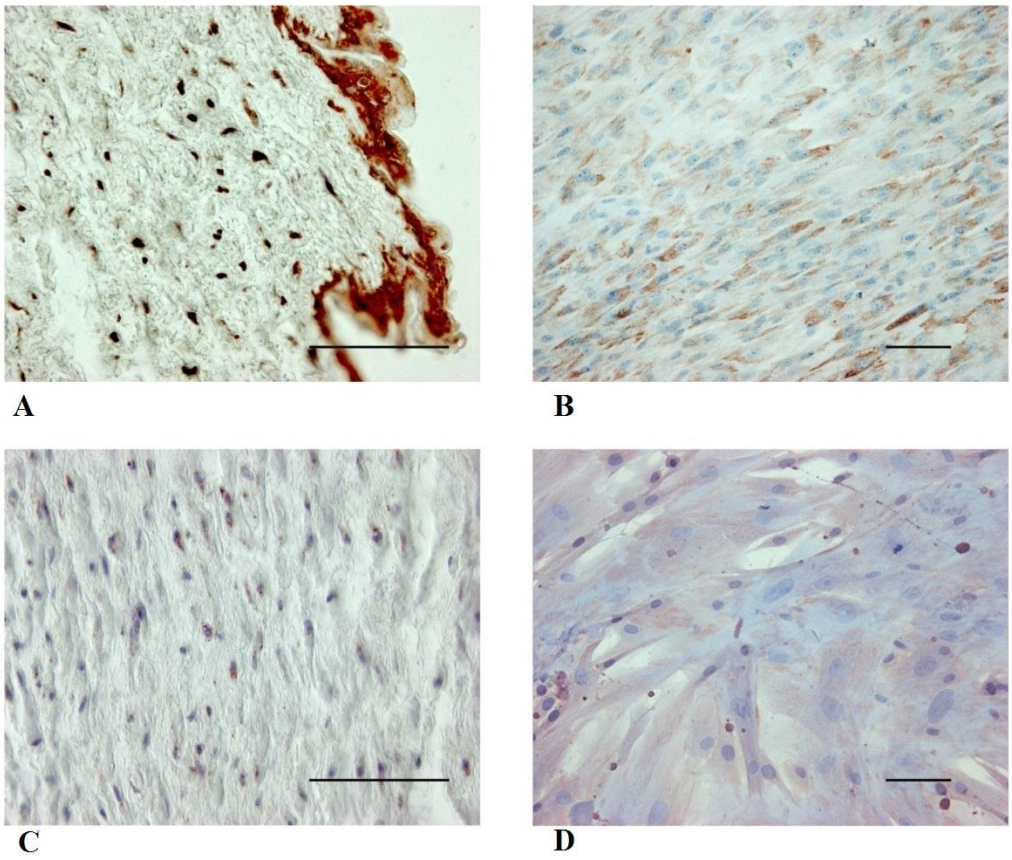

Fig.19: Representative panels of immunohistochemical / immunocytochemical detection of immune-related molecules in umbilical cord specimens and paired cultured cells. Both UC tissue (A,C) and WJCs (B.D) were positive for IDO (A, B) and EPF (C and D). Magnification 20x and 10x.

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

We analyzed some of the previously assessed markers by western blot, in order to confirm the data on proteins expression. 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 eventual supra-molecular complexes, which are not influenced by SDS presence. Figure 20 A shows that HLA-E is markedly present in all the passages considered, therefore confirming the results obtained with previous experiments. Interestingly, apart 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 20 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 20 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 20 D) showed that the 22 kDa precursor band is expressed in all the cell passages, while at passage 3 is also observable a 40 kDa band (the mature glycosylated form) and a higher molecular weight band (around 160 kDa).

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.

