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MESENCHYMAL STROMAL CELLS AS IMMUNOMODULATORS AND TROPHIC MEDIATORS FOR THE TREATMENT OF TYPE 1 DIABETES AND ITS COMPLICATIONS

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ABSTRACT

In Type 1 Diabetes (T1D), insulin-producing pancreatic islet beta cells are lost due to a T-cell mediated autoimmune attack. We hypothesized that Mesenchymal Stromal Cells (MSCs) derived from Pancreas (P) and from Pancreatic Islet (PI) modulate immunity, sustain beta cell function and survival. We generated a sizable cell bank with human and murine samples, now available to qualified researchers. We confirmed the mesenchymal nature of human PI-MSC (hPI-MSC) via immunophenotypic profiling and *in vitro* differentiation towards mesenchymal lineages. We observed that a subset of these cells expresses NG2, a marker of pericytes, and HLA-G, a molecule involved in immunomodulation. We studied the immunomodulatory activity of hPI-MSC *in vitro*. We observed that these cells can inhibit the proliferation of activated Peripheral Blood Mononuclear cells (PBMC), via mechanisms that include increased expression of HLA-G. Moreover, we found that hPI-MSCs have a potent effect on T cells, stimulating an expansion of Regulatory T cells (T regs). We studied the effect of hPI-MSC on beta cells. Remarkably, we found that hPI-MSC can boost the glucose-responsive insulin release function of islet beta cells, and they do so via release of soluble factors. We studied the effect of co-transplantation of murine P-MSC with islets in a stringent murine model of autoimmune T1D, via transplantation in the anterior chamber of the eye. This study allowed us to perform *in vivo* imaging with resolution at the cellular level. We performed a longitudinal study on the changes occurring in islets, GFP-tagged P-MSCs, revascularization, and immune cell trafficking. We observed a remodeling of the vascular structures, with contribution from mP-MSC. Ongoing and future *in vivo* experiments will be focused on long-term analyses of islet survival and immune cell trafficking. Considered altogether, these finding indicate that the pancreas and pancreatic islets represent a source of MSC that modulate immunity, sustain beta cell function, and could promote survival of islet endocrine cells in the setting of autoimmune diabetes.

In a separate project, we analyzed the effect of the secretome of placenta-derived MSCs in an *in vitro* Scratch Wound assay. The supernatant from one tissue source (undisclosed due to intellectual property rights) significantly accelerated wound healing in our *in vitro* model. This could have important potential for translational applications in the treatment of diabetic ulcers.

INTRODUCTION

The Pancreas

The pancreas is a glandular organ involved both in the endocrine system and in the digestive system. It is located in the abdominal cavity, it lies behind the stomach and it is connected to the duodenum by the ampulla of Vater, where the main pancreatic duct connects with the common bile duct (fig. 1).

The adult human pancreas measures 12-15 cm and weighs 70-150 grams. In humans, from proximal to distal, the organ is subdivided into four regions: head, neck, body and tail. Differently, in mice the shape of the pancreas is less well defined (fig. 2) (1, 2).

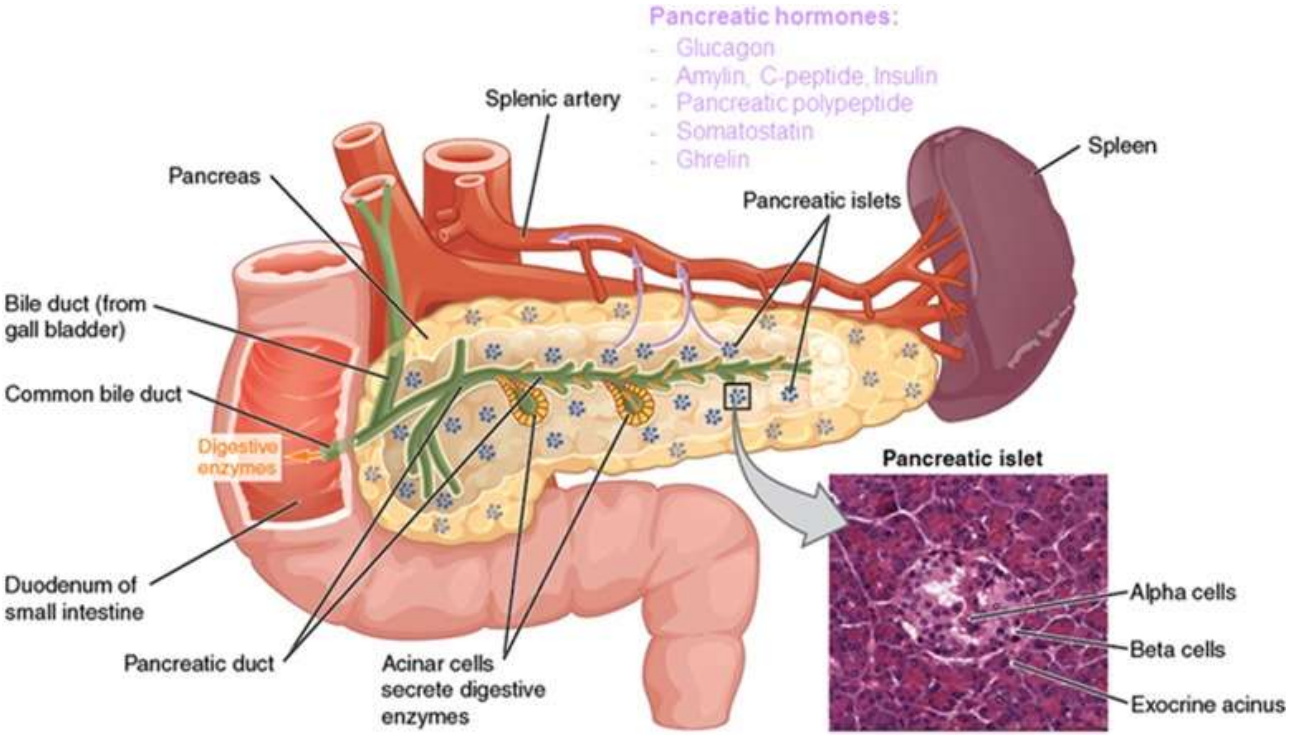


Fig. 1 - Anatomical organization of the human pancreas (3).

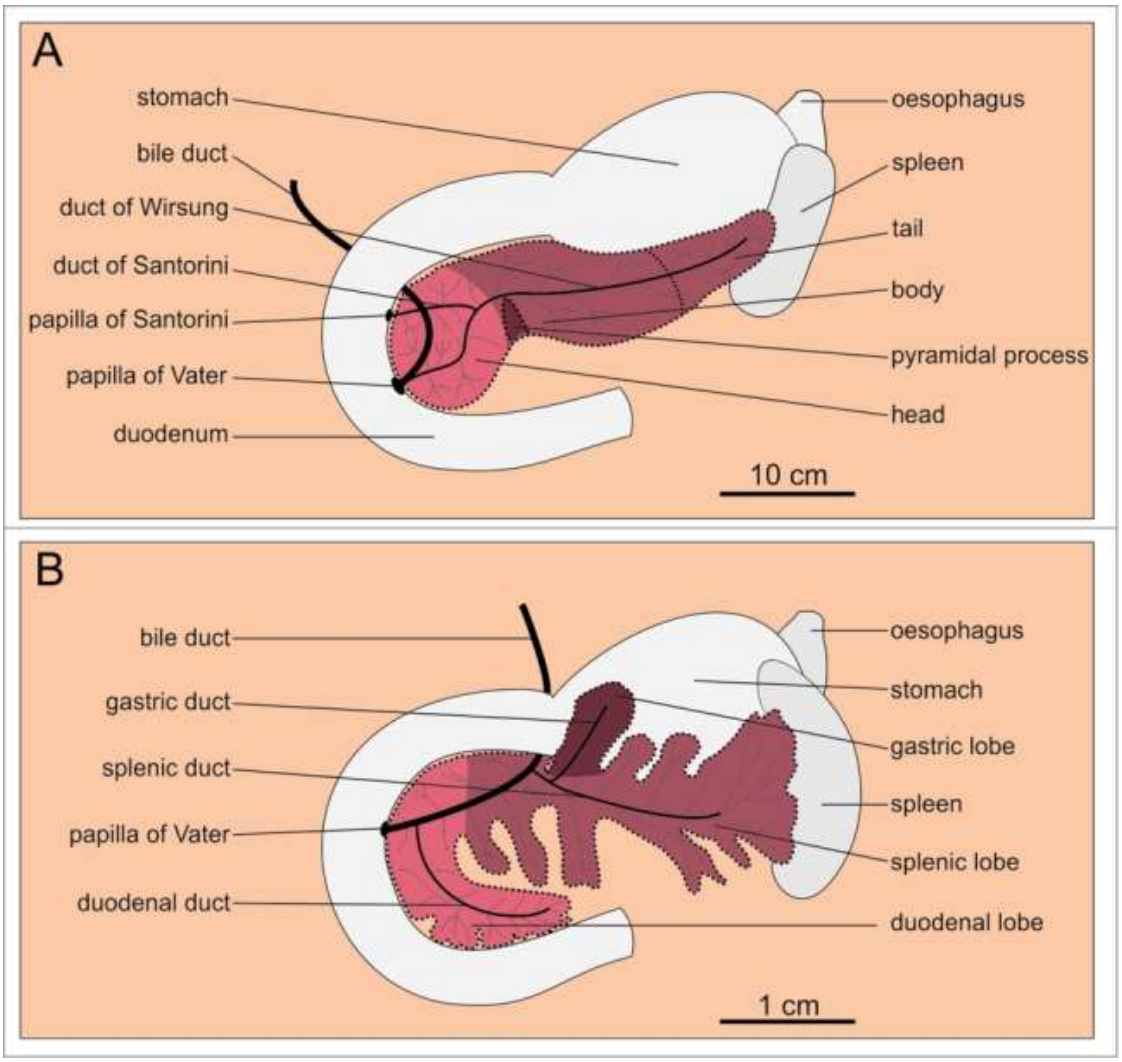


Fig. 2 - Macroscopic anatomy of the human pancreas (A) and of the mouse pancreas (B). The color coding indicates homologous parts (4).

The pancreas is characterized by a combination of exocrine cells that secrete enzymes in the digestive tract, and endocrine cells that secrete hormones in the bloodstream.

Both endocrine and exocrine cells arise from the same endoderm rudiment as a ventral and dorsal bud, which merge together (1).

The main portion of the pancreas consists of exocrine cells grouped into acini with several secretory granules containing the digestive enzymes that catalyze the breakdown of proteins, lipids and carbohydrates. After release from the acini, the pancreatic juice is transported through a ductal network into the duodenum (2). This is a hormonal-stimulated release, driven by secretin, cholecystokin, gastrin, and neural stimuli (1, 2).

The endocrine pancreas consists of the islets of Langerhans, clusters of hormone-producing cells embedded in the exocrine tissue. Pancreatic islets constitute 1-2% of the total mass of the organ, and most of them present diameters ranging between 50 and 200 µm.

There are approximately 1000 islets in a mouse pancreas, whereas the human adult pancreas may contain more than a million islets. Islets are highly vascularized, so that endocrine cells secrete hormones in a network of capillaries to exert systemic effect in response to change in blood glucose. Islets are composed by five main endocrine cell types (fig. 3): beta cells producing insulin, alpha cells producing glucagon, delta cells producing somatostatin, PP cells producing pancreatic polypeptide, and epsilon cells producing ghrelin (5). Endocrine cells are in contact with a dense network of endothelial and pericytic cells: while islets constitute only 1 to 2% of the pancreas volume, they receive 10-15% of the blood flow of the organ.

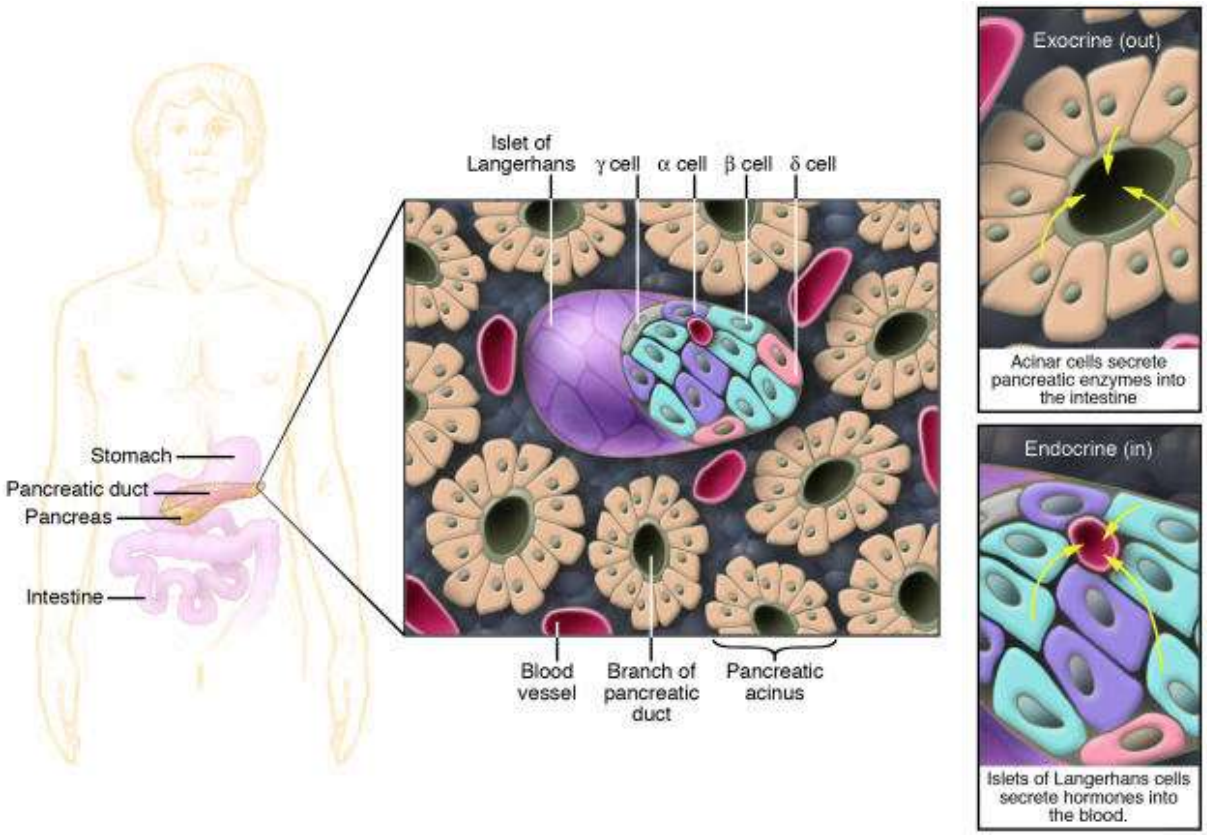


Fig. 3 - Cross section of the pancreas. The pancreas houses exocrine tissue, made up of acinar cells that secrete pancreatic enzymes delivered to the intestine to facilitate the digestion of food, and endocrine cells, aggregated in as islets of Langerhans, where glucagon, insulin, somatostatin, pancreatic polypeptide, and ghrelin are produced and delivered to the blood (6).

Cytoarchitectural differences between human and mouse islets have been described. Beta cells are the main cellular component of islets in most species, representing 60% to 80% of islet cells in rodents and 50% to 70% in humans. Mouse beta cells are located predominantly in the center of the islets, whereas alpha and delta cells are periphery. Instead, in human islets alpha cells are interspersed throughout the islet, and beta cells are intermixed with other islet cells.

These cytoarchitectural differences could explain why in primate organisms beta cell function is increased, as primate beta cells respond to low concentrations of glucose (1 mM) to which normal mouse islets are blind (7, 8).

Human Pancreas development and differentiation of Stem Cells

Mesoderm and definitive endoderm arise from the epiblast during gastrulation. The definitive endoderm then forms the primitive gut tube, embedded in mesenchyme and the notochord. The primitive gut tube is segmented into midgut and hindgut - that will form the colon and small intestine, and foregut - that will give rise to the lungs, esophagus, thyroid, stomach, liver and pancreas (9).

Fibroblast growth factor (FGF) 4 and retinoic acid, mediate the commitment of foregut endoderm to the pancreatic lineage (10, 11).

The notochord secretes activin and FGF2, which repress sonic hedgehog (Shh) signaling, leading to the appearance of PDX1 positive epithelium (12, 13).

The epidermal growth factor (EGF) stimulates the proliferation of early pancreatic progenitor cells, but inhibits endocrine differentiation. FGF ligands have a role consistent with that of FGF10 in mice, acting as mitogens of progenitor cells in the developing pancreas (14). WNT signaling also promotes the proliferation of human pancreatic progenitors (15, 16), as it does in mice (17).

The roles of Notch, retinoic acid and bone morphogenetic protein (BMP) signaling during human pancreas development are less well defined. Notch signaling is activated by FGF10 and affects the expansion of undifferentiated pancreatic progenitor cells expressing Pdx1, Nkx6–1 and p48/Ptf1a, as experiments in mice have shown (18). These cells give rise to the endocrine and exocrine compartments of the human pancreas. The acinar and ductal cells containing the exocrine tissue are specified by Wnt-activating ligands and release of FGF10, FGF7, laminin-1 and follistatin, in addition to Notch signals. Acinar differentiation is regulated by transcription factors including Ptf1a

and Mist1 (19). Ptf1a forms a complex with Tcf12 and Rbpjl, which allows the expression of genes for the secretory enzymes present in the mature acini (20).

Ductal cell-specific transcription factors are not well known, but HNF1B and HNF6 are thought to be active in this cell type. They express cytokeratin-19, cystic fibrosis transmembrane receptor, and carbonic anhydrase 2. Expression of the transcription factor neurogenin 3 (NGN3) increases with the emergence of human fetal β-cells whereas SOX9 is absent in endocrine cells (21) but not in acinar cells. The expression of NGN3 in human fetuses is transient and peaks toward the end of the first trimester and becomes undetectable after week 35 (22). Transcription factors, such as PDX1, NKX6-1, PAX6, NEUROD1 and NKX2-2, are also showed by endocrine cells starting at 8 weeks post-conception (10).

Epithelial progenitor cells migrate into the mesenchyme and form islets composed by of alpha, beta, delta, pancreatic polypeptide and epsilon cells.

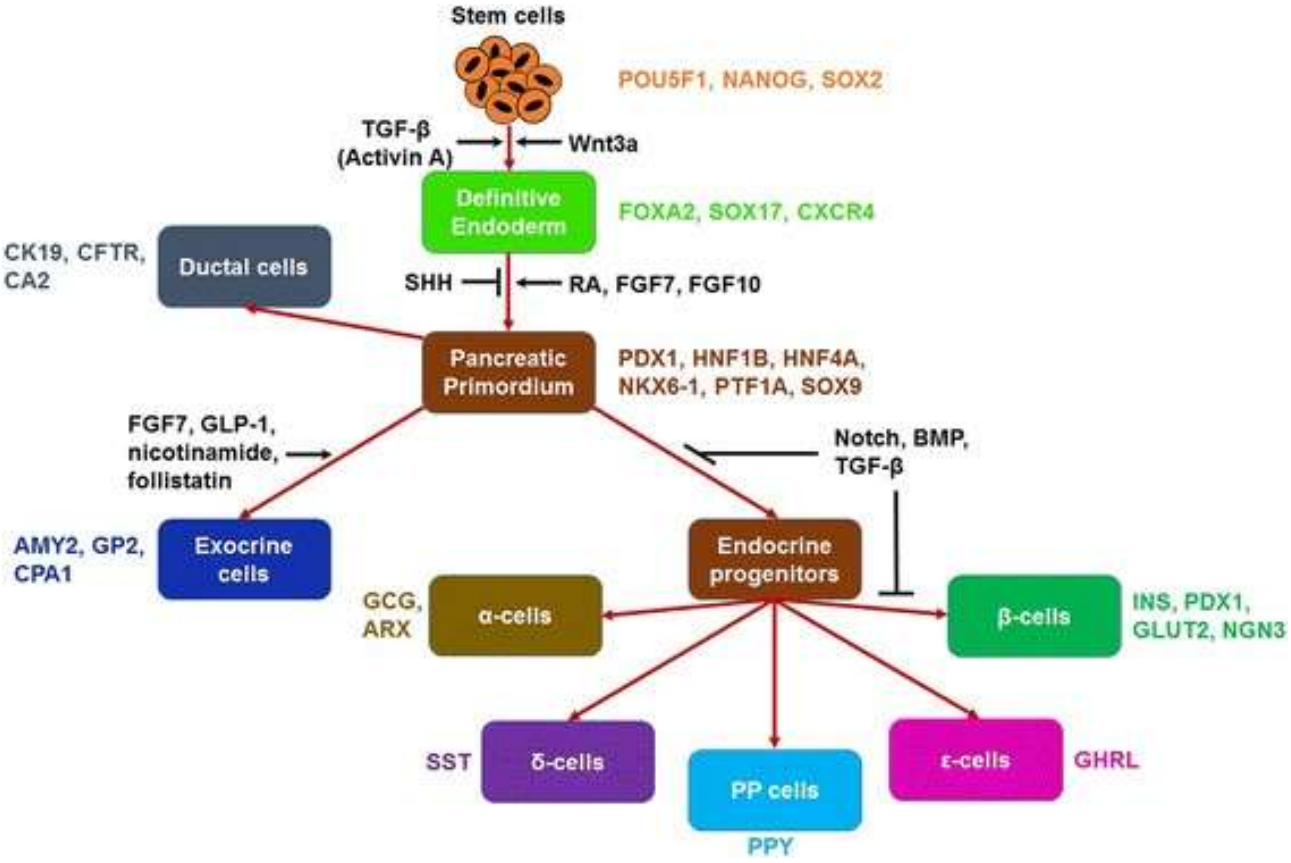


Fig. 4 - Pancreatic differentiation of stem cells. SHH: sonic hedgehog, RA: retinoic acid, FGF7: fibroblast growth factor-7, FGF10: fibroblast growth factor-10, GLP-1: glucagon-like peptide-1, BMP: bone morphogenetic protein (9).

Beta Cell function and Insulin

In humans, each islet of Langerhans contains 1000-3000 cells, with 50%- 80% of beta cells (23). These cells are centrally involved in the regulation of glucose metabolism. Beta cells produce and store insulin. In response to increased blood glucose levels, beta cells secrete insulin. Small increases of plasma glucose concentration (4.5-8 mM in human) lead to changes in insulin secretion within minutes. Insulin regulates both the uptake of glucose by muscle and fat cells, and the hepatic glucose output.

On the other hand, low levels of glucose lead to the production and release of glucagon by alpha cells: in liver cells, glucagon stimulates glucose mobilization from glycogen deposits, which leads to an increase in glycemia to bring levels back to normoglycemia (24).

Insulin is a dimeric SS linked protein composed by 51-aminoacids.

It is synthesized as a single chain precursor, the prohormone proinsulin (fig. 4), which loses its signal peptide firstly, and then loses also a segment known as the C-peptide, before becoming the mature hormone insulin.

The prohormone proinsulin is converted into mature insulin through the action of prohormone convertases (PC1, PC2, encoded by Pcsk1 and Pcsk2, respectively) during trafficking through the secretory pathway (25).

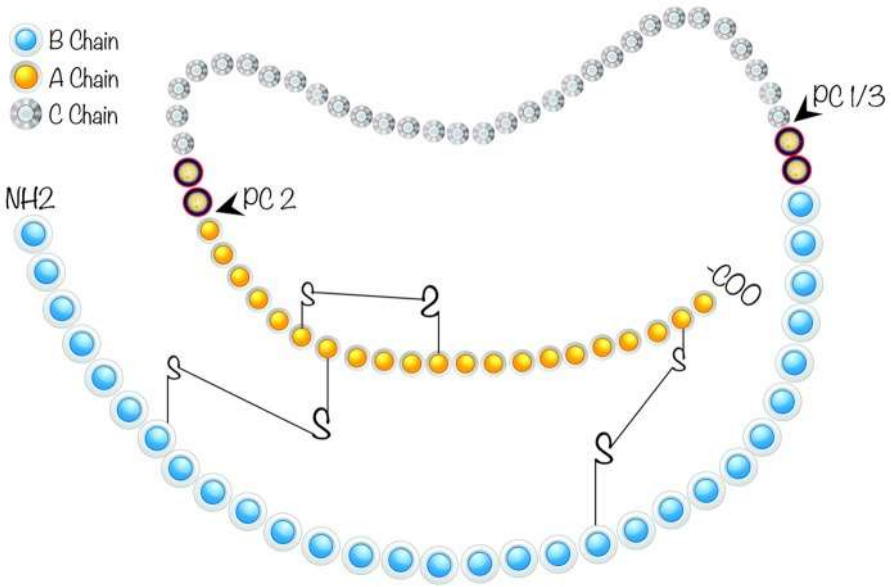


Fig. 5 - Schematic representation of human proinsulin. C-peptide, a 31 amino acid residue peptide, is depicted between A (21 aa) and B (30 aa) chains (26).

Mature insulin is stored in secretory granules, each containing at least 300000 molecules of insulin, and its release is tightly controlled by the level of glucose in the blood perfusion.

Glucose uptake from the extracellular environment is mediated by glucose transporters: GLUT1, 2 and 3 in humans, GLUT2 in mouse (27). Glucose is taken up via glucose transporters: it is metabolized in glycolysis and Krebs cycle, resulting in an increase of the cytoplasmic ratio ATP/ADP, leading to the closure of ATP-sensitive K+ (KATP) channels.

Highly localized changes in free calcium are believed to be important in this process. Calcium release from intracellular organelles including the endoplasmic reticulum and Golgi (mediated via inositol 1,4,5-trisphosphate, IP3), as well as secretory granules and lysosomes also appear to be involved in insulin secretion (25, 28).

The process described above represents the canonical pathway for glucose-stimulated insulin secretion, but further intracellular signaling events, independent of KATP channel closure, are also important.

Besides glucose, other important secretory 'potentiators' also exists: incretin hormones glucagon-like peptide-1 (GLP-1), glucose-dependent insulinotropic peptide (GIP), cholecystokinin (CCK), peptide YY (PYY) and oxyntomodulin, released from the gut in response to food transit (25).

In particular, GLP-1 reduces blood glucose through inhibition of glucagon secretion from pancreatic alpha cells. Moreover, GLP-1 is involved in the refreshment of the intracytoplasmatic insulin depots through enhancement of cAMP-mediated proinsulin gene transcription and mRNA stabilization. GLP-1 stimulates Pdx-1 gene synthesis and its binding to the insulin gene promoter. Pdx-1 is also responsible for the anti-apoptotic properties of GLP-1 (and its agonists) on beta cells. These gut-derived factors that enhance glucose-stimulated insulin secretion from islet beta cells are responsible for the augmentation of insulin release in response to food intake versus an identical change in glycemia imposed by intravenous injection of the sugar (25).

Human and rodent beta cells share many similarities, but there are also some differences between species. There is only one insulin gene in humans, whereas two genes encode insulin in rodents. Furthermore, in human islets alpha beta and delta cells are dispersed throughout the islet; differently, in murine islets, beta cells are found as a core surrounded by alpha and delta cells (23).

In addition, even if rodent and human beta cells share many common transcription factors, some differences are observed. Human beta cells also have a lower set point for glucose-stimulated insulin secretion compared with rodent beta cells (23).

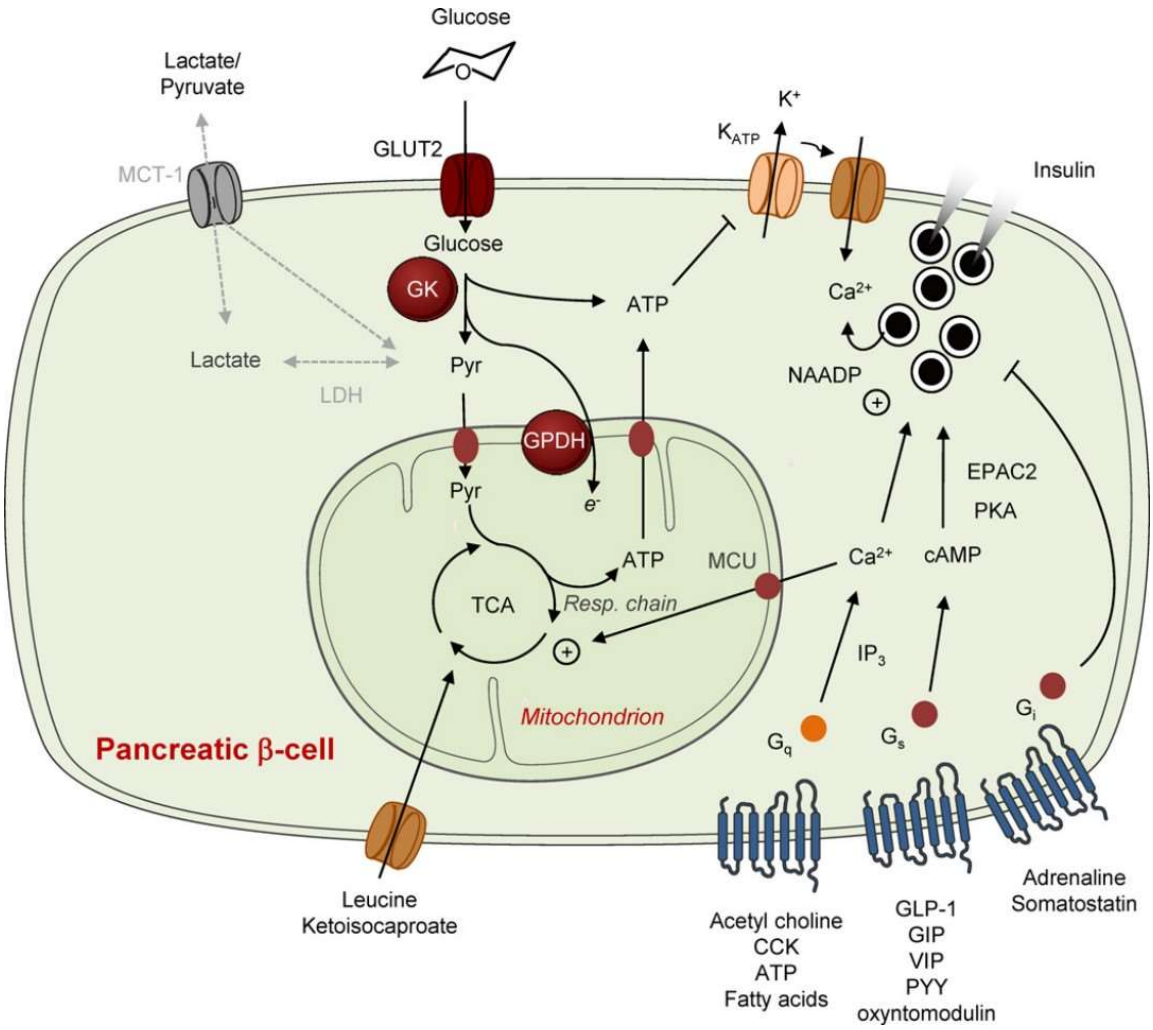


Fig. 6 - Overview of canonical signaling mechanisms involved in β-cell glucose sensing, and responses to secretory potentiators or inhibitors (25).

Type 1 Diabetes (T1D)

Type 1 diabetes mellitus (T1D) is a chronic disorder characterized by an immune-mediated destruction of insulin-producing pancreatic beta-cells.

T1D is one of the most common chronic diseases of childhood, slightly more common in males than females. T1D is increasing both in incidence and prevalence, with overall annual increases in incidence of about 2‐3% per year (29, 30). US data (31) suggest an overall annualized incidence from 2001 to 2015 of about 22.9 cases per 100 000 people among those younger than 65 years. Accordingly, data from other regions suggest similar incidences (32). The greatest increases in incidence of T1D are among children younger than 15 years, most of all in those younger than 5 years (33).

Although traditional T1D is classified as juvenile onset, it can occur at any age. Up to 50% of cases

occur in adulthood, and such cases might be initially misclassified as type 2 diabetes (T2D) (34, 35). The diagnosis of diabetes is based on a fasting blood glucose concentration higher than 126 mg/dL, a random blood glucose concentration above 200 mg/dL with symptoms, or an abnormal result from an oral glucose tolerance test (36). In 2009, the American Diabetes Association had also included glycated hemoglobin (HbA1c) concentration above 48 mmol/mol (6.5%) (37).

Despite the effort to standardize the diagnosis, diagnosis of T1D versus T2D can be challenging. An important distinguishing feature between T1D and T2D is the presence of autoantibodies against β-cells. More than 90% of individuals with a recent diagnosis of T1D have one or more of the following autoantibodies at disease onset: antibodies reactive to insulin (IAA), glutamic acid decarboxylase (GADA), insulinoma-associated autoantigen 2 (IA2A) and zinc transporter 8 (ZnT8A). Development of type 1 diabetes-associated autoimmunity may occur months or years before the onset of symptoms, and therefore autoantibodies screening can help in the identification of people with an increased risk of disease.

T1D is a polygenic disorder, with at least 40 loci known to affect disease susceptibility.

Most of these loci are thought to involve immune responses, so genetic influences include mechanisms that collectively contribute to aberrant immune responsiveness, as the development and maintenance of tolerance (38, 39).

The HLA region of chromosome 6 provides maybe one-half of the genetic susceptibility, and among HLA types, HLA class II shows the strongest association with T1D.

Pancreas or islet transplantation can revert diabetes, but require chronic immunosuppression, and both chronic rejection and recurrence of islet autoimmunity may affect long-term graft survival (40).

In most patients, autoantibodies targeting islet cells appear in peripheral blood (41) long before the clinical onset of diabetes, and they are believed to mark the activation of the autoimmune process. The risk of T1D increases when the number of autoantibodies increases (41, 42).

Insulitis, the inflammatory lesion affecting pancreatic islets in T1D, consists mostly of an autoreactive T lymphocytes population reacting against islet cell autoantigens (43, 44). Beta cells become progressively impaired and this leads to the progression towards of T1D (45).

Beta cell turnover can be detected even several decades after T1D onset (46), suggesting that repair and regeneration processes occur in the diseased pancreas. Unfortunately, this turnover is insufficient to counterbalance the loss of beta cells resulting from autoimmunity (40).

In autoimmune T1D, beta cells seem to have an important role in generating autoantigens. Their secretory granules become available to the immune system, as beta cells transfer vesicles containing insulin and its catabolites to islet phagocytes for presentation to T cells (40, 47).

The reason why we find immune cells targeting beta cell antigens is not completely understood, but there is growing evidence that autoimmunity may be triggered by modified forms of self-antigens. Chemical reactions, inflammation, cell death, viral infection or other environmental factors may facilitate the generation of such modified forms of self-peptides (40).

The non-obese diabetic (NOD) mouse is a particularly well characterized experimental model for T1D. In female NOD mice, insulitis starts at 3 weeks of age, concomitantly with the appearance of initial thymic alterations, and the majority these mice spontaneously develop autoimmune diabetes at 10–12 weeks (48).

The thymus is a primary lymphoid organ where T cells are generated. Starting from precursor cells of hematopoietic derivation, T cells mature into CD4 or CD8 single-positive thymocytes ready to emigrate to peripheral lymphoid organs and properly finish their maturation (49).

Although the exact mechanism underlying how differentiating thymocytes evolve to autoreactive Tcells destroying beta cells are not elucidated, several alterations are observed in the NOD mouse thymus about the expression and role of molecules involved in cell adhesion and migration, peptide hormones under control of the AIRE gene and miRNAs (48).

Lower insulin levels in the thymus are related with increased reactivity to insulin in the periphery, including in NOD mice (50). Together, these mechanisms can shape the T cell repertoire and change the frequency of Tregs and the ratio of Treg and effector T cells (51, 52).

Regulatory T cells are important for the maintenance of immune tolerance in the periphery, and therefore their dysfunction has been implicated in the pathogenesis of both T1D and other autoimmune diseases (53). NOD Mice that lack Tregs due to Foxp3 deficiency rapidly develop autoimmune diabetes due to an immediately destructive insulitis (54). *In vivo* ablation of FoxP3+ T regs also unleashes autoimmune lesion within the pancreatic islets (55). Reduced numbers of regulatory T cells (Treg, CD4+ CD25+ FOXP3+) are observed in peripheral lymphoid organs and pancreas in NOD mice; this appear to affect the function of effector T cells (48). In T1D patients, FoxP3+ Treg seem to occur in (slightly) lower numbers (56-58) and their suppressive function appear to be impaired compared to healthy controls (58-60). These observations are still limited to analyses of peripheral blood and are still a matter of debate (61-63). Studies using both mouse models and human samples have demonstrated that the failure of Treg-mediated tolerance in autoimmune

diabetes is largely attributable to an increased resistance of effector T cells to Treg-mediated suppression rather than a direct defect of the Tregs (64-67). T reg functions could thus be bolstered to move the tip of the balance. Studies of T reg modulation have highlighted the potential of this strategy in limiting autoimmune diabetes and controlling immune responses targeting islet beta cells.

Despite the advances in T1D research and therapy, researchers and clinicians are disappointed by a perceived lack of progress. The discovery of insulin in 1921-22 was the most important event in the history of type 1 diabetes therapies. However, exogenous insulin replacement does not always provide the metabolic regulation necessary to avoid complications associated with T1D. Diabetes management in modern countries often includes the use of insulin analogs and mechanical technologies like insulin pump.

The first pancreatic transplantation was attempted by Kelly et al in 1966 (68); afterward more than 25.000 transplantations have been conducted worldwide. Rapid control of hyperglycemia with consequential discontinuation of the exogenous insulin supplementation was noted in successful cases. However, the major drawbacks were significant morbidity and mortality related to the major surgery and digestive nature of the organ, as well as requirement of life-long immunosuppression, which has severe side effects - including recurrence of diabetes. Another approach studied and developed by Ricordi and other investigators is the isolation and transplantation of pancreatic islets (69). Safety of this protocol is significantly superior to whole pancreas transplantation, but there are limitations that make insulin-free survival for more than 5 years extremely rare (70). Other modalities of islet encapsulation, immune modulation, and delivery techniques are currently being developed.

Long-term restoration of immune tolerance is paramount to the survival of residual endogenous or implanted islets. Two types of approaches have been evaluated to achieve this goal. The first type aims to eliminate parts of the immune system, such as T and B cells. This kind of approach is quite useful, but patients became susceptible to infections and cancers. Furthermore, autoimmunity usually resurges after the discontinuation of the treatment. Considering the safety and efficacy of current diabetes care, this is not a viable long-term solution for the majority of T1D subjects. The second type of approach aims to specifically eliminate the immune cells responsible for the β-cells destruction by exploiting their beta cell antigen specificity. A combined approach will probably be necessary: on one hand it will be important to control the immune system to avoid recurrence of

autoimmunity, and on the other hand, a source of insulin will be required to control blood glucose (71).