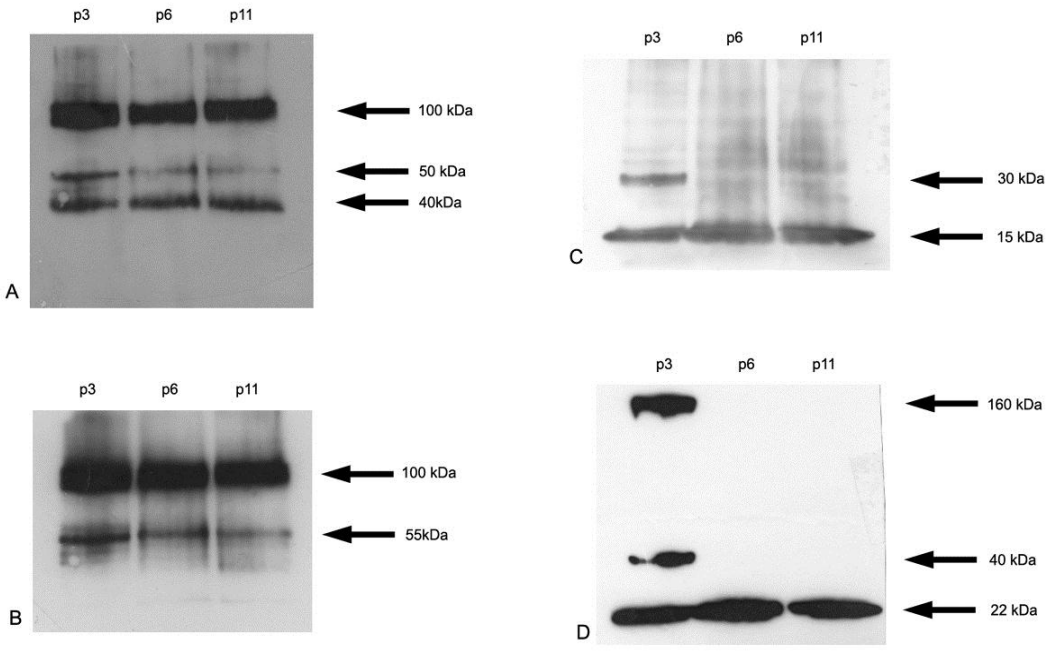

Figure 20: Panel of Western Blotting analyses for the expression of immunomodulatory molecules in WJ-MSCs. A: HLA-E; B: B7-H3; C: Galectin-1; D: LIF.

7.6 Neuronal markers expression of Wharton's jelly Mesenchymal stem cells (WJ-MSCs) by ICC

Another interesting datum, which can emerge from the analysis of the phenotype of undifferentiated WJ-MSCs, is their ability to express molecules belonging to several mature lineages, such as the neuro-ectodermal one. 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 21 shows the results of immunocytochemical analyses on cultured WJ-MSCs: cells were positive for the expression of Nestin (figure 21 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 is not surprising to find a very low number of NF68-expressing cells (figure 21 D) scattered between the mainly nestin-positive ones. WJ-MSCs do express also other markers of the neural lineage, as shown for NSE, neuron-specific enolase, (figure 21 A) and GFAP (glial fibrillar acidic protein, 21 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 represent a secreted molecule associated with the differentiation of islet cells in pancreas, as observed in cat development [107]. 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 [108].

Figure 22 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. As shown, 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 [109] was detected only in mouse tissues. The most abundant bands referred to the higher supra-molecular complexes that GDNF forms with heparin, 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.