Cell-based strategies for Diabetes

As previously mentioned, glucose metabolism and glycemia are controlled by the secretion of insulin from pancreatic islet beta cells. Beta cells can be lost, can be impaired, or can become impaired due to very different mechanisms. The lack or insufficiency of their insulin release function leads to a group of diseases with characteristic pathological features: abnormal metabolism of carbohydrates and elevated levels of glucose in the blood and urine (72).

Type 1 Diabetes (T1D) is a multifactorial chronic disorder that is characterized by a T-cell mediated autoimmune attack of insulin-producing pancreatic beta cells: the disease becomes clinically overt when the vast majority of beta cell lose function or are lost (38, 42, 45). To date, there is no definitive cure for this disease and life-long exogenous insulin replacement is required (73).

Differently, Type 2 Diabetes (T2D) is characterized by insulin resistance, hyperglycemia and eventually dysfunction of the insulin-producing cells, and it is mainly caused by diet and lifestyle choices (74).

In the most severe cases of T1D, treatment with exogenous injected insulin is not sufficient to reach an effective control of glycemia and glucose metabolism. Severe hypoglycemic events and hyperglycemic states become common in these patients, which can lead to life-threatening complications. Allogenic transplantation of cadaveric pancreas or pancreatic islets can correct diabetes restoring normo-glycemia in T1D patients (75-79). Unfortunately, several factors limit these transplantation strategies: lack of donors, need for immunosuppression (often characterized by serious side effects (80)), immune rejection, recurrence of autoimmunity, and limited islet graft (81, 82). The identification of an inexhaustible source of transplantable insulin producing beta cells is an important hurdle that still needs to be overcome (81), but the recent activation of clinical trials testing the safety of Embryonic Stem Cell-derived Pancreatic Progenitor cells represents an important milestone in that direction (NCT 02239354, ClinicalTrials.gov). Beta cell replacement would be extremely beneficial for T1D patients, and beta cell supplementation could be beneficial for a subset of T2D patients. Nevertheless, patients with T1D and T2D would benefit from strategies that modulate immunity and inflammation, that protect or sustain beta cells, that improve islet transplantation and that stimulate angiogenesis. During the last decade, a cell population has

catalyzed significant interest and has been tested in a number of clinical trials for diabetes: Mesenchymal Stem/Stromal Cells (MSCs) (83). MSC possess important characteristics that could be exploited in cell-based strategies for T1D and for complications of T2D. These cells showed a good safety profile in initial clinical trials for T1D and T2D. In order to maximize their therapeutic efficacy, important considerations related to the harvesting site and to donor derivation need to be made.

Mesenchymal Stem/Stromal Cells

Mesenchymal Stem/Stromal Cells (MSCs) were first described in the 1970s by Friedenstein et al (84) who isolated a population of cells from mouse bone marrow (BM) and showed these had the ability to form colonies. About twenty years later Caplan (85) defined the corresponding terminology, 'Mesenchymal Stem Cells', and approximately ten years later MSCs were identified in human adult BM (86, 87). MSCs are characterized by the adherence to plastic in culture, expression of a set of surface markers in the absence of lineage-specific marker expression, and potential to differentiate into multiple mesodermal lineages (osteoblasts, adipocytes, and chondroblasts) (88, 89). MSCs are potent immunomodulators, exerting suppressive functions on immune effector cells and orchestrating the action of other regulatory cells (90-98). MSCs are able to migrate to sites of inflammation and to regulate the traffic of different hematopoietic cells (99). Moreover, MSCs have been shown to promote repair and regeneration of endogenous and transplanted islets (83, 100, 101). Furthermore, they have shown a good safety profile in clinical trials, including a very limited risk of tumor formation (101-103).

The functional capacity of MSCs, together with their responsiveness to inflamed or damaged micro-environments, have made them an attractive potential agent for many regenerative, anti-inflammatory, and auto-immune applications for a wide range of disorders (104). MSC-based therapies for T1D are mostly focused on alleviating hyperglycemia by inhibiting autoimmunity, stimulating pancreatic beta cell regeneration and function. MSC-based therapies for T2D are more focused on the treatment of co-morbidities (105). The main therapeutic effect of MSCs seems to derive from their release of cytokines and soluble factors - molecules determining immunosuppressive, anti-inflammatory, pro-angiogenic and pro-regenerative changes (106). Alleviation of hyperglycemia seems to be the net result of a dampening of the immune responses, along with a stimulation of the survival and of the proliferation of pancreatic progenitors and/or

beta cells (107). MSC transdifferentiation towards insulin producing beta cells is not considered a major therapeutic mechanism.

The pathways determining anti-inflammatory and immunosuppressive effects have not been completely elucidated, but inhibitory interaction via direct contact with effector cells, the production of soluble mediators and the activation of regulatory cell subtypes, all may contribute to the MSC effect (90-98, 108, 109).

MSCs can inhibit dendritic cells differentiation and maturation, suppress the proliferation of CD4+ and CD8+ T cells, impair the cytotoxic activity of cytotoxic lymphocytes, induce and expand T regulatory cells, and can balance T helper subsets (110, 111). Thanks to the interaction of MSC receptors with ligands indicating inflamed environments, MSCs selectively home in inflamed tissues and promote tissue repair and regeneration (106). MSCs secrete several molecules (such as IL-6, IL-8, TGF-beta, TIMP-2, VEGF, HGF) which can stimulate tissue repair and act as chemo-attractants, recruiting macrophages and endothelial cells at the site of injury or inflammation (112, 113). MSCs also appear to have angiogenic and trophic potential that improve, in a co-transplant setting, the ability of pancreatic islets to survive the first few days after transplantation (81).

In fact, several models of islet transplantation showed positive effects of MSCs in promoting engraftment and increasing survival and function of beta cells (114). MSCs from recipient rats mediated such an effect when co-transplanted with allogeneic islets, resulting in long term survival and sustained normoglycemia (115). The effect of MSCs in this model could be due either to an anti-inflammatory effect or an immunomodulatory effect, or to a combination of both.

The positive effect observed in this study was paralleled by increased neoangiogenesis at the implant site, a key observation that highlights the multiple mechanisms of action of MSCs (115, 116).

In stringent models of transplantation in fully allogeneic recipients, the co-administration of MSCs with islets led to highly significant prolongation of graft survival in rodent models (117, 118). In nonhuman primates, allogeneic MSCs significantly enhanced engraftment and function of co-transplanted islets (119). In a subset of animals, additional infusions of MSCs resulted in reversal of rejection episodes and prolongation of islet function (119).

Several clinical trials are currently testing MSCs transplantation in patients with T1D and T2D (83, 101, 120). So far, MSC transplantation has showed a good safety profile with a very limited risk of tumor formation (101).

An open label pilot trial (121) enrolled T1D patients with recent onset of diabetes. Twenty patients were randomized to the group receiving transplantation of autologous bone marrow-derived MSCs

(BM-MSCs), or to the control group which only received insulin therapy. The treatment was found to be safe; moreover, at one year after MSCs infusion, most of the patients treated with MSC transplantation showed the preservation of stimulated C-peptide secretion, a key marker of the insulin release from residual beta cells, whereas the control patients showed a decline in C-peptide levels. Patients in both the treated and control groups continued to require insulin therapy and there were no statistically significant differences in insulin requirements and glycated hemoglobin levels between the two groups.

Ongoing clinical trials are also testing the safety and efficacy of MSCs transplantation in patients with T2D (83, 100) in order to treat common complications of diabetes such as ulcers, limb ischemia, and nephropathy, and to improve metabolic control (100, 122, 123). Treatment with MSCs was reported to be safe, it appeared effective in facilitating wound closure of diabetic foot ulcers (124) and in inducing T reg cells in T2D patients (125). Allogeneic placenta (PL)-derived MSCs were transplanted in 10 patients with T2D and the infusion was reported to be safe. The patients experienced a reduction in daily insulin requirement, showed a better control of blood glucose fluctuations, and experienced improvements in quality of life (126). Interestingly, a recent meta-analysis on clinical reports highlighted that the tissue source of the MSCs impacts the outcome of the cell therapy (127).

The effect of MSCs is largely mediated by their secretome (Fig. 7). MSCs can promote the survival and function of islet beta cells. Moreover, they can stimulate islet revascularization and oxygenation. Furthermore, MSCs can protect islet beta cells from allogeneic and autoimmune responses. MSCs exert multiple immune-modulatory functions, including an inhibition of Effector T cell functions and of DC differentiation, and a stimulation of T reg functions. MSCs could be employed in therapeutic strategies for T1D and T2D: they could be utilized to protect and sustain endogenous or transplanted islet beta cells. Moreover, MSCs could be employed to stimulate angiogenesis and inhibit inflammation in complications of T1D and T2D.

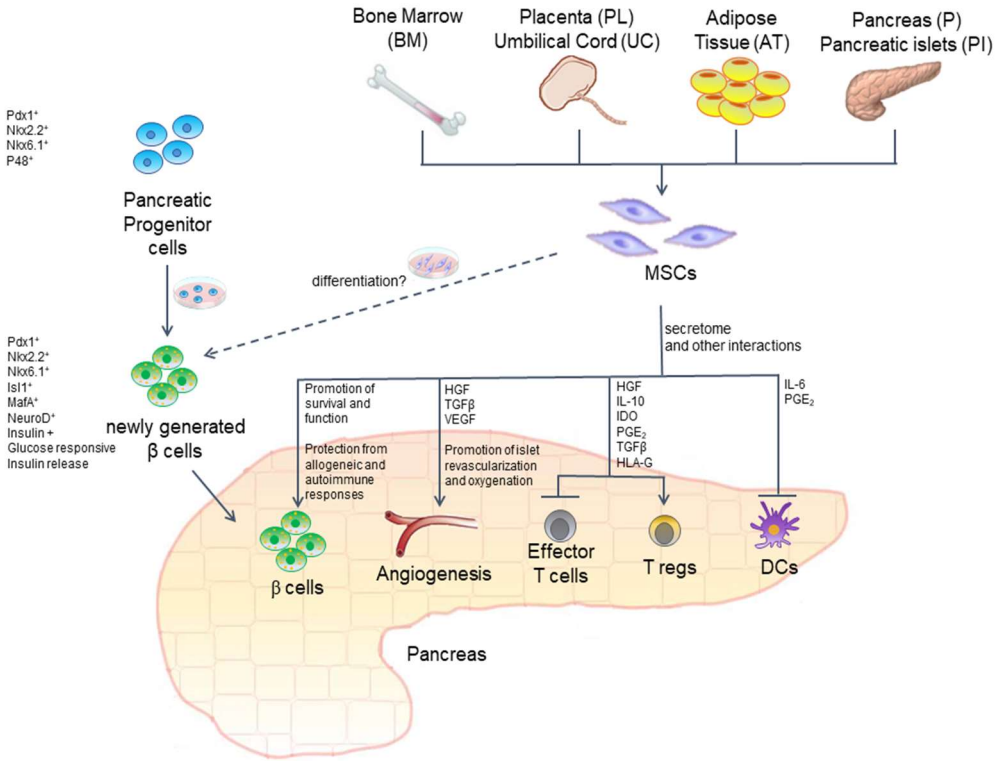


Fig. 7 - Effect of MSCs mediated by their secretome (128).

MSC-derived exosomes and Diabetes

Exosomes (EXOs) are nanoscopic (30–100 nm) biological entities that are secreted as vesicles in the extracellular environment by many different types of cells (129), including MSCs. MSC-derived exosomes (MSC-EXOs) are emerging as a new important paracrine mechanism for cell-to-cell communication, implicated in wound healing, injury and tissue repair. They are known to contain proteins, mRNAs and microRNAs (130, 131); moreover, they have immunostimulatory and immunoregulatory functions (129, 132, 133). Certain EXOs and cargos present molecular signatures of pathological processes and could be implicated in the pathogenesis of multiple pancreatic diseases, such as T1D, T2D, Diabetic nephropathy, Diabetic retinopathy, Gestational diabetes mellitus, and Pancreatic cancer (134, 135). EXOs can be easily isolated from different body fluids collected by non-invasive methods and therefore have the potential to be utilized for the analysis of disease biomarkers. EXOs can also be easily collected from the supernatant of *in vitro* cell cultures. EXOs derived from MSC cultures were shown to promote regulatory T cell (T reg) activity, inhibit Effector T cell, Natural Killer (NK) cells and Dendritic Cells (DCs) activities (136, 137). The advantages of using EXOs instead of live cells are connected to their minimal immunogenicity

(allowing an allogenic use), low inherent toxicity (138), and potentially lower risk for tumor formation (139). Moreover, because of their chemical composition and small size, EXOs may easily diffuse across the biological barriers reaching target cells. A common assumption in the context of T1D is that imbalances between Effector T cells and T regs, as well as DC presentation of islet auto-antigens, play a major role in the destruction of islet β cells (140, 141). The beneficial effect of MSCs for the treatment of T1D derives largely from their immune-modulatory and anti-inflammatory secretome. Therefore, MSC-EXOs might be employed as immune modulators in MHC-mismatched recipients, overcoming the potential immunogenicity of MSC in an allogenic setting (138). EXOs/microvescicles derived from endothelial progenitor cells combined with islets can activate angiogenesis improving revascularization and pancreatic beta cell function (142). The same study observed that EXOs/microvescicles also inhibited endothelial-leukocyte interaction. MSC-EXOs could have similar proangiogenic effects. Sheng et al (143) showed the other side of the coin: insulinoma-derived EXOs contain diabetes-triggering autoantigens that may stimulate autoreactive T cells inducing inflammatory cytokine secretion and activating antigen presenting cells. In accordance with this study, suggesting that exosomes could serve as triggering factors for specific autoimmunity events leading to diabetes , also Rahman et al (144) and Lukic et al (145) propose a possible causative role of the islet MSCs and their EXOs in triggering the islet-specific autoimmunity in the NOD mouse strain. During beta cell apoptosis in the islet, MSCs might be activated or recruited in islets to repair the damage, and therefore could become a source of EXOs able to initiate autoimmune response (144).

MSCs harvesting site

An important open issue is represented by the site of MSCs harvest. The bone marrow has been long investigated as a source of stem cells, and MSCs were first isolated from the bone marrow, therefore a large proportion of the studies on MSCs in the context of diabetes were conducted on Bone Marrow-derived (BM)-MSCs. However, the clinical applicability of BM-MSCs is limited due to the relatively invasive procedure required for sample collection as well as the marked reduction in cell number, proliferation, and differentiation capacity with the age of the donor (146). Thus, various different tissues have been studied as alternatives sources of MSCs.

It is now accepted that MSCs can be harvested from multiple anatomical locations, and it has been widely assumed that MSCs derived from different sources are largely equivalent, at least in terms

of surface marker expression and differentiation potential. On the other hand, evidence suggests that there are differences in term of marker and gene expression profiles; these differences may have a profound impact on MSCs function (81, 147, 148) and clinical efficacy (127).

In recent years, multiple alternative sources of MSCs have shown a great potential, including umbilical cord (UC), umbilical cord blood (UCB), and adipose tissue (AT). Cells derived from UC and UCB are easily bankable and offer the theoretical advantage of youth (74)**.** The advantages of using MSCs from birth-associated tissues have been highlighted by Hass et al (149): the use of parts of the neonatal placenta and umbilical cord/Wharton's jelly is not invasive and raises no ethical concerns, MSCs from these tissues possess increased proliferative capacity *in vitro*, especially under hypoxic conditions, in comparison to certain MSCs populations obtained from adult tissues.

Another MSCs source that currently commands great attention is the adipose tissue (AT), which can be readily collected and processed for autologous use. AT-MSCs have been found to have proliferative ability and differentiation potential comparable to those of BM-MSCs (150). Therefore, adipose tissue offers important advantages when compared to bone marrow, given its availability and ease of collection.

It is now evident that MSCs from these tissues and from BM are morphologically and immunophenotypically similar, but not identical (151). UCB-derived MSCs form the fewest colonies and show the highest proliferative capacity, whereas AT-MSCs form the greatest number of colonies, and BM-MSCs have the lowest proliferative capacity. MSCs from AT and UCB (152) may gain more popularity because of the versatility of the tissue sources and because of their great potential for a wide range of clinical applications.

Jeon et al (153) isolated MSCs from the placenta (PL) and adipose tissue, and showed significant molecular differences in the properties of the MSCs according to their cellular source. The cytoskeleton proteins were abundantly expressed in BM-MSCs and in AT-MSCs, while the oxidative stress proteins and apoptosis proteins were abundantly expressed in PL-MSCs. Therefore, the authors suggest that PL-MSCs may be more appropriate for treatments that aim to increase therapeutic ability.

In the context of diabetes, the source of the MSCs is considered important. Pancreas and Pancreatic islet-derived MSCs (first isolated in 2001 (154)) could be considered a better option than other commonly used MSCs, because of their potential for stimulation of islet-specific functions (155). Pancreatic-islet derived MSCs may have the peculiar ability to enter the pancreatic endocrine differentiation path, although the level of transcriptional and functional maturation is still far from

that expected of true beta cells (81). The increasing interest in pancreas-derived MSCs is due to their potential use for the modulation of immune function, stimulation of angiogenesis, and potentiation of islet endocrine functions; moreover, these cells may differentiate into beta like cells with a yield superior to that of MSCs from different sources, without the need of additional genetic engineering – but this differentiation potential is still debated (155-159). As stated, the tissue source of the transplanted MSCs seems to impact the outcome of the therapy in the clinical setting: importantly, UC-MSCs appeared to be superior to BM-MSCs in improving C-peptide levels in T1D patients (127). The main characteristics of MSC harvested from different sources are summarized in Table 1.

MSC Source	Main characteristics
BM	Long investigated. Invasive procedure for sample collection. Yield and expansion potential may be limited in aging individuals.
AT	Morphologically and immunophenotypically similar to BM-MSC. Proliferative ability and differentiation potential similar to BM-MSC. Easy accessible, highly available, easily bankable, no invasive procedure for sample collection. Important advantages for autologous applications.
UC, UCB	Morphologically and immunophenotypically similar to BM-MSC. Young natural age. Increased proliferative capacity. Increased expression of oxidative stress proteins. Easy bankable, no invasive procedure for sample collection.
PL	Morphologically and immunophenotypically similar to BM-MSC. Young natural age. Increased proliferative capacity. Increased expression of oxidative stress proteins. Easy bankable, no invasive procedure for sample collection. Risk of co-isolation of maternal and fetal cells.
P, PI	Features and differentiation capacity in line with those of MSC from other sources; potential for the stimulation of islet-specific functions, potentially easier differentiation into beta cells.

Table 1 - Main characteristics of MSC harvested from different sources.

Abbreviations: BM, bone marrow; AT, adipose tissue; UC, umbilical cord (including Wharton`s Jelly); UCB, umbilical cord blood; PL, placenta; P, Pancreas; PI, Pancreatic Islets.