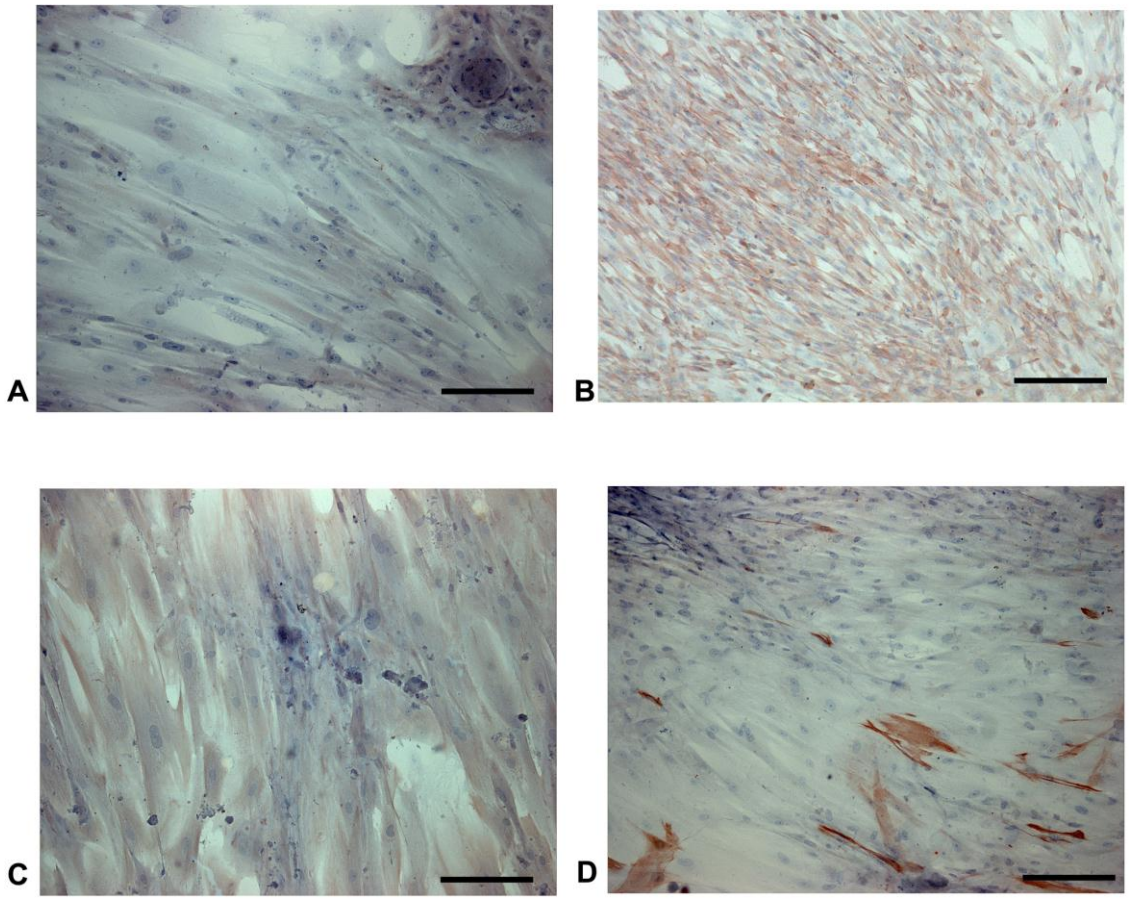

Figure 21: Representative panels of immunocytochemical detection of neural markers in WJ-MSCs. Cells were amply positive for the expression of NSE (A), Nestin (B) and GFAP (C). Scattered cells were also positive for the expression of the mature intermediate filament NF68 (D). Magnification: 10x.

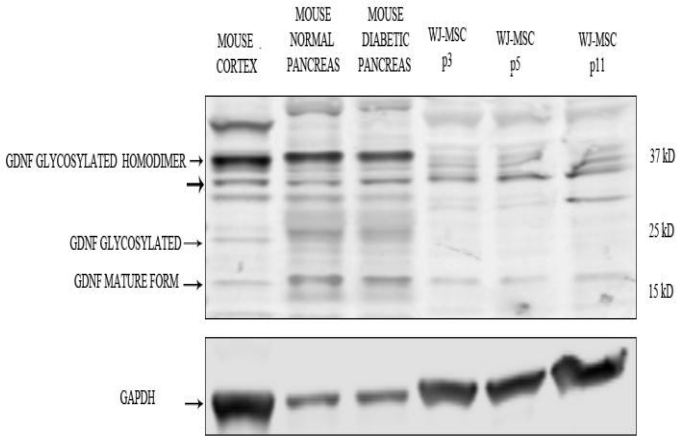

Figure 22: Western blotting analysis of GDNF expression in mouse tissues. GDNF was detectable as the mature form, which weighs 16kDa in all the considered specimens, the free glycosylated isoform was detected in mouse tissues but not in WJ-MScs. In all samples, the predominant forms were the numerous supra-molecular complexes with heparin, which range in MW between 33 to 45 kDa. GAPDH was used as control for protein expression.