Pancreatic Islet-derived MSCs

Isolated human pancreatic islets (hPI) and murine pancreatic islets (mPI) can be cultured *in vitro* in conditions that stimulate the expansion of MSCs. The primary adherent culture is composed of a slow-growing endocrine cell population and a rapidly expanding spindle-shaped fibroblast-like cell population - which becomes predominant after few weeks of expansion in culture (160). This latter culture-expanded population satisfies the criteria established by the International Society for Cellular Therapy for defining a cell population as multipotent mesenchymal stromal cells (89). Pancreatic Islet-derived Mesenchymal Stromal/Stem Cells (PI-MSCs) exhibit self-renewal and high clonogenic potential. They have the ability to differentiate *in vitro* toward osteocytes, chondrocytes, and -with a relatively lower yield- adipocytes. These cells express mesenchymal cell surface markers (CD44, CD90, CD73, CD105) and do not express hematopoietic markers (CD14, CD34, CD45) and HLA-DR (157, 160-162).

PI-MSCs have been investigated as a potential source of islet endocrine cells (157) and as a supportive cell population for combination with pancreatic islets (163). Proteomic analysis highlighted that there are differences in MSCs protein expression depending on the site of cell origin (157). Along with these, differences in disposition towards commitment have been observed (157). It was proposed that PI-MSC cultures originate from insulin-producing β cells undergoing epithelial-to-mesenchymal transition (164), but subsequent studies based on lineage tracing in mice suggested that these cells predominantly derive from pre-existing mesenchymal cells, such as islet pericytes and islet stromal cells (165, 166). Specific culture conditions, mediators, or genetic modification could facilitate the differentiation of MSCs into insulin-producing beta-like cells, but robust *in vivo* differentiation appears to be difficult to obtain. Pancreatic-islet derived MSCs (or MSC-like cells) seem to have the ability to enter the pancreatic endocrine differentiation path, but the level of transcriptional and functional maturation is far from that expected for true β cells (154, 156, 161, 164, 167, 168). Contamination from residual endocrine cells in PI-MSC cultures could account for some of the early findings.

Besides their potential for maturation or reprogramming into pancreatic islet beta cells, PI-MSCs could find application in the treatment of T1D because of their beneficial immunomodulatory and pro-angiogenic properties,(102, 103, 127, 128, 169). In Type 1 Diabetes, autoreactive T cells exhibit pro-inflammatory cytokine profiles (170). Additionally, there are defects in peripheral tolerance, including decreased numbers and impaired functions of regulatory T cell (T reg) function (170). It has been reported that in T1D patients there are defects in T reg phenotype and suppressive

capacity, yet it is still unknown how these cells are acting or maturing in the islet microenvironment (63). Inflammatory environments have shown to destabilize Tregs, specifically upon stimulation with IFN-γ *in vitro*: Tregs downregulate CD25 and FoxP3, and exhibit limited expansion (63). Bone Marrow-derived MSC have been shown to exert immunomodulatory effects: they have the ability to suppress allogenic T cells proliferation and to expand regulatory T cells, an effect mediated by HLA-G secretion (171). These characteristics render MSCs promising for applications in autoimmune diseases. The ability of human pancreatic islet-derived MSCs to stimulate T regulatory cells remains a matter of investigation. Experimental data also indicate that MSCs can protect islet beta cells via production of paracrine factors that stimulate engraftment and modulate the immune response (160, 163, 172-174). An increasing amount of evidence suggests that PI-MSCs could aid in the restoration of normoglycemia, and could support neovascularization when co-transplanted with islets (160). Pancreatic islets-derived MSC and bone marrow (BM)-derived MSCs present largely similar metabolism and immunomodulatory properties (163). One interesting finding at the gene expression level is that FGF9, a factor involved in pancreatic regeneration (175, 176), is expressed at higher levels in PI-MSCs (163). This could therefore make PI-MSCs more appropriate for pancreatic islet protection and regeneration, compared with MSCs from other sources. In basal conditions, cultured PI-MSCs do not express HLA-DR (160-163), a major histocompatibility complex class II cell surface receptor. This suggests that the cells could evade a set of immunological responses. Nevertheless, similarly to BM-MSCs, PI-MSCs respond to IFN-γ stimulation by increasing their level of HLA-DR (163). In a way similar to BM-MSCs, PI-MSCs suppress proliferation of allogeneic T lymphocytes stimulated with anti-CD3 and anti-CD28 antibodies (163, 177). PI-MSCs can inhibit both CD4+ and CD8+ T lymphocyte in a dose-dependent manner. Yet, PI-MSCs appear more potent than BM-MSCs, as they suppress proliferation of lymphocytes at lower MSC-to-lymphocytes ratios (163). The ability of BM-MSCs to suppress the proliferation of activated lymphocytes is widely recognized, and the fact that PI-MSCs show similar, if not superior, characteristics is very intriguing. Compared with MSCs from other sources, PI-MSCs possess advantages for co-transplantation with islet endocrine cells: they promote engraftment, create a favorable immune microenvironment, and sustain long-term islet cell survival (163).

PI-MSC could play a role during the development of T1D (178), and could be utilized in therapeutic strategies for this disease.

Autologous or allogenic MSCs in Diabetes

Another important matter of debate is whether autologous or allogeneic MSCs are more suitable for therapeutic strategies in T1D and T2D. Under pathological conditions, MSCs can become functionally compromised. Autologous MSCs may present abnormal functions due to the autoimmune process in T1D, or due to the diabetic microenvironment in both T1D and T2D. Allogeneic MSCs may be recognized and may be rejected by the competent immune system of the recipient (179), may transmit donor-derived infections or diseases (121).

Studies addressing potential abnormalities in MSCs derived from patients with autoimmune or inflammatory disorders are scarce and somewhat contradictory. To date, available evidence is still not strong enough to support a recommendation, and more studies should be performed in order to fully establish advantages and weaknesses of autologous or allogeneic MSCs.

Thus, studies that investigate characteristics of autologous MSCs isolated from both T1D and T2D patients are essential to improve the knowledge of the effect that the host environment has on stem cell function, and therefore to guide future clinical applications.

Recent studies analyzing functions of T1D BM-MSCs demonstrated that T1D and healthy BM-MSCs exhibit no differences in term of morphology, immune-suppressive activity, and migration capacity (180-182). However, some studies revealed differential expression of genes related to cytokines, immunomodulation, and wound-healing potential, which would be important to investigate further. A study by Yaochite et al (182) evaluated the *in vitro* properties and the *in vivo* therapeutic efficacy of BM-MSCs isolated from newly diagnosed (6 weeks, corresponding to early stages after clinically overt disease) T1D patients. T1D BM-MSCs showed morphology, immunophenotypic profile, and adipocyte differentiation capacity comparable to healthy MSCs. MSCs in inflammatory environments develop immunosuppressive functions by molecules of acute phase inflammation, especially tumor necrosis factor alpha (TNFα) and interferon gamma (IFN-γ), or toll-like receptor (TLR) ligands (183). In the study by Yaochite and colleagues (182), microarray analysis was performed and no significant differences were observed in the expression of immunomodulatory genes (PDL1, NOS2, IL10, PTGES, TGFB1, PDL2, HLAG, and TGS6) and licensing-related genes (IFNGR2, TNFR1, IFNGR1, TNFR2, TLR4, and TLR3). However, the HGF gene was significantly downregulated in T1D BM-MSCs (182).

When administered to diabetic mice, both T1D-MSCs and healthy donor-derived MSCs showed equal contribution to improving β-cell mass, increasing insulin production and glucose tolerance (182). Therefore, it seems that T1D-MSCs do not present functional abnormalities (182).

Accordingly, Dong et al (184) reported that MSCs isolated from diabetic rats decreased blood glucose levels and prevented body weight loss when transplanted into diabetic animals, suggesting that diabetes does not influence MSCs properties and supporting the use of autologous MSCs in the treatment of T1D patients.

On the contrary, Fiorina et al (185) supported the hypothesis that transplantation of MSCs derived from nondiabetic donors, rather than autologous MSCs, would be the best option for the treatment of T1D; in fact, they reported that MSCs isolated from non-obese diabetic (NOD) mice were unable to delay the onset of diabetes when administered to pre-diabetic NOD mice and did not reverse hyperglycemia with already established diabetes.

Studies have demonstrated the beneficial role of MSCs on *in vivo* and *in vitro* induction/proliferation of Treg cells (186, 187), but neither the study conducted by Yaochite et al (182), nor the study by Fiorina et al (185) observed significant modifications. Opposite results were reported by Madec et al (188). Yaochite suggested that their analyses were performed 35 days after MSCs administration, which may represent too long a period of time to detect alterations in Treg cell frequency (182). Therefore, on the one hand further experiments should be performed earlier after cell transplantation, and on the other hand the beneficial effects promoted by administration MSCs are not related to late or long-standing expansion of Treg cells (182).

Another recent study by Davies et al (180) investigated whether BM-MSCs from T1D patients offer a therapeutic cell source equivalent to healthy donors BM-MSCs. Differences in gene expression were observed between healthy and late-stage T1D donors in relation to cytokine secretion, immunomodulatory activity, and wound-healing potential - suggesting a state of disease memory in these cells. Long-term exposure to the diabetic environment has been suggested to induce disease memory in BM-MSCs (189). Despite differential gene expression, T1D-MSCs did not demonstrate a significant difference from healthy controls in immunosuppressive activity, migratory capacity, or hemocompatibility. Therefore, the authors concluded that MSCs from T1D donors are phenotypically and functionally similar to healthy control MSCs indicating their suitability for use in autologous cell therapy (180).

In another recent study by de Lima et al (181), BM-MSCs from newly-diagnosed T1D patients (within 6 weeks from diagnosis) were compared with those from healthy individuals for morphological

characteristics, immunophenotypical characteristics, differentiation potential, and gene expression profile. T1D-MSCs and control MSCs showed similar morphology, immunophenotype, and multipotent differentiation, as reported by others, but T1D-MSCs showed an increased migratory capacity. Importantly, T1D-MSCs showed abnormalities in mRNA expression, including a downregulation of the immunomodulatory molecules VCAM-1, CXCL12, CCL2, CCL24, CXCL5, of the pro-regenerative molecule HGF, of the stemness-related EGFR and FGFR, along with the activation of sympathetic nervous system and JAK STAT signaling (181). This gene expression profile suggests that human T1D-MSCs may have impairments in their interactions with immune/hematopoietic cell populations and in their ability to suppress immune effector functions. In accordance with what Davies et al(180) had found, the study by de Lima et al (181) also confirmed the down-modulation of HGF in T1D-MSCs. HGF is associated with angiogenesis and cell survival (190, 191), it can stimulate kidney and liver regeneration. Moreover, HGF is believed to be a protective factor for pancreatic β cells, and consequently its downregulation may indicate a decreased potential for the stimulation of pancreatic islet regeneration. Additionally, EGFR and FGFR were also found downregulated in T1D-MSCs: these receptors regulate stemness, inhibit senescence, are essential for cell growth, tissue repair, and homeostasis (192, 193); a downregulation of EGFR signaling may determine the downregulation of HGF (192, 194, 195). This study analyzed MSCs after *in vitro* culture, therefore the abnormalities found could be influenced by culture conditions beyond the exposure to the altered diabetic bone marrow milieu. Further functional experiments will be required in order to better elucidate how these gene expression alterations may affect therapeutic efficacy of autologous MSCs in T1D patients (181).

The studies focused on autologous T2D BM-MSCs suggest that long term exposure to the disease-related inflammatory and hyperglycemic environment affect their functions.

Shin and Peterson (196) examined the influence of T2D on the therapeutic potential of endogenous BM-MSCs, showing that the diabetic mice had BM-MSCs occurring in lower numbers, with impaired proliferation and survival *in vitro*.

The study conducted in 2009 by Phadnis et al (197) investigated the characteristics of BM-MSCs derived from T2D patients. As it was described by the articles cited in this review about T1D (180-182), also T2D-MSCs appear similar to healthy MSCs in phenotype, morphology, and multilineage differentiation potential. However, the diabetic environment seems to have an impact on MSCs: C-peptide and insulin transcripts can be detected in T2D-MSCs (197). Kojima and colleagues had

previously observed that hyperglycemia, with or without established diabetes, activates insulin gene transcription and proinsulin production in multiple extrapancreatic and extrathymic tissues (198).

However, unlike in β-cells, MSCs from T2D exclusively produced proinsulin and very little mature insulin, and did not contribute significantly to insulin production *in vivo* (197). Although high glucose concentration induces proinsulin transcription, it also stimulates the secretion of cytokines such as interleukin1, which cause β-cell apoptosis *in vitro* and *in vivo* (199). Kojima et al (198) hypothesized that these cells may mediate the ill effects of hyperglycemia, and may contribute to chronic diabetic complications such as diabetic neuropathy.

Although the amount of proinsulin produced by the BM cells exposed to hyperglycemia *in vivo* was extremely small, the appearance of proinsulin-producing cells outside the pancreas may represent the body's attempt to reverse hyperglycemia (197). Thus, chronic exposure to hyperglycemia may be important for the decreased potential of these BM-MSCs, precluding them for autologous stem cell therapy in T2D patients. In fact, these cells appear to be terminally differentiated, therefore leading to a loss of stemness and failure of further propagation (197).

Furthermore, the persistent hyperglycemic milieu in T2D is also associated with several pathological complications, mostly related with compromised vascularization and/or aberrant angiogenesis (200). By releasing growth factors and cytokines such as IGF-1, BM-MSCs stimulate endothelial cell migration (201), inhibit endothelial apoptosis, stimulate angiogenesis, promote neovascularization and tissue regeneration (201-203). The influence of T2D on the secretome and pro-angiogenic functions of BM-MSCs deserves thorough investigations. Ribot et al (200) analyzed the impact of T2D on BM-MSCs secretome and functions, hypothesizing that in the diabetic milieu these could have different composition and properties. The results obtained provided the evidence that short-term T2D alters the BM-MSC secretome composition and promotes angiogenic capabilities (200).

Angiogenesis-related genes are differentially expressed in BM-MSCs from diabetic fatty rats (ZDF, a T2D model) when compared with lean animals (control). In particular, several pro-angiogenic genes were found to be overexpressed, while anti-angiogenic genes were downregulated. The up-regulated genes included IGF-1 and TIE1, which are critical regulators of angiogenesis (204), MCP-1 and IL-6, homing factors for BM-MSCs and Endothelial Cells/Endothelial Progenitor Cells (205, 206), and IL-6 and TNFa, critical mediators of the inflammatory process. Moreover, proteomic analysis of the T2D BM-MSC secretome showed decreased levels of ab-crystallin, a chaperone for VEGF-A, and increased levels of LTBP1 and LTBP2, regulators of TGF-b availability (207), as well as of OSTP and

FMOD, which are components of the extracellular matrix and might be involved in the paracrine action of T2D BM-MSCs on endothelial cells (208-211). In addition, the proteomic analysis of T2D BM-MSC demonstrated a specific secretory phenotype of extracellular matrix remodeling and glucose metabolism, showing overexpressed proteins involved in extracellular matrix homeostasis and remodeling-related molecules (212); in contrast, proteins involved in the metabolism of glucose (such as ALDOA, LDHA, KPYM, G6P, PTMA, OAS2, ALD1, and IBP2) were secreted at lower levels.

Functional impairment of T2D MSCs is evident from preclinical and clinical studies that have been performed to determine their efficacy in the treatment of peripheral arterial disease (PAD). PAD is frequently associated with diabetes, hypertension, atherosclerosis, and aging - all of which could damage the regenerative function of stem cells and progenitor cells (213-218). Yan et al (219) have shown that experimental T2D causes hyperinsulinemia-induced oxidant stress in murine MSCs, a stress that restricts their multipotency and impairs their capacity to promote neovascularization.

The same authors observed that MSCs harvested from T2D mice show several dysfunctions deriving from oxidative stress (220). Rather than increasing post-ischemic neovascularization and limb blood flow, injection of MSCs from T2D mice impaired blood flow recovery. Should human MSCs display similar oxidative stress-induced impairment of function, these findings recommend a therapeutic approach aimed maximizing the potential of MSC transplantation, particularly in the increasingly common setting of diabetes or other cardiovascular risk factors. The authors propose that either *in vivo* systemic treatment with an antioxidant and/or *ex vivo* treatment of MSCs with antioxidants could significantly increase the intended clinical benefit (220).

A recent study by Rezabakhsh et al (221) investigated the impact of T2D sera on the angiogenic differentiation capacity of primary healthy BM-MSCs. The study showed that T2D serum decreased the angiogenic properties of MSCs via direct effect on angiogenesis pathways or via induction of autophagy signaling (221).

Taking all these considerations together, the pathophysiology of T2D and the associated changes in the bone marrow microenvironment seem to affect multiple aspects of BM-MSCs biology and function. T2D seems to exacerbate the impairment of these stem cells to an extent greater than T1D. It is however still largely unknown whether distinct mechanisms underlie BM-MSCs dysfunction in T1D compared to T2D (105).

De Vyver et al (105) argued that strategies focused on restoring stem/progenitor cells mobilization in autologous cell therapy are limited in that stem cell damage can occur at the bone marrow niche

before mobilization into the peripheral blood. This hypothesis was also confirmed by an observation by Januszyk et al (222), who affirmed that the pathogenesis of both T1D and T2D may deplete specific subpopulations of BM-MSCs and this defect cannot be corrected by restoring glucose homeostasis.

In addition to affecting BM-MSCs viability and functional capacity, long term exposure to the pathological bone marrow niche environment can induce a certain degree of disease memory in MSCs (189).

Future studies are required to provide a strict assessment of the efficacy of MSCs transplantation in T1D, T2D, and related complications.

Autologous T1D BM-MSCs	Autologous T2D BM-MSCs
No differences compared to healthy BM-MSCs in term of morphology, immune-suppressive activity, and migration capacity. Differential expression of genes related to cytokines, immunomodulation, and wound-healing potential.	No differences compared to healthy BM-MSCs in term of phenotype, morphology, and multilineage differentiation potential. Decreased potency, these cells appear to be terminally differentiated. Dysfunctional secretome composition, affecting pro-angiogenic functions. Several oxidative stress-dependent dysfunctions.

Table 2 - Main characteristics of autologous BM-MSCs isolated from T1D and T2D patients.

Pericytes

Pericytes are mural cells present in all vascular beds. They interact with endothelial cells and form the vessel wall. They are embedded within the basement membrane, wrapped around the endothelial layer (223).

Pericytes are a heterogeneous and plastic population of cells with different properties depending on their localization through the vascular tree. They are identified by their anatomic localization and

expression of markers, such as α-SMA (smooth muscle actin), NG2, and PDGFRβ (platelet-derived growth factor receptor β) (223).

Pericytes are well known to be involved in angiogenesis (224) and are essential for the maintenance of the blood brain barrier (225, 226). The crosstalk between pericytes and endothelial cell is essential in both remodelling and quiescent vasculature, and this complex interaction is often altered in disease condition.

Evidence is emerging that pericytes are also involved in regulating leukocyte function in inflammation process (227, 228).

Leukocytes are believed to be the first responders to infectious and sterile inflammatory processes, but it is increasingly documented that there is also an important contribution of stromal cells and extracellular matrix (229, 230).

Pericytes reside in a strategic position between the blood stream and the interstitial space. The physical contact between pericytes and leukocytes has a significant impact on the phenotype and function of the leukocytes, increasing their immunosurveillance as well as effector functions (231).

Arteriolar NG2 pericytes regulate interstitial leukocyte migration during sterile inflammation in the skin *in vivo* (231). They are able to sense inflammatory process via pattern recognition receptors (PRRs) and react by secreting chemokines (such as MIF, CCL2, CXCL8) and by exposing adhesion molecules on their surfaces (231-234).

The function of pericytes in immunosurveillance has been investigated extensively in the brain and in the lungs. These cells are gatekeepers to the brain and regulate blood flow, leukocyte infiltration and the entry of circulating mediators and metabolites (235).