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

As detailed in methods, mice of the two experimental groups were rendered diabetic with STZ injection. After three days, the 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 the blood glucose levels and body weight. As expected, the negative control mouse, which received only the 200 IEq transplantation, failed to revert diabetes, and died within three weeks from transplantation. Of the three mice that received WJ-MSCs together with 200 IEq islets, two reverted diabetes since two weeks after transplantation. Body weight, which is another important parameter to be monitored for diabetic animals, due to their tendency to progressive lose weight, also confirmed this tendency, with the control mouse losing weight progressively until death, and the mice receiving the WJ-MSCs co-transplantation gaining weight after transplant and remaining to normal levels until the end of the experiment.

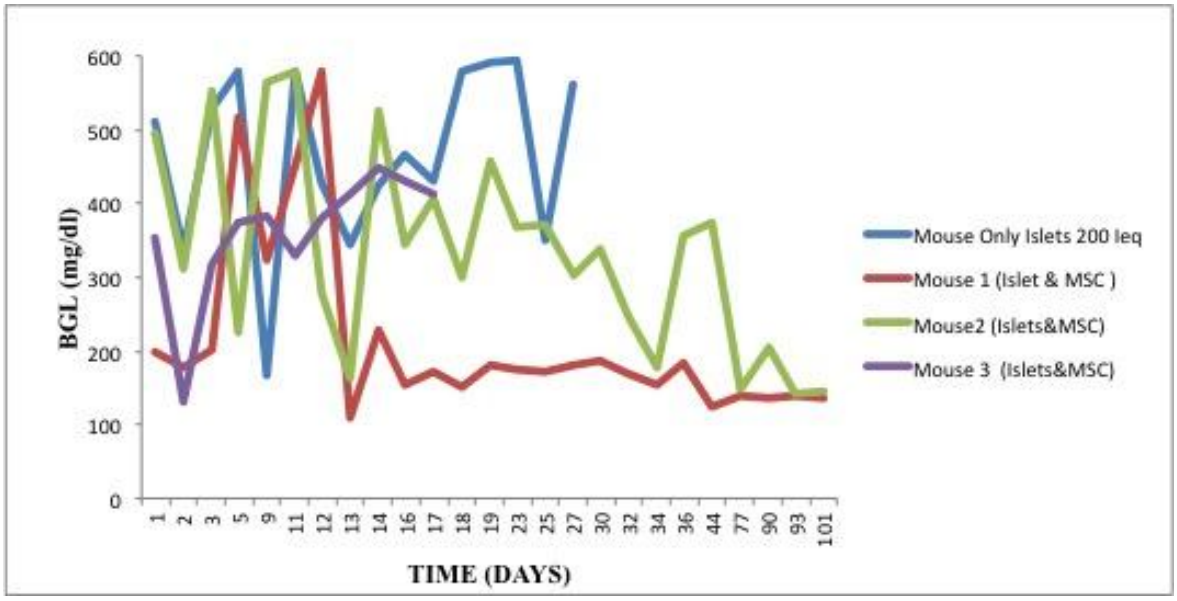

Figure 23: Graph depicting the blood glucose levels (BGL) of the animals during the co-transplantation experimental period.

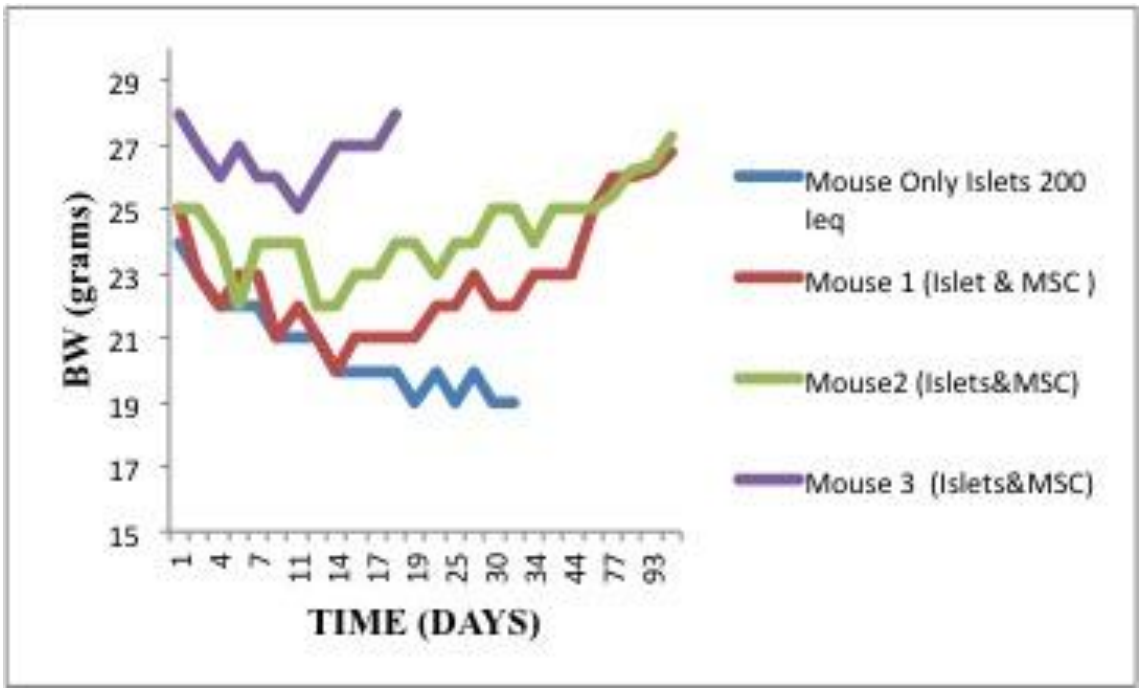

Figure 24: Graph depicting the body weight of the mice of the two experimental groups.

7.8 IPGTT results

To confirm the diabetes reversion of the two mice which were normoglycemic after three months, we tested the intra-peritoneal glucose tolerance test (IPGTT). Dextrose injection caused a peak in BGL after around 30 minutes, and then decreased slowly for the subsequent 1,5 hours. The day after, the values were fully normalized. There are contrasting data in literature [110] regarding the interpretation of the BGL after IPGTT, since fasting (which was the case of 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 were completely normoglycemic.