When leukocyte adhere to the endothelium and start to transmigrate, they meet the network of pericytes, which usually limits further infiltration into the brain parenchyma. In the setting of inflammation, pericytes increase the permeability of the blood brain barrier not only regarding macromolecules, but also for immune cells (236, 237).

Within the interstitial space, immune cells migrate to the focus of inflammation to clear necrotic areas and to limit the dissemination of pathogens. Neutrophils can also perform reverse transmigration and reenter the systemic circulation to systemically disseminate inflammation (238). However, the contribution of pericytes to the process of reverse transmigration is still unclear.

Crisan et al (239) documented that MSCs are derived from pericytes from a number of human tissues. If one sorts culture expanded human pericytes for the *in vivo* marker CD146 or α-SMA, the cells obtained have all of the classic markers described for MSCs (CD105, CD90, CD73, etc.).

Caplan (240) strongly take the stance that pericytes give rise to MSCs. Since the vascular endothelial cells have well documented progenitors not connected to pericytes (241, 242), Caplan excludes the vascular endothelial cells as possible progenitors of MSCs.

When a vessel is inflamed, the pericytes detach from the blood vessel and differentiate into activated MSCs (243) which sense the microenvironment and respond by secreting molecules (240). Pancreatic islets are highly vascularized through a capillary network of endothelial cells and pericytes (244, 245), but the role of pericytes remains largely unclear (245-247).

In embryo, beta cells are formed by differentiation of pancreatic endocrine precursors, followed by proliferation of differentiated beta cells (248, 249). After birth, pericytes constitute a major part of the mesenchymal cell population in the pancreas (245, 250). In this period, the major route of beta cell generation is their replication (251-253), which decline with age.

Interactions between endothelial cells and pericytes was shown to support beta cell proliferation and function (247, 254). Therefore, the low beta cell proliferation rate during adulthood (255) could suggest that pericytes do not support beta cell proliferation after the neonatal period.

Epshtein et al (256) showed that pericytes isolated from neonatal pancreatic tissue released factors that can stimulated beta cell proliferation *in vitro*. Thus, they proposed that age-dependent changes in pancreatic pericytes affect their ability to promote beta cell proliferation in adult age, or that physiological levels of pericytic components might be insufficient to drive adult beta cell proliferation *in vivo* (256).

An increased metabolic demand lead to phenotypic changes in islet pericytes. Hypertrophy of islet pericytes, which accompanies vessel dilation, has been reported in obesity (257, 258). However, the contribution of diabetes associated changes in islet pericytes to disease progression remains unknown.

Sasson et al (250) observed impaired glucose stimulated insulin secretion and insulin content in isolated islets after pericyte depletion, accompanied by a reduced expression of mature beta cell phenotype genes. *Ex vivo* depletion of islet pericytes resulted in similar impairment in beta cell gene expression, implicating an intra-islet role. These findings suggest that pericytes are pivotal components of the islet niche, as they are essential for beta cell maturity and functionality (250).

Pericytes are believed to regulate islet vascular permeability and blood flow via affecting endothelial cells (257). The dysfunction of beta cells upon pericyte depletion may be secondary to abrogated vascular function, but it was recently shown that adult beta cell function is independent of blood flow (259). Moreover, the decreased beta cell gene expression after *in vitro* pericyte depletion in

isolated islets confirms a direct intra-islet role for these cells in maintaining beta cell phenotype, independent of blood flow regulation.

Recent findings point to beta cell dedifferentiation as a key in diabetes progression (25).

In response to increased metabolic demand, islet pericytes undergo phenotypic changes implicated in islet fibrosis during diabetes (257, 260, 261). This raises the possibility that diabetes-associated changes in islet pericytes may interfere with their ability to properly support beta cells, contributing to dedifferentiation of the latter (250).

Changes in pericyte biology are believed to be directly associated with biochemical changes in diabetes, and pericyte loss is an early hallmark of diabetes-associated microvascular disease, including retinopathy and nephropathy (262).

Diabetic retinopathy, characterized by pericyte loss, is the most frequent diabetes-associated microvascular complication. A third of patients with diabetes develop ocular complications, eventually resulting in blindness (262).

Pericytes are sensitive to hyperglycemia-induced oxidative stress, hence this stress contributes to pericyte apoptosis (263).

Interestingly, pericytes derived from diabetic donors have an altered cytoskeletal organization and contractility, which are associated with alterations in their secretome, including increased secretion of pro-angiogenic factors such as VEGF (264).

Diabetic nephropathy is the main cause of end-stage renal disease and is characterized by pathological alterations within glomeruli and the tubular system, associated also with a decrease in pericyte coverage, leading to disruption of the filtration barrier and proteinuria (265).

There is therefore clear evidence that pericytes are important in the pathogenesis of some diabetes-associated vascular complications, and not just passively: they actively contribute to the progression of vascular dysfunction (262).

AIMS

The overarching goal of our studies is to modulate deleterious immune functions and stimulate pancreatic islet regeneration in the context of Type 1 Diabetes (T1D). In this project, we aimed at testing the potential of pancreas-derived Mesenchymal Stromal Cells (MSCs) to act as immunomodulators and as trophic mediators in disease-relevant models. We tested pancreatic MSCs in a comparison with MSCs from other tissue sources. MSCs from other sources have shown potential to modulate T1D autoimmunity and enhance engraftment of transplanted pancreatic islet cells. We propose that Pancreas-derived MSCs (P-MSCs) and Pancreatic Islet-derived MSCs (PI-MSCs) could sustain islet-specific functions, including promotion of beta cell function and survival, regulation of beta cell regeneration, and islet immunomodulation.

We initially aimed at isolating, characterizing, and banking MSCs from murine and human pancreatic tissues. We generated a cell bank for use in multiple model systems. We studied the immunomodulatory potential of the cells *in vitro.* Subsequently, we performed *in vitro* studies to test the MSC effect on islet beta cell function. Moreover, we tested the effect of Pancreatic MSC co-transplantation with islets in a murine model of autoimmune diabetes.

To enable longitudinal studies in mice with autoimmune diabetes, we developed a method of islet and MSC co-transplantation in the anterior chamber of the eye, with follow-up via *in vivo* imaging. We hypothesized that locally delivered MSCs could protect islet beta cells from recurrence of autoimmunity and stimulate beta cell survival. To test this hypothesis, we co-transplanted MSC with islets in NOD mice with recent onset of autoimmune diabetes, and we analyzed islet survival, revascularization, and immune cell trafficking via *in vivo* imaging. The model of transplantation in the anterior chamber of the eye enables an unprecedented view of the local immunomodulatory and paracrine effects of MSCs, along with analyses of islet beta cell survival and function.

In a separate project, relevant to the field of diabetic ulcers and wound healing, we analyzed the effect of the secretome of placenta-derived MSC in an *in vitro* model of wound healing.

MATERIALS AND METHODS

Isolation and culture of Mouse Bone Marrow-MSCs (mBM-MSCs)

Non-diabetic NOD male mice (age 4-5 months, n=6), NOD GFP+ male mice (age 4-5 months, n=6), NOD-SCID female mice (age 4-5 months, n=6), were humanely euthanized via asphyxia in a CO_2 chamber followed by cervical dislocation. The bodies of the animals were subsequently submerged in a 70% ethanol solution and maintained in incubation for 15 minutes. Surgery was performed to harvest different organs and tissues. Femurs and tibiae were dissected and the muscles and tendons were removed in sterile conditions. The bones were opened with scissors and the bone marrow was flushed by utilizing 10 mL of PBS 1X per bone, injected with a syringe with needle Gauge 27 G 1/2. The mineralized portion of the bones was removed and the bone marrow was washed twice with PBS, then washed once with DMEM-L (Glucose 1g/L) for 5 minutes at 1500 RPM. The bone marrow was resuspended in DMEM 10% heat inactivated Fetal Bovine Serum (FBS, ThermoFisher Scientific Gibco # 10082-147) with the addition of antibiotics and antimycotics (Sigma A5955-100mL), 12 mL of medium per 75 cm^2 flask (Corning # 430641U). Cells were incubated at 37°C 5% CO_2. The cells were maintained in the initial T-75 flask and initial culture medium for 7 days. Culture Medium was changed with DMEM-L 10% FBS without trypsin inhibitors starting from day 7. Medium was subsequently replaced every 3-4 days. After reaching 80% confluence, the cells were harvested with TrypLE Express (ThermoFisher Scientific Gibco 12604013), then re-seeded at a ratio of 1:3, and/or cryopreserved with CryoStor CS10 Freeze media (BioLife Solutions #210102).

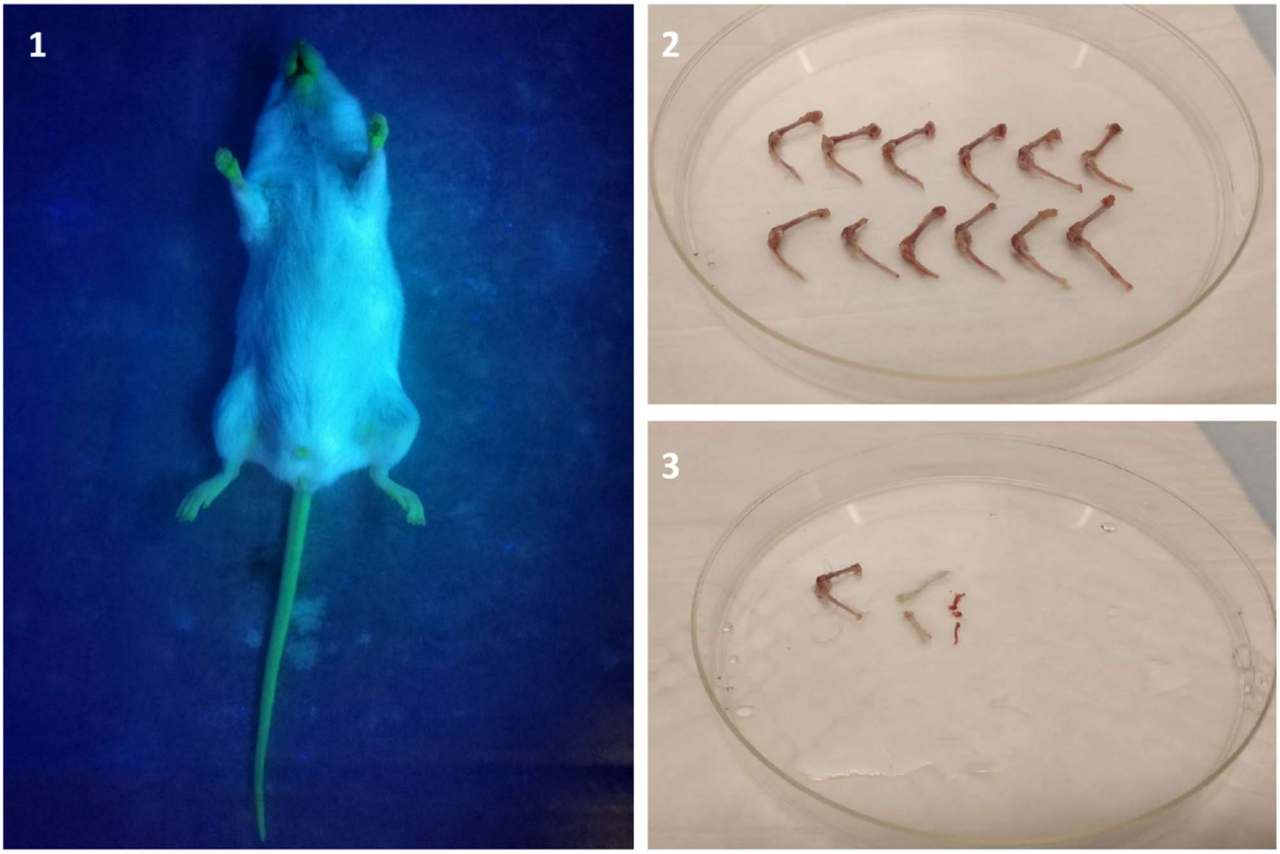


Fig. 8 – Isolation of mBM-MSCs. Femurs and tibiae from NOD GFP+ male mouse (8.1) were dissected, the muscles and tendons were removed in sterile conditions (8.2), and the bone marrow was flushed by injecting PBS 1X with a syringe with needle in the bone marrow cavity (8.3).

Isolation and culture of Mouse Pancreas-MSCs (mP-MSCs)

The pancreas harvested from the same animals was processed for Pancreas-MSC isolation. The whole pancreas was dissected by opening the abdomen and extracting the spleen. The pancreas was cleaned and minced on a Petri dish in sterile conditions. The tissue was digested with 10mL of sterile Collagenase type II 2 mg/mL prepared in Advanced RPMI (ThermoFisher Scientific Cat#12633012). Enzymatic digestion lasted 30 minutes at 37°C with 10 minutes of pipette resuspension. The digestion was stopped by the addition of 10 mL of DMEM-L (1g glucose/L; ThermoFisher Scientific Cat# 10567022) 10% FBS (ThermoFisher Scientific Cat# 16000044). After a 5 minutes centrifugation at 1500rpm, the pellet was washed twice with 10mL of PBS (ThermoFisher Scientific Cat# 10010049) and once with DMEM-L. The whole pancreas cell pellets were resuspended in 12mL of DMEM-L 10% FBS with antibiotics and antimycotics (Sigma A5955-100mL)

and trypsin inhibitors (Sigma T-64114-100 mL). Cells were seeded in a 75 cm^2 flask (Corning # 430641U) and they were incubated at 37°C 5% CO_2.

The cells were maintained in the initial T-75 flask and initial culture medium for 7 days. Culture Medium was changed with DMEM-L10% FBS without trypsin inhibitors starting from day 7.

Medium was subsequently replaced every 3-4 days. After reaching 80% confluence, the cells were harvested with TrypLE Express (ThermoFisher Scientific Gibco 12604013), then re-seeded at a ratio of 1:3 in tissue-culture treated polystyrene flasks (Corning), and/or cryopreserved with CryoStor CS10 Freeze media (BioLife Solutions #210102).

Isolation and culture of Mouse Pancreatic Islet-MSCs (mPI-MSCs)

Healthy Balb/c mice (age 14 weeks, n=10), C57BL/6 mice (age 14 weeks, n=10), NOD-SCID mice (age 4-5 months, n=10), were euthanized via asphyxia in a CO_2 chamber followed by cervical dislocation. Murine pancreatic islets were isolated following a method previously established at the Diabetes Research Institute Animal Core Facility. Viability and purity of pancreatic islets were analyzed via Trypan blue exclusion and Dithizone staining.

Before the release of each islet batch, isolated islets were cultured overnight in CMRL 1066 medium supplemented with 10% heat inactivated Fetal Bovine Serum, L-Glutamine, and antibiotics. Murine Pancreatic Islet-derived Mesenchymal Stromal Cells (mPI-MSC) were isolated by culturing isolated islets in DMEM-L 10% FBS. The cells were maintained in the initial T-75 flask and initial culture medium for 7 days. Culture Medium was changed with DMEM-L 10% FBS starting from day 7, and subsequently replaced every 3-4 days. After reaching 80% confluence, the cells were harvested with TrypLE Express (ThermoFisher Scientific Gibco 12604013), then re-seeded at a ratio of 1:3 in tissue-culture treated polystyrene flasks (Corning), and/or cryopreserved with CryoStor CS10 Freeze media (BioLife Solutions #210102).

Isolation and culture of MSCs from Human Placenta (extraembryonic) tissues: Chorion Membrane, Amniotic Membrane, Umbilical Cord

Flasks were coated leaving them with 1mg/mL Prime XV Fibronectin (IrvineScientific #31002) solution in the incubator at 37°C for 10 minutes.

Chorion Membrane / Amniotic Membrane / Umbilical Cord were resected from a fresh human placenta from a cesarean section.

1 gram of Chorion Membrane / Amniotic Membrane / Umbilical Cord tissue was minced with Nelson Metzenbaum round scissors until the fragments appeared invisible by eye.
The minced tissue was resuspended with 15 mL of 2mg/ml Collagenase II solution (Collagenase Type II, Gibco Life Techologies, # 9001-12-1) with 37°C warm DMEM-L, 1x PenStrep (P/S) without FBS, and transferred to a 50mL tube for the first round of dissociation.

The resuspension was vortexed at maximum speed for 1 minute and then transferred in the warm water bath at 37°C for 10 minutes. After 10 minutes of incubation, the suspension was vortexed once again 1 minute at the maximal speed. The tube was left 5 minutes under the hood to enable sedimentation of the digested tissue at 1g.

The supernatant was transferred to a 50 ml new tube (collection #1) and PBS was added to stop the collagenase digestion, using a volume equal to the double of the collected supernatant. The tube with the precipitated tissue was digested again with Collagenase II 2mg/mL solution, adding the same volume of the supernatant just removed.

The same steps were repeated for 2 additional cycles of digestion (collection #2, collection #3). After the last cycle of digestion, the undigested tissue was removed via filtration through a 100µm Nylon cell strainer (Corning, cell strainer #431752). After addition of PBS, the suspensions were centrifugated 5 minutes at 1500 rpm. After the centrifugation, the supernatant was removed and the cell pellet was resuspended fresh MSC Nutristem XF Basal Medium (BI, #05-200-1A) with supplements. The cells were then cultured in fibronectin-coated flasks. When 90% confluence was reached, cells were harvested with CTS™ TrypLE™ Select Enzyme (ThermoFisher Scientific, Gibco Cat # A1285901) then re-seeded at a ratio of 1:3 in tissue-culture treated polystyrene flasks (Corning), and/or cryopreserved with CryoStor CS10 Freeze media (BioLife Solutions #210102).

Fig. 9 - Amniotic Membrane / Chorion Membrane / Umbilical Cord were resected from a fresh human placenta. It is possible to separate amnion and chorion by a simple mechanical traction.

Isolation and culture of Human Pancreatic Islet-MSCs

Cadaveric pancreata (n=7, see donor characteristics in Table 3) were obtained via Organ Procurement Organizations. After receipt of the pancreas, pancreatic islets were isolated at the cGMP Cell Processing Facility of the Diabetes Research Institute – University of Miami following the Ricordi method (266). Viability and purity of pancreatic islets were analyzed via Trypan blue exclusion and Dithizone staining. Before the release of each islet batch, isolated islets were cultured overnight in CMRL 1066 medium supplemented with 10% heat inactivated Fetal Bovine Serum, L-Glutamine, and antibiotics.

Human Pancreatic Islet-derived Mesenchymal Stromal Cells (hPI-MSC) were isolated after islet batch release. 4000 Islet equivalents (IEQ) were collected from each batch, centrifugated for 5 minutes at 250 rpm, washed once with PBS (ThermoFisher Scientific Cat# 10010049), and subsequently dissociated with 4 mL of Accutase (Bioscience, Cat# 00-4555-56) pre-warmed at 37°C. Islets were incubated in Accutase solution for 10 minutes at 37°C, with 1 minute vortexing repeated at minute 0 and minute 9 of digestion. Enzymatic digestion was blocked with the addition of 10 mL of CMRL

complete islet medium. Cells were subsequently centrifugated for 5 minutes at 1500 rpm, washed once with PBS. After an additional centrifugation at 1500 rpm for 5 minutes, the supernatant was discarded and the cell pellet was resuspended in 12 mL of Dulbecco`s Modified Eagle`s Medium (DMEM, 1g glucose/L; ThermoFisher Scientific Cat# 10567022) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS, ThermoFisher Scientific Cat# 16000044). The cells were seeded in this culture medium in T-75 tissue-culture treated polystyrene flasks (Corning Catalog # 430725U) and incubated at 37°C in 5% CO_2. To allow cell adhesion, the culture medium was not replaced during the first 7 days of cell culture. At day 7 the medium was replaced with fresh DMEM 10% FBS, and medium changes were subsequently performed every 3 days. Foci of adherent fibroblast-like cells grow in culture within 7 days. When 90% confluence was reached, cells were harvested with CTS™ TrypLE™ Select Enzyme (ThermoFisher Scientific, Gibco Cat # A1285901) and subcultured at 1:3 ratio in tissue-culture treated polystyrene flasks (Corning).