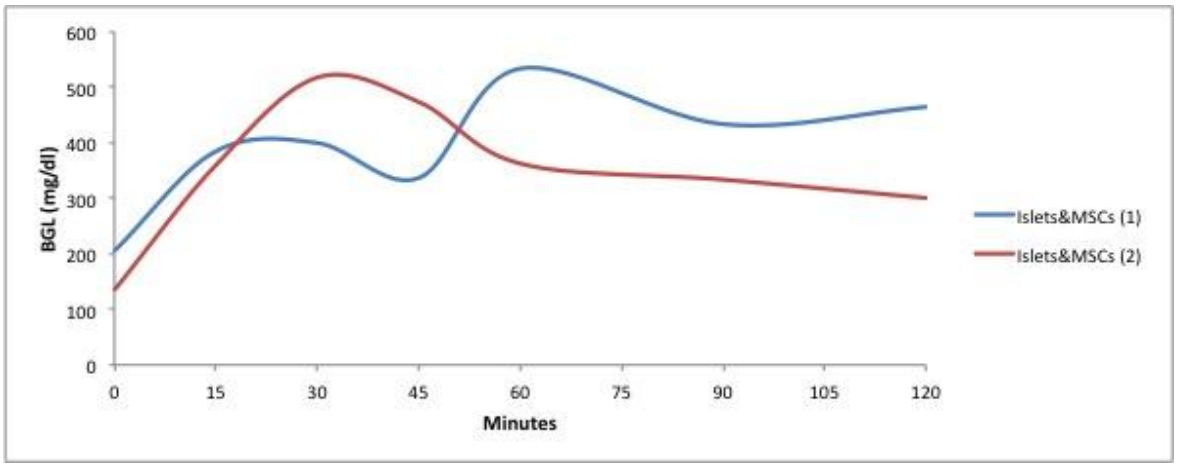

Figure 25: Graph depicting the results of IPGT test for the two mice of the experimental group performed after three months. The two mice reacted differently to the stimulation, with only one showing the characteristic peak around 30 minutes after injection. Both mice showed fully normal BGL values since the day after the test.

7.9 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 26, 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 revert diabetes, it is manifest that the presence of the co-transplanted cells favored islets survival and engraftment and ultimately diabetes reversal.