Sample	Age	Gender	BMI	Cause of Death	Islet Purity %
HP2232	44	F	17,2	CVA	90%
HP2233	59	F	29	CVA	90%
HP2271	63	M	26,4	STROKE	85%
HP2288	51	F	32,2	STROKE	95%
HP2291	45	F	32,1	ICH	80%
HP2292	58	F	26,8	STROKE	95%
HP2293	42	F	32	CVA	90%

Table 3 - Characteristics of the human donors. CVA : Cerebrovascular accident; ICH: Intracerebral hemorrhage.

Cell growth kinetics

In order to evaluate the cell proliferation activity, PI-MSCs at passage 5 (P5) were plated at 5.000 cells/cm2 in 24-well plates and cell growth was assessed using Alamar Blue assay (Invitrogen, Cat# DAL1100). Alamar Blue 10% was added to the basal medium and incubated for 4 hours. Fluorescence was measured using a Victor Multilabel Counter (Perkin Elmer, Boston, MA, USA). The same procedure was repeated on days 2, 3, 5, and 7 after seeding. Data were plotted as the percentage of Alamar reduction analyzed at specific time points.

Cell growth curves based on cell confluence were generated via imaging time series with an Incucyte Zoom instrument and software (Essen Bioscience).

MSC immunophenotypic characterization

hPI-MSCs and mPI-MSCs were analyzed via flow cytometry to investigate their immunophenotypic profile. For hPI-MSCs we utilized the following antibodies: anti-CD44-FITC, anti-CD73-PE, anti-CD90-phycoerythrin-cyanine 5 (PC5), anti-CD105-PE (Beckman-Coulter, Brea CA, USA), anti-CD34-FITC, anti-CD45-allophycocyanin (APC); anti-PanCytokeratin-PE (Santa Cruz Biotechnology, Cat# sc-8018), anti-HLA-DR. For mPI-MSCs we utilized the Mouse Mesenchymal Stem Cell Surface Markers panel (R&D Systems - SC018). A CytoFLEX flow cytometer (Beckman-Coulter, Fullerton, CA, USA) and a Navios flow cytometer (Beckman-Coulter, Fullerton, CA, USA) were utilized for data collection. Data were analyzed by Kaluza FC Analysis software.

In vitro differentiation of MSCs towards Mesenchymal lineages

hPI-MSCs (Passage 5) were tested for their ability to differentiate into the three classical mesenchymal lineages: osteogenic, adipogenic and chondrogenic lineages. Control cells were cultured in standard medium, DMEM-L 10% FBS. For adipogenic differentiation, PI-MSCs were cultured with adipogenic medium (hMSC, Mesenchymal Stem Cell Adipogenic Differentiation Medium, Lonza, Cat# PT-3004). For osteogenic differentiation, hPI-MSCss were cultured with osteogenic medium (StemPro Osteogenesis Differentiation Kit, ThermoFisher Scientific, Gibco, Cat #A10072-01). To induce chondrogenic differentiation, aliquots of 250,000 cells were pelleted in polypropylene conical tubes in chondrogenic medium (StemPro Chondrogenesis Differentiation Kit, ThermoFisher Scientific, Gibco, Cat# A10071-01). The medium was then changed twice a week for three weeks. At the end of the induction, adipogenic differentiation was assessed using Oil Red O staining (Merck, Cat# 105230) and osteogenic differentiation using Alizarin Red stain (AR-S, Sigma-Aldrich Co., Cat# A5533). Finally, for chondrogenic differentiation pellets were formalin-fixed, embedded in paraffin, examined morphologically with hematoxylin eosin staining and assessed using Alcian Blue stain (Polysciences, Cat# 0234).

mPI-MSC were characterized for their ability to differentiate into the three classical mesenchymal lineages by utilizing the MesenCult™ Adipogenic Stimulatory Supplement (Mouse) (Stem Cell Technologies #05503), MesenCult™ Osteogenic Stimulatory Kit (Stem Cell Technologies #05504), MesenCult™-ACF Chondrogenic Differentiation Medium (Stem Cell Technologies #05455). At the end of the induction, adipogenic differentiation was assessed using Oil Red O Staining Kit (ScienCell #08439); osteogenic differentiation using Alizarin Red staining Staining Kit (ScienCell #0223), and chondrogenic differentiation was assessed with Alcian Blue Staining Kit (ScienCell #8378).

Immunofluorescent staining

Cells were seeded onto glass coverslips or 8-well glass chamber slides (Falcon #354118), cultured overnight in DMEM 10% FBS, then washed twice with PBS and fixed for 10 min in either 2% Paraformaldehyde or ice-cold Acetone:Methanol (1:1) at room themperature. After three washes in PBS, cells were permeabilized for 10 min with PBS 1% Triton X-100 (Sigma-Aldrich Co., Cat # T-6878), then incubated in blocking solution 1X PBS 1% BSA (Sigma-Aldrich Co., Cat # A8022) for 30 min. Primary antibody anti-PanCytokeratin (1:100, Santa Cruz, Cat # Sc-8018), anti-Vimentin (1:100, Santa Cruz, Cat # Sc-32322), anti-NG2 (Thermo Fisher Scientific Life Technologies # 37-2300; Millipore # AB5320; Santa Cruz # sc-53389), anti-HLA-G (Exbio # 11-499-C100) diluted in blocking solution was added and incubated for 1h at RT. After three washes in 1X PBS, secondary antibodies diluted in blocking solution were added and incubated for 45 min at room temperature. Coverslips were mounted after washes in PBS in ProLong Gold Antifade Mountant with Dapi (ThermoFisher Scientific, Cat # P36935).

Aggregation of PI-MSCs and Islets

Aggregation of PI-MSC and islets cells was obtained by harvesting MSCs and combining them with isolated islets in hanging drops at the desired ratio (1 MSC : 5 islet cells; 1 MSC : 15 islet cells - calculating 1560 islet cells/IEQ). Each hanging drop had a volume of 30uL and contained 2.5 IEQ. Hanging drops were incubated overnight at 37°C, 5% CO_2.

For experiments requiring large numbers of islets, the hanging drop method is not convenient, hence we combined MSCs and islets at the desired ratio (1:5, 1:15) in Aggrewell 800 6 well plates

(StemCell Technologies Cat # 34821, 6 well, 1800wells/plate; 800 µm wells) following the manufacturer`s indications.

Glucose-stimulated Insulin Release (GSIR)

hPI-MSCs were seeded at P5 with DMEM (1g/L glucose) 10% FBS and cultured overnight on top of transwells (COSTAR Cat# 3413) or in the bottom of wells of a 24 multiwell plate, tissue culture treated (FALCON Cat# 353047). 4160 cells per well were seeded in 600µl of DMEM-L (1g/L glucose) 10% FBS medium (600 µl total, loading a maximum of 200 µl on the top of the transwell).

The second day, 40 IEQ (corresponding to approximately 62400 islet cells, yielding a hPI-MSCs:islet cells Ratio of 1:15) were loaded on top of the transwells. Medium with no glucose was additioned with glucose to obtain 2.8 and 28 mM glucose concentration.

To test glucose stimulated insulin release (GSIR) via static incubation, cells were incubated in 4 different steps of 45 minutes each in the following media: low glucose (2.8 mM, Phase 1), high glucose (28 mM, Phase 2), wash step in low glucose, additional step in low glucose (2.8 mM, Phase 3). We chose these glucose concentrations following the gold standard GSIR used for the CIT clinical trial of islet transplantation. Following each incubation, the supernatants were collected. The amount of human insulin present in each supernatant was measured using a chemistry analyzer ROCHE Cobas 6000/Inmu:e601 insulin kit. The stimulation index was calculated by dividing insulin concentration of the islet sample stimulated with high glucose (28.0 mM glucose, Phase 2), by the insulin concentration in samples with low glucose (2.8 mM glucose, Phase 1).

For reproducibility, all samples were run in triplicate.

Immunomodulation Assay: PI-MSCs and PBMCs

In order to investigate the immunomodulatory properties of PI-MSCs on activated Peripheral Blood Mononuclear Cells (PBMCs), hPI-MSCs were plated in 6-well plates at a density of 50,000 cells/cm^2 and allowed to stabilize in culture for 1 day. PBMCs were isolated by density gradient centrifugation (Ficoll-Paque, Sigma-Aldrich Co., Cat# 10771) from healthy donors and co-cultured on PI-MSC monolayers at a PBMCs: PI-MSC ratio of 2:1 in RPMI (Lonza, Cat# 12-167F) with 10% FBS. PBMCs were activated by the addition of Phytohemagglutinin (PHA, 5 µg/mL, Sigma-Aldrich Co., Cat #L9640) and incubated for 5 days at 37°C, 5% CO_2. PBMCs without PHA stimulation were used as

negative control and PBMCs stimulated by PHA in the absence of PI-MSCs as positive control. The immunomodulatory ability of PI-MSCs was quantified by different assay. In order to study the ability of PI-MSCs to inhibit the PBMCs proliferation, we analyzed BrdU incorporation by activated PBMCs. After 5 days of co-cultures between PI-MSC and PBMCs, the latter were recovered, 100 µl were seeded in triplicate in a 96-well plate and then BrdU incorporation levels were quantified using a colorimetric immunoassay, according to the manufacturer's instructions (Cell Proliferation ELISA, BrdU colorimetric kit, Roche, Cat #11647229001).

PI-MSCs cultured with and without activated PBMCs were fixed with intraPep kit (Beckman-Coulter, Cat #A07803) and the HLA-G expression (Abcam, ab7904) was measured through flow cytometric analysis.

Immunomodulation Assay: PI-MSCs and purified T cells, T reg expansion Assay

We tested the ability of MSCs to stimulate the proliferation of human T regulatory cells *in vitro*. We seeded hPI-MSCs at P5 from 7 different donors in in 1mL of DMEM-L 10% FBS in wells of 12 well Plates (Grenier Bio-one CELL STAR Cat# 665180). The day after, human Pan-T cells (Stem Cell Technologies, Catalog # 70024) were stained with CFDA-SE (Stem Cell Technologies, Catalog #75003 – cell permeable precursor to CFSE). We then co-cultured Pan T cells with MSCs, at a ratio of 2:1. CD3/CD28 T cell activator tetrameric antibodies (Stem Cell Technologies, Catalog # 10971) were utilized to activate T cells. Cells were cultured in ImmunoCult™-XF T Cell Expansion Medium (Stem Cell Technologies, Catalog #10981) in the presence of 20 IU/mL IL-2 (Stem Cell Technologies, Catalog #78036). After 72h of coculture, cells were harvested and stained for flow cytometry analyses. We analyzed cell proliferation based on CFSE dilution, and we analyzed the population of T regulatory cells via CD4/CD25/FoxP3 staining (CD4 PE-Cy7, BioLegend, Catalog # 300512; CD25 BV421, BD Biosciences, Catalog # 562442; FOXP3 PE/Dazzle 594, BioLegend, Catalog # 320126). Flow cytometry analysis was performed with a CytoFLEX Flow Cytometer and CytExpert software.

Statistical Analyses

Statistical analyses were performed with GraphPad Prism version 8. The experimental design for comparing the means of two samples was based on Unpaired t test, parametric (gaussian distribution), with Welch`s correction when appropriate (for those cases where we cannot assume equal Standard Deviations). Results are expressed as mean +/- standard deviation. *p* is presented.

Animals and management of spontaneous diabetes

A proportion of NOD mice spontaneously develops autoimmune diabetes. Approximately 80% of female NOD mice develop diabetes between 8 and 25 weeks of age. We maintained 100 female NOD mice (Stock # 001976 - NOD/ShiLtJ, female, The Jackson Laboratory) in normal conditions starting from 9-10 weeks of age. We chose this number of animals with the aim of obtaining approximately 13 newly diabetic animals each month, to enable experiments in parallel. Blood glucose values (glycemia) were assessed on peripheral blood samples obtained from tail vein pricking using a portable glucometer. The procedure is quick and well tolerated by the animals without need for anesthesia. Glycemia was analyzed twice a week in all animals. To preserve the health of animals developing spontaneous diabetes, we placed insulin pellets (Catalog # Re-1-T, LinShin) under the skin as soon as diabetes was detected. Insulin pellets can control glycemia for up to 1 month in these animals.

Female NOD-SCID animals (Stock # 001303 - NOD.CB17-Prkdc<scid>/J, The Jackson Laboratory) were utilized as islet donors.

Considerations on immune responses for *in vivo* studies

Pilot studies of human MSC transplantation in non-immunodeficient mice showed that an immune response is raised against xenogenic cells. To avoid such confounding factor, we decided to focus our attention on the effect of murine male NOD-GFP+ MSCs in recipient autoimmune-diabetic female NOD mice. This setting enables us to monitor GFP+ cells *in vivo*, as well as *to* analyze their presence and phenotype after tissue preparation - via either GFP or Y chromosome product

detection. Immune responses targeting the GFP protein from *Aequorea victoria* or Y-chromosome products remain as a potential confounding factors.

Concerning islets, to minimize the risk of transplanting functional passenger immune cells with isolated islets, female NOD-SCID animals were utilized as islet donors. Male NOD-GFP+ MSCs were thus combined with female NOD-SCID islets. NOD autoimmunity targeting beta cells is expected to recur at approximately 8 days post transplantation of NOD-SCID islets in autoimmune-diabetic NOD mice.

Isolation of pancreatic islets for transplantation

For each islet isolation session, Female NOD-SCID mice (n=10) were humanely euthanized via CO_2 asphyxia and cervical dislocation. Islets were isolated as previously reported.

MSC and Islet aggregation pre-transplantation

Murine NOD-GFP+ MSCs from multiple tissues (Bone Marrow, Adipose Tissue, Pancreas, and Panreatic Islets) were aggregated with Islet cells as previously described in a 1:5 ratio.

Transplantation in the Anterior chamber of the Eye (ACE) of diabetic NOD mice

Diabetic NOD mice with recent onset of disease (under 30 days) were utilized recipient of transplantation in the anterior chamber of the eye. The transplantation of islets in this site was not aimed at controlling diabetes, but rather at imaging *in vivo* the transplanted cells, as well as at monitoring revascularization and immune cell trafficking. Mice were anesthetized via controlled-rate isoflurane inhalation in an induction chamber, to effect, and maintained anesthetized with a controlled-rate isoflurane inhalation face mask. The animals were treated with a single injection of extended release buprenorphin sub cute. Under general anesthesia, the cornea was perforated using the tip of a 27-33G needle. A small cannula was introduced into the anterior chamber of the eye through the hole created by the needle. The saline solution containing Islets, MSCs, or

Islets+MSC aggregates was delivered using a precision syringe with pedal controls. Excess solution volume was allowed to drain out of the eye to avoid increases in intraocular pressure. The procedure on each animal lasted less than 15 minutes. Both eyes received cell transplants: MSC and Islet aggregates were transplanted in the Right eyes, whereas either Islets alone or MSC alone were transplanted in the Left eyes (controls). Mice were followed for up to 30 days to analyze islet and MSC engraftment, revascularization, survival, recurrence of autoimmunity, and immune cell trafficking.

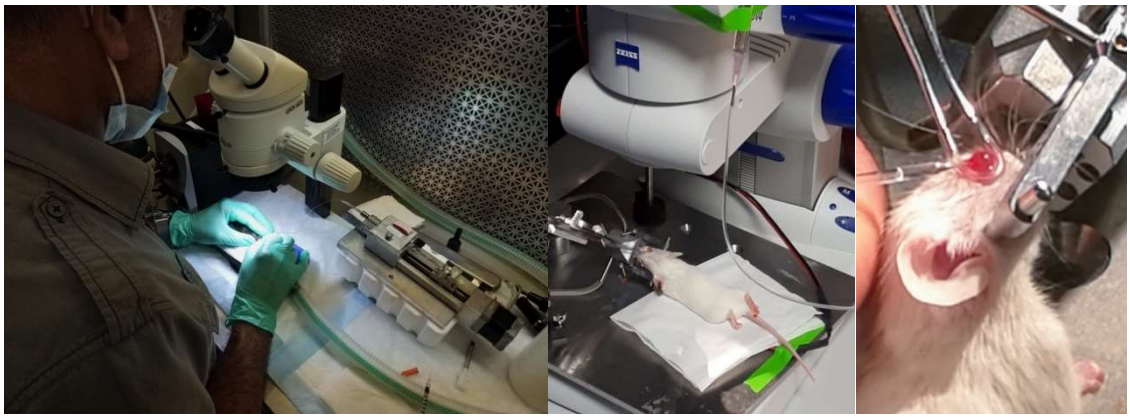


Fig. 10 - Transplantation in the anterior chamber of the eye: setup and procedure.

In vivo imaging in the eye

Mice were anesthetized via controlled-rate isoflurane inhalation in an induction chamber, to effect, and maintained anesthetized with a controlled-rate isoflurane inhalation face mask. The animals were placed on a warming pad and a stereotaxic holder under a multiphoton/confocal fluorescence microscope. In animals transplanted intraocularly, one eye at the time was imaged. Briefly, the transplanted eye was exposed using custom forceps facing the objective lens. Both eyes were kept humidified during the imaging session. After each session, animals are allowed to recover on a warm pad and returned to their cage.

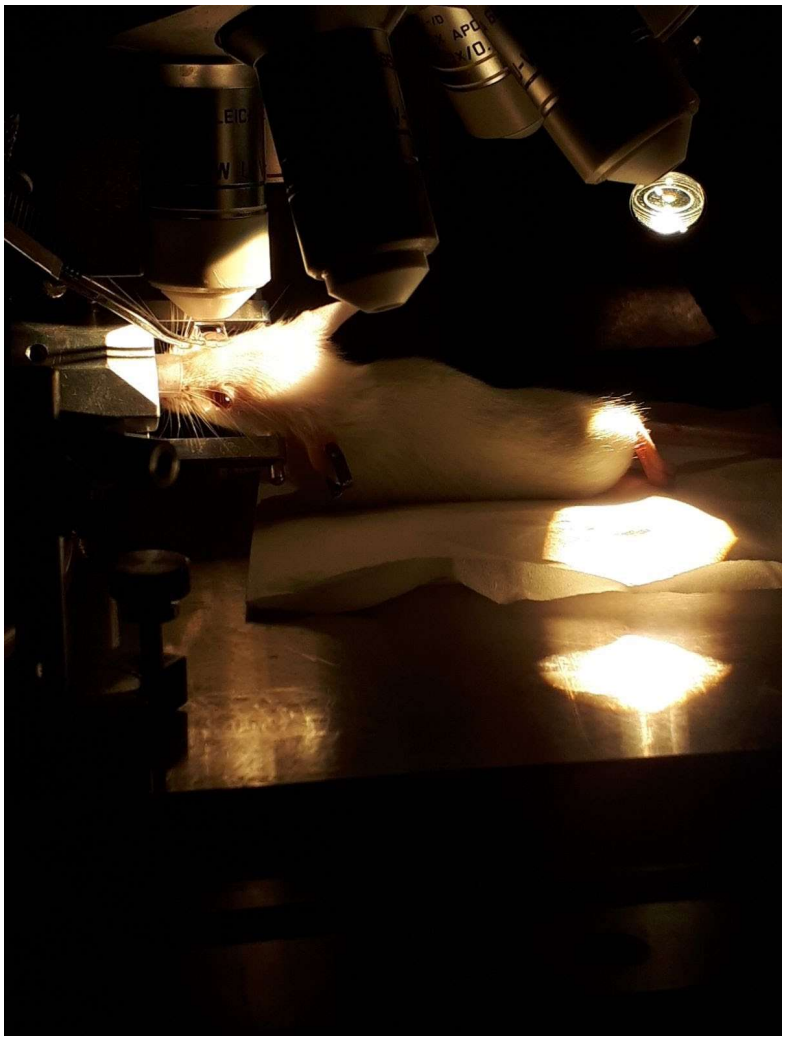


Fig. 11 - In vivo imaging in the eye.