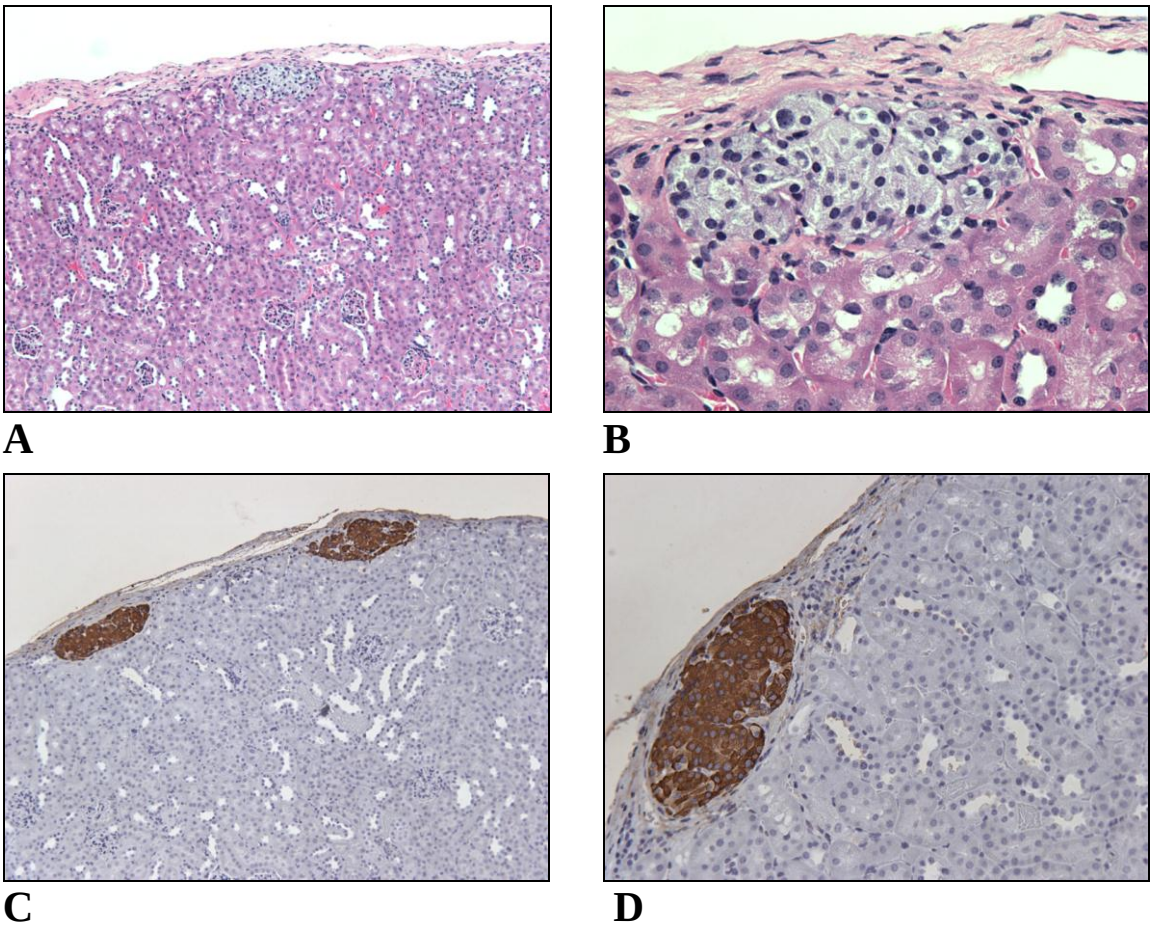

Figure 26: Localization of islets transplanted under the kidney capsule of mice receiving co-transplantation of WJ-MSCs. A, B: Hematoxylin/Eosin staining of islets detectable under the capsule of the transplanted kidneys. The islets show standard morphology. C, D: panels of immunohistochemical detection 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

Diabetes constitutes one of the major global health problems worldwide. Despite the advances in medical therapy, transplantation (either orthotopic or islet) remains a viable option. Stem cells have been proposed either as direct replacement of differentiated beta cells, or as supporting cells to stimulate the organ self-repair. The immunological milieu in which type I diabetes develop, constitutes a major challenge for any cell type to be infused in patients. Perinatal stem cells have recently emerged as promising populations, which may be reliably sourced from tissues discarded at birth. These cells, apart being able to differentiate in several mature types, have hypoimmunogenicity and immune modulation features that render them promising for transplantation in immunocompetent hosts without concurrent immune suppression. In particular, WJ-MSCs have been highlighted as cells which retain most of the immunomodulatory features of the perinatal tissues, which are fundamental for embryo and fetus survival.

This project stemmed from the need of a stem cells population, which may be successfully used in treating type I diabetes. We chose to explore a different mechanism of action, with respect to the classical repopulation-type approach, with WJ-MSCs to be used in co-transplantation with pancreatic islets, therefore maximizing their contribution in terms of anti-inflammatory and immunomodulatory molecules which may help islets engraftment and revascularization after transplantation.

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 terms of classical markers expression as well as the expected 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 for example 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 been limitedly attempted in the past, strongly suggested that the cells maintain,

even in the culture conditions, the same phenotype which they feature in the umbilical cord and warrants their properties *in vivo*. More importantly, since our aim was to use these cells as support populations in islets co-transplantation, we wanted to characterize in detail the expression of immune modulatory/anti-inflammatory molecules both in cultured cells and in the umbilical cord. Surprisingly, lot of 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 some new molecules expressed by the isolated cells, again in a conserved fashion with respect to the in organ situation, which may justify part of the properties these cells have in vivo when transplanted in immunocompetent 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 immunolocalization, 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. 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 and en route 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.

Finally, the last aim of this research project was the demonstration of the efficacy of WJ-MSCs in co-transplantation experiments with pancreatic islets in mice rendered diabetic by STZ. We explored the worst scenario, in which a sub-optimal marginal mass dose of 200 IEq (not effective alone to revert diabetes as shown by controls) was coupled to $3x10^6$ un-differentiated WJ-MSCs. 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 arrived at the end of the experiment with normal BGL and body weight parameters, therefore providing a formal proof of the efficacy of a limited number of stem cells in warranting the survival of islets in numbers sufficient to revert diabetes. IPGT test showed that also a massive dextrose injection, 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 coming from this research project confirmed the usefulness of WJ-MSCs even besides an eventual differentiation towards insulin-producing cells. 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 may open new paths for WJ-MSCs to be used in the therapy of type I diabetes and certainly open new questions on the many ways in which support to local populations by these cells may culminate in the promotion of self-repair.

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