Intravenous and intraocular injection of imaging reagents

In selected animals, fluorescently labeled agents were given systemically or locally under general anesthesia to reveal structures and cells, to acquire static and dynamic data. To reveal blood vessels, a solution containing fluorescently labeled (Cy-3, red) dextran was administered systemically via tail vein injection. Flurescently labeled dextran can subsequently be captured by monocytes/macrophages, thus enabling imaging of such cells.

To visualize immune cell subsets, fluorescently labeled antibodies directed to specific antigens expressed on the surface of immune cells were introduced by direct injection of the antibodies in the anterior chamber of the eye. We utilized anti-mouse CD4 (anti mouse CD4 Alexa Fluor 647, BD Pharmingen clone RM4-5, Catalog # 557681), CD8 (Rat anti mouse CD8 Alexa Fluor 405, AbD

SEROTEC clone YTS169.4, Catalog # MCA1768A405), and CD25 (CD25 PE-CF 594 BD, Bioscience Catalog # 562695) antibodies. Antibodies were premixed in PBS and prepared for a single injection. Micropipettes or needles were introduced into the ACE by penetrating the cornea at shallow angles on opposite sides of the eye using two micromanipulator units. One micropipette was attached to a reservoir filled with solution via polyethylene tubing, and injection was performed. Confocal/multi-photon microscopy allows for imaging of immune cell interactions at high definition. The antibodies are cleared and immunofluorescence is completely lost within hours from the injection.

Euthanasia, Necropsy, and tissue preparation for Histology

At the conclusion of the experiments, the animals were humanely euthanized via CO_2 asphyxia and cervical dislocation, and the eyes were harvested via enucleation, embedded in O.C.T., frozen at -80°C, and sectioned for histological analysis.

Collection of MSC-Conditioned Medium for Scratch Wound Assay

We expanded MSCs from 3 different tissues of human term placenta. We will define these sources A, B, and C due to intellectual property rights. Placental tissue-derived MSCs were cultured as previously described, expanded *in vitro* via harvesting and re-seeding at a 1:3 ratio in T175 tissue culture treated flasks. When the cells at passage 5 reached 100% confluence, the medium was replaced with an animal component-free medium: Mesencult ACF + Supplements (StemCell Technologies #05449). After 48h of accumulation, the conditioned medium (CM) from three different samples was collected.

To eliminate dead cells and debris, supernatant fractions collected from MSC cultures were centrifuged at 300 g for 10 min. The supernatant was transferred to a new tube and centrifugated at 2,000 g for 20 min. The supernatant was transferred to a new tube and frozen at -20 °C ready to use. In this way, the conditioned medium contained the secretome released by cells, without cellular debris.

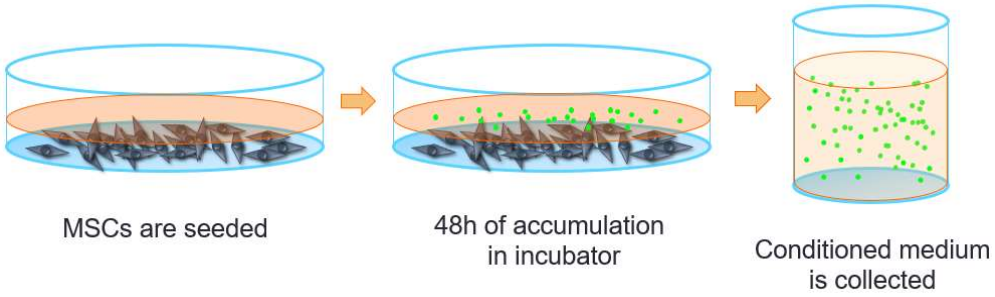


Fig. 12 – Schematic representation of the collection of MSC-conditioned medium.

Scratch Wound Assay

Skin Fibroblasts (HSF-CRL-2201 Skin Fibroblasts, P11) were seeded at a density of 2.5×10^4 cells/well in a 96-well ImageLock™ plate (Essen Bioscience #4379). A Scratch Wound was created on confluent Skin Fibroblasts monolayer using a WoundMaker™ (Essen BioScience) and following the manufacture's protocol. The 96-pin WoundMaker simultaneously creates wounds in all wells of a 96-well ImageLock plate by gently removing the cells from the confluent monolayer using an array of 96 pins. After creating the scratches, the medium was aspirated, and the wells were washed to remove the cells from the scratched area.

Test samples, including the MSC-conditioned media and control basal medium (Mesencult ACF + Supplements) were added in quadruplicate wells and the plate was placed inside the IncuCyte for time-lapse image acquisition.

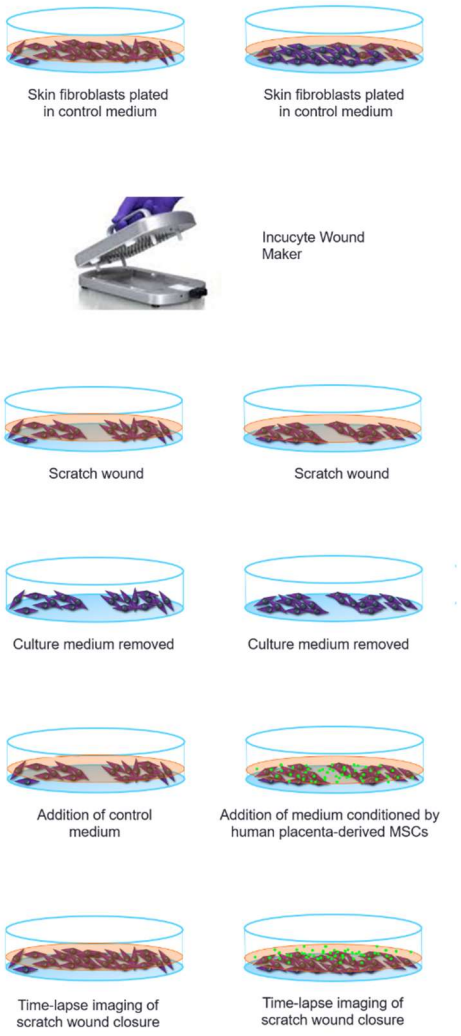


Fig. 13 – Schematic of the Scratch Wound Assay procedures.

Test samples included:

- Mesencult ACF + Supplement (control);
- Undisclosed source (A)-MSCs (P5) supernatant (2 days accumulation at confluence);
- Undisclosed source (B)-MSCs (P5) supernatant (2 days accumulation at confluence);
- Undisclosed source (C)-MSCs (P5) supernatant (2 days accumulation at confluence);

MSC supernatants from these different sources were tested at different concentrations (33% - 66% - 100%), diluted in control medium (Mesencult ACF + Supplements).

MESENCULT ACF+Suppl	MESENCULT ACF+Suppl	MESENCULT ACF+Suppl	MESENCULT ACF+Suppl	MESENCULT ACF+Suppl	MESENCULT ACF+Suppl	MESENCULT ACF+Suppl	MESENCULT ACF+Suppl	MESENCULT ACF+Suppl	MESENCULT ACF+Suppl	MESENCULT ACF+Suppl	MESENCULT ACF+Suppl	MESENCULT ACF+Suppl	positive control
33.3% (A)-MSC SUPERNATANT, 66.6% MESENCULT ACF+Suppl	33.3% (A)-MSC SUPERNATANT, 66.6% MESENCULT ACF+Suppl	33.3% (A)-MSC SUPERNATANT, 66.6% MESENCULT ACF+Suppl	33.3% (A)-MSC SUPERNATANT, 66.6% MESENCULT ACF+Suppl	33.3% (B)-MSC SUPERNATANT, 66.6% MESENCULT ACF+Suppl	33.3% (B)-MSC SUPERNATANT, 66.6% MESENCULT ACF+Suppl	33.3% (B)-MSC SUPERNATANT, 66.6% MESENCULT ACF+Suppl	33.3% (B)-MSC SUPERNATANT, 66.6% MESENCULT ACF+Suppl	33.3% (C)-MSC SUPERNATANT, 66.6% MESENCULT ACF+Suppl	33.3% (C)-MSC SUPERNATANT, 66.6% MESENCULT ACF+Suppl	33.3% (C)-MSC SUPERNATANT, 66.6% MESENCULT ACF+Suppl	33.3% (C)-MSC SUPERNATANT, 66.6% MESENCULT ACF+Suppl		
66.6% (A)-MSC SUPERNATANT, 33.3% MESENCULT ACF+Suppl	66.6% (A)-MSC SUPERNATANT, 33.3% MESENCULT ACF+Suppl	66.6% (A)-MSC SUPERNATANT, 33.3% MESENCULT ACF+Suppl	66.6% (A)-MSC SUPERNATANT, 33.3% MESENCULT ACF+Suppl	66.6% (B)-MSC SUPERNATANT, 33.3% MESENCULT ACF+Suppl	66.6% (B)-MSC SUPERNATANT, 33.3% MESENCULT ACF+Suppl	66.6% (B)-MSC SUPERNATANT, 33.3% MESENCULT ACF+Suppl	66.6% (B)-MSC SUPERNATANT, 33.3% MESENCULT ACF+Suppl	66.6% (C)-MSC SUPERNATANT, 33.3% MESENCULT ACF+Suppl	66.6% (C)-MSC SUPERNATANT, 33.3% MESENCULT ACF+Suppl	66.6% (C)-MSC SUPERNATANT, 33.3% MESENCULT ACF+Suppl	66.6% (C)-MSC SUPERNATANT, 33.3% MESENCULT ACF+Suppl		
100% (A)-MSC SUPERNATANT,	100% (A)-MSC SUPERNATANT,	100% (A)-MSC SUPERNATANT,	100% (A)-MSC SUPERNATANT,	100% (B)-MSC SUPERNATANT	100% (B)-MSC SUPERNATANT	100% (B)-MSC SUPERNATANT	100% (B)-MSC SUPERNATANT	100% (C)-MSC SUPERNATANT	100% (C)-MSC SUPERNATANT	100% (C)-MSC SUPERNATANT	100% HU-MSC SUPERNATANT		repeated
MESENCULT ACF (No Suppl)	MESENCULT ACF (No Suppl)	MESENCULT ACF (No Suppl)	MESENCULT ACF (No Suppl)	MESENCULT ACF (No Suppl)	MESENCULT ACF (No Suppl)	MESENCULT ACF (No Suppl)	MESENCULT ACF (No Suppl)	MESENCULT ACF (No Suppl)	MESENCULT ACF (No Suppl)	MESENCULT ACF (No Suppl)	MESENCULT ACF (No Suppl)		negative control
33.3% (A)-MSC SUPERNATANT, 66.6% MESENCULT ACF+Suppl	33.3% (A)-MSC SUPERNATANT, 66.6% MESENCULT ACF+Suppl	33.3% (A)-MSC SUPERNATANT, 66.6% MESENCULT ACF+Suppl	33.3% (A)-MSC SUPERNATANT, 66.6% MESENCULT ACF+Suppl	33.3% (B)-MSC SUPERNATANT, 66.6% MESENCULT ACF+Suppl	33.3% (B)-MSC SUPERNATANT, 66.6% MESENCULT ACF+Suppl	33.3% (B)-MSC SUPERNATANT, 66.6% MESENCULT ACF+Suppl	33.3% (B)-MSC SUPERNATANT, 66.6% MESENCULT ACF+Suppl	33.3% (C)-MSC SUPERNATANT, 66.6% MESENCULT ACF+Suppl	33.3% (C)-MSC SUPERNATANT, 66.6% MESENCULT ACF+Suppl	33.3% (C)-MSC SUPERNATANT, 66.6% MESENCULT ACF+Suppl	33.3% (C)-MSC SUPERNATANT, 66.6% MESENCULT ACF+Suppl		
66.6% (A)-MSC SUPERNATANT, 33.3% MESENCULT ACF+Suppl	66.6% (A)-MSC SUPERNATANT, 33.3% MESENCULT ACF+Suppl	66.6% (A)-MSC SUPERNATANT, 33.3% MESENCULT ACF+Suppl	66.6% (A)-MSC SUPERNATANT, 33.3% MESENCULT ACF+Suppl	66.6% (B)-MSC SUPERNATANT, 33.3% MESENCULT ACF+Suppl	66.6% (B)-MSC SUPERNATANT, 33.3% MESENCULT ACF+Suppl	66.6% (B)-MSC SUPERNATANT, 33.3% MESENCULT ACF+Suppl	66.6% (B)-MSC SUPERNATANT, 33.3% MESENCULT ACF+Suppl	66.6% (C)-MSC SUPERNATANT, 33.3% MESENCULT ACF+Suppl	66.6% (C)-MSC SUPERNATANT, 33.3% MESENCULT ACF+Suppl	66.6% (C)-MSC SUPERNATANT, 33.3% MESENCULT ACF+Suppl	66.6% (C)-MSC SUPERNATANT, 33.3% MESENCULT ACF+Suppl		
100% (A)-MSC SUPERNATANT,	100% (A)-MSC SUPERNATANT,	100% (A)-MSC SUPERNATANT,	100% (A)-MSC SUPERNATANT,	100% (B)-MSC SUPERNATANT	100% (B)-MSC SUPERNATANT	100% (B)-MSC SUPERNATANT	100% (B)-MSC SUPERNATANT	100% (C)-MSC SUPERNATANT	100% (C)-MSC SUPERNATANT	100% (C)-MSC SUPERNATANT	100% HU-MSC SUPERNATANT		*repeated

Table 4 –Test samples Scratch Wound Assay plate.

Images were acquired with the IncuCyte ZOOM™ livecell imaging system (Essen BioScience) at 10x magnification. The cells were photographed automatically, at the same place each time, every 4 h until 55h. The imaging system software enables analyses of the area of the scratch in real time and enables automated analyses of the scratch area, scratch width, and cell density on the scratch area at each time point.

There are three integrated metrics that the software can calculate based on the processed images:

1. *Wound Width* represents the average distance between the edges of the scratch wound mask in each line of resolution within an image.
2. *Wound Confluence* measurement relies on the initial scratch wound mask to differentiate the wounded from the non-wounded region. The resulting value represents the percentage of wound area that is occupied by cells.
3. Like Wound Confluence, *Relative Wound Density* (RWD) also relies on the initial scratch wound mask to differentiate between cell-occupied and cell-free regions of the image. Once these regions are defined, a second image analysis algorithm is applied to calculate the density of both the cell region and the wound region as defined by the initial scratch wound mask.

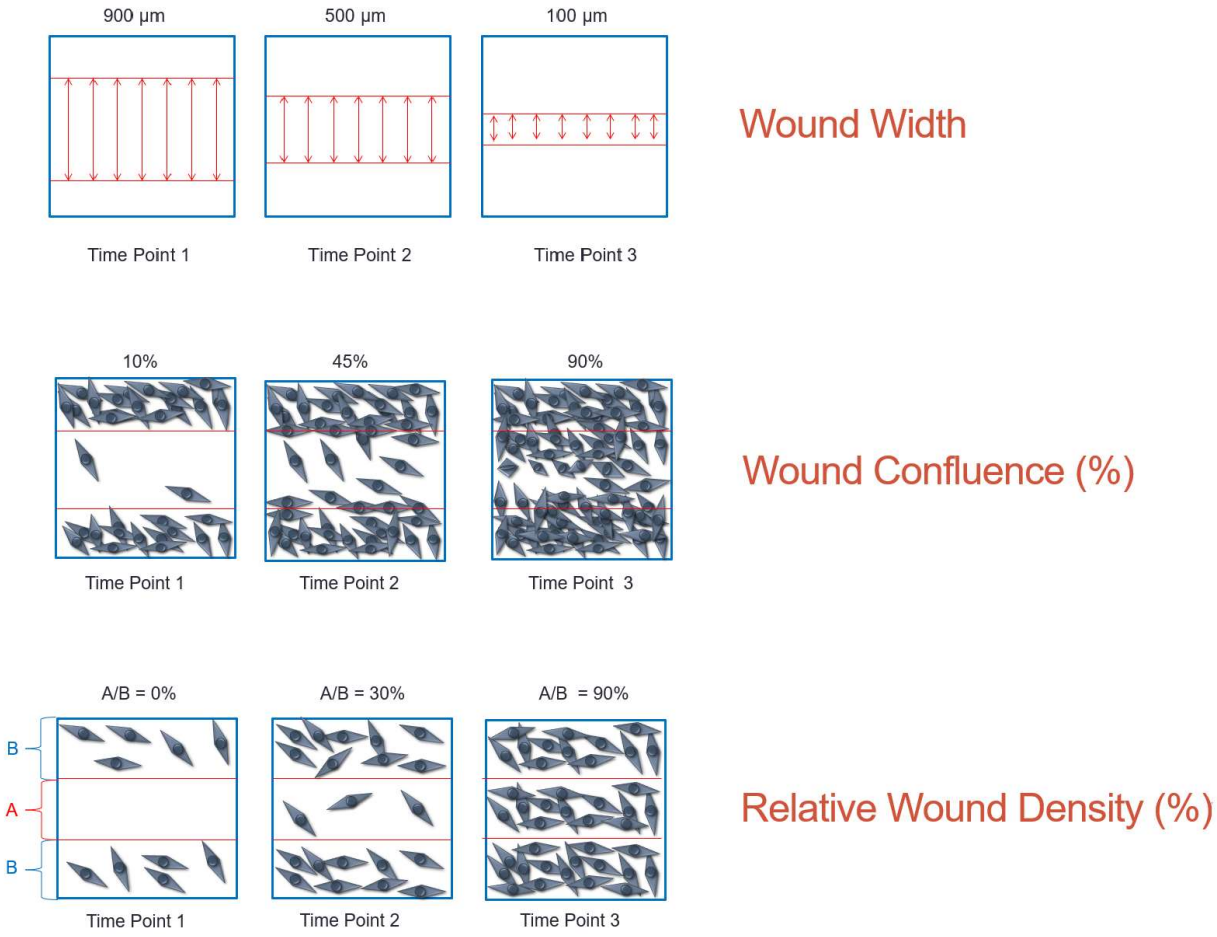


Fig. 14 - The IncuCyte ZOOM™ livecell imaging system (Essen BioScience) enables quantification of the scratch wound closure area at each timepoint. It enables calculation of the wound width (um), wound confluence (%), and relative wound density (%) at each time point.

RESULTS AND DISCUSSION

Isolation and culture of MSCs from different sources

My activity was initially focused on the isolation and culture of MSCs from different sources in order to set up a cell bank ready to use for experiments.

We have harvested and cultured MSCs from human and murine donors, as described in the Material and Methods section. In particular, we harvested and cultured MSCs from human placental tissues such as Umbilical Cord (hUC), Amniotic Membrane (hAM), Chorion Membrane (hCM), Pancreas (hP), and Pancreatic Islets (hPI). We have also derived cells from murine Bone marrow (mBM), Adipose Tissue (mAT), Pancreas (mP), pancreatic islets (mPI).

Our cell bank, based at the Diabetes Research Institute of the University of Miami, currently includes more than 850 samples of MSCs from human and murine sources, cryopreserved at different culture passages. These cell cultures, as well as their derivatives (e.g. conditioned supernatants, purified exosomes), have been obtained from well-phenotyped donors and are made available to qualified research scientists throughout the world.

We generated a searchable database with the characteristics of the samples, including: species, cell type, sample number, culture passage, number of vials, date, culture medium, harvesting reagent, freezing medium, and number of cells.

Specie	Cell Type	Sample N	Passage	Number of via	Date	Culture Medium	Harvesting reagei	Freezing medium	Number of cells/surfac	Location
h	PI-MSC	2271	5	1	14/05/2018	DMEM-L 10% FBS	TrypLE Select	CS10	718k cells	-80C 5th fl
m	P-MSC NOD	0359	2	4	14/05/2018	DMEM-L 10% FBS	Trypsin-EDTA 0.05%	10% DMSO, 50% FBS, 40% DMEM-L	1/3 T175	-80C 5th fl
h	PI-MSC	2293	1	6	15/05/2018	DMEM-L 10% FBS	TrypLE Select	CS10	1/3 T175	-80C 5th fl
h	PI-MSC	2233	5	10	15/05/2018	DMEM-L 10% FBS	TrypLE Select	CS10	87500 cells/35µl	-80C 5th fl
h	PI-MSC	2292	4	6	17/05/2018	DMEM-L 10% FBS	TrypLE Select	CS10	1/3 T175	-80C 5th fl
h	PI-MSC	2271	3	5	16/05/2018	DMEM-L 10% FBS	TrypLE Select	CS10	1/3 T175	-80C 5th fl
h	PI-MSC	2293	2	6	18/05/2018	DMEM-L 10% FBS	TrypLE Select	CS10	1/3 T175	-80C 5th fl
h	PI-MSC	2293	3	5	21/05/2018	DMEM-L 10% FBS	TrypLE Select	CS10	1/3 T175	-80C 5th fl
h	PI-MSC	2271	4	6	21/05/2018	DMEM-L 10% FBS	TrypLE Select	CS10	1/3 T175	-80C 5th fl
h	AM-MSC	0001	1	4	22/05/2018	BIMSC Nutritem XF + supplement	TrypLE Select	CS10	1/2 T75	-80C 5th fl
h	Chorion-MSC	0001	0	9	23/05/2018	BIMSC Nutritem XF + supplement	TrypLE Select	CS10	2/15 T175	DRI cGMP
h	Chorion-MSC	0001	0	5	23/05/2018	BIMSC Nutritem XF + supplement	TrypLE Select	CS10	2/15 T175	-80C 5th fl
h	Chorion-MSC	0001	0	1	23/05/2018	BIMSC Nutritem XF + supplement	TrypLE Select	CS10	1/19 T175	-80C 5th fl
h	PI-MSC	2293	4	6	24/05/2018	DMEM-L 10% FBS	TrypLE Select	CS10	1/3 T175	-80C 5th fl
h	PI-MSC	2292	5	3	24/05/2018	DMEM-L 10% FBS	TrypLE Select	CS10	1*10^6 cells	-80C 5th fl
h	PI-MSC	2292	5	15	24/05/2018	DMEM-L 10% FBS	TrypLE Select	CS10	125k/50µl	-80C 5th fl
h	AM-MSC	0001	2	4	25/05/2018	BIMSC Nutritem XF + supplement	TrypLE Select	CS10	1/2 T75	-80C 5th fl
h	Chorion-MSC	0001	1	6	28/05/2018	BIMSC Nutritem XF + supplement	TrypLE Select	CS10	1/3 T175	-80C 5th fl
h	Chorion-MSC	0001	1	6	28/05/2018	BIMSC Nutritem XF + supplement	TrypLE Select	CS10	1/3 T175	-80C 5th fl
h	PI-MSC	2293	5	14	28/05/2018	DMEM-L 10% FBS	TrypLE Select	CS10	125k/50uL	-80C 5th fl
h	PI-MSC	2293	5	7	28/05/2018	DMEM-L 10% FBS	TrypLE Select	CS10	1*10^6 cells	-80C 5th fl
h	PI-MSC	2271	5	15	28/05/2018	DMEM-L 10% FBS	TrypLE Select	CS10	125k/50uL	-80C 5th fl
h	AM-MSC	0001	3	6	28/05/2018	BIMSC Nutritem XF + supplement	TrypLE Select	CS10	1/3 T75	-80C 5th fl
h	PI-MSC	2293	5	1	28/05/2018	DMEM-L 10% FBS	TrypLE Select	CS10	1*10^6 cells	-80C 5th fl
h	PI-MSC	2271	5	3	28/05/2018	DMEM-L 10% FBS	TrypLE Select	CS10	780k cells	-80C 5th fl
h	PI-MSC	2233	4	3	31/05/2018	DMEM-L 10% FBS	TrypLE Select	CS10	87500 cells/35µl	-80C 5th fl
h	PI-MSC	2288	4	3	31/05/2018	DMEM-L 10% FBS	TrypLE Select	CS10	87500 cells/35µl	-80C 5th fl
h	PI-MSC	2293	4	6	31/05/2018	DMEM-L 10% FBS	TrypLE Select	CS10	87500 cells/35µl	-80C 5th fl
h	PI-MSC	2293	4	1	31/05/2018	DMEM-L 10% FBS	TrypLE Select	CS10	685k cells	-80C 5th fl
h	PI-MSC	2232	5	5	31/05/2018	DMEM-L 10% FBS	TrypLE Select	CS10	87500 cells/35µl	-80C 5th fl
h	PI-MSC	2271	4	6	31/05/2018	DMEM-L 10% FBS	TrypLE Select	CS10	87500 cells/35µl	-80C 5th fl
h	PI-MSC	2271	4	1	31/05/2018	DMEM-L 10% FBS	TrypLE Select	CS10	418k cells	-80C 5th fl
h	PI-MSC	2291	4	8	31/05/2018	DMEM-L 10% FBS	TrypLE Select	CS10	87500 cells/35µl	-80C 5th fl
h	PI-MSC	2291	4	1	31/05/2018	DMEM-L 10% FBS	TrypLE Select	CS10	277k cells	-80C 5th fl
h	PI-MSC	2292	4	1	31/05/2018	DMEM-L 10% FBS	TrypLE Select	CS10	78k cells	-80C 5th fl
h	PI-MSC	2292	4	8	31/05/2018	DMEM-L 10% FBS	TrypLE Select	CS10	87500 cells/35µl	-80C 5th fl

Fig. 15 - The Cell Bank generated, based at the Diabetes Research Institute, currently includes more than 850 samples of MSCs from different human and murine sources, cryopreserved at different culture passages. The Cell Bank is now available to qualified research scientists throughout the world.

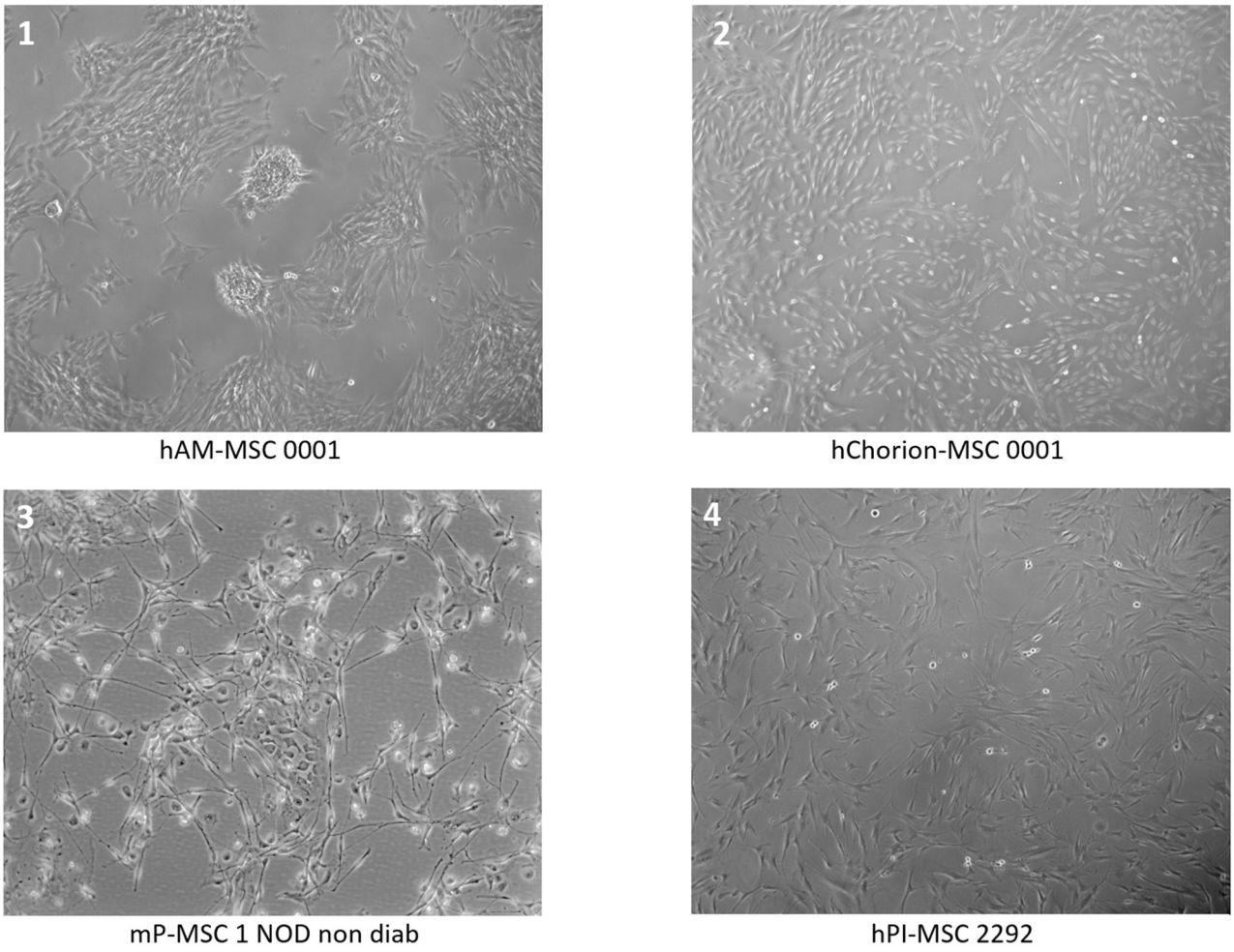


Fig. 16 - Cultures of MSCs from different sources. The culture-expanded cells present different morphologies.

1 – human Amniotic Membrane-derived MSCs;
2 – human Chorion Membrane-derived MSCs;
3 – murine Pancreas-derived MSCs;
4 – human Pancreatic Islet-derived MSCs.

Human Pancreatic Islet-derived MSCs culture and characterization

Cadaveric donor pancreata (n=7) were obtained via Organ Procurement Organizations and pancreatic islets (PI) were isolated following the Ricordi method at the cGMP Cell Processing Facility of the Diabetes Research Institute – University of Miami.

Pancreatic Islets were seeded and cultured in tissue treated flask with DMEM-L (Dulbecco`s Modified Eagle`s Medium 1g glucose/L) supplemented with 10% heat-inactivated FBS (Fetal Bovine Serum) and incubated at 37°C in 5% CO2.

These culture conditions facilitate the proliferation of MSCs. Cells with fibroblast-like morphology grow out of pancreatic islets structures within 7 days, and we can rapidly obtain cultures of fibroblast-like morphology that appears morphologically similar to MSCs derived from other sources. Culture expansion determines the selection of a homogeneous mesenchymal population with loss of endocrine cells.

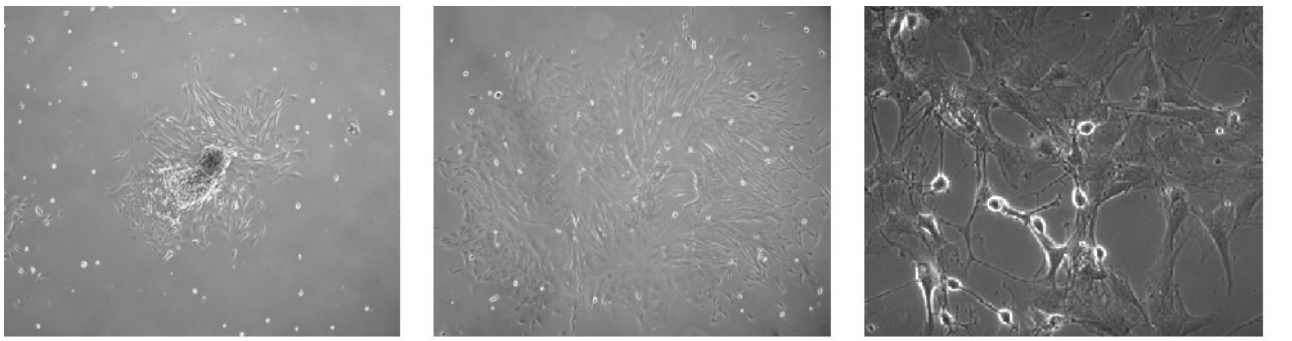


Fig. 17 - hPI-MSCs culture.

In order to evaluate the cell proliferation activity, a cell growth assay was performed with PI-MSCs at passage 5. Cells were plated at 5.000 cells/cm2 in 24-well plates and cell growth was assessed using Alamar Blue assay and fluorescence was measured using a Victor Multilabel Counter. The same procedure was repeated on days 2, 3, 5, and 7 after seeding. Data were plotted as the percentage of Alamar reduction analyzed at specific time points.

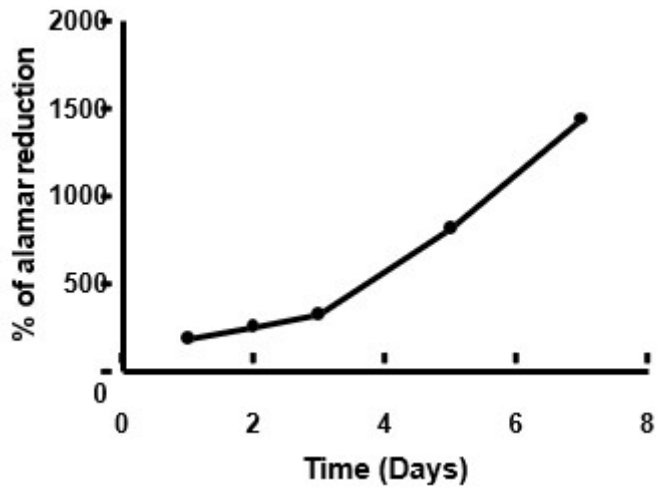


Fig. 18 - PI-MSCs (P5) growth kinetics.

The immunophenotypic analysis performed via Flow Cytometry confirmed the mesenchymal nature of the isolated cells and showed that PI-MSCs are similar to MSCs from other sources.

PI-MSCs do not express hemopoietic markers CD34 and CD45, nor the epithelial marker PanCitokeratin. Instead, PI-MSCs show a high expression of mesenchymal specific markers, such as CD44, CD73, CD90, and CD105. PI-MSCs are negative for HLA-DR, a major histocompatibility complex (MHC) class II cell surface receptor. This suggests the ability of PI-MSCs to evade recognition from the immune system, a characteristic that could facilitate allogeneic use.

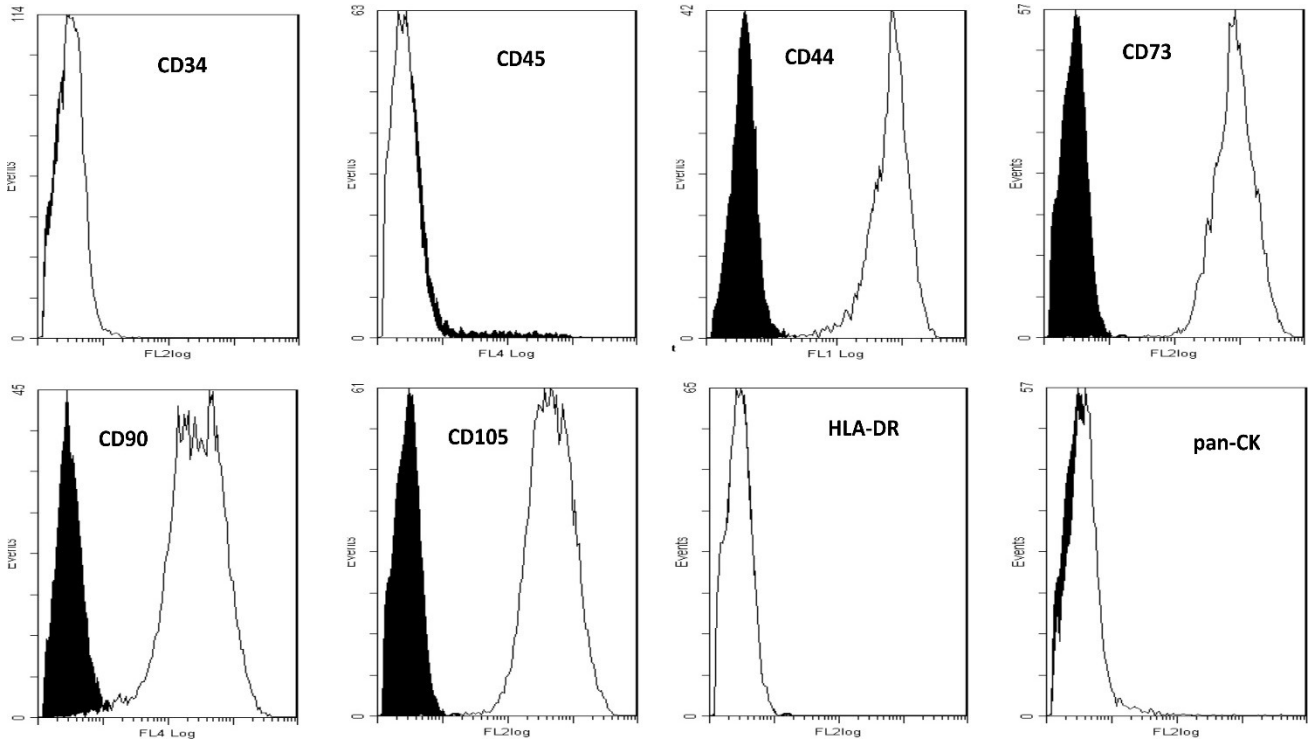


Fig. 19 - PI-MSCs immunophenotypic characterization. Cytofluorimetric analysis of the expression of hemopoietic

markers (CD34 and CD45), of mesenchymal markers (CD44, CD73, CD90 and CD105), HLA-DR and epithelial marker Pan-CK. Unstained controls are presented as filled black histograms, the specific cell markers as white histograms.

Moreover, the following immunofluorescence confirmed that hPI-MSCs do not express the epithelial marker Cytokeratin, whereas they express the mesenchymal marker Vimentin.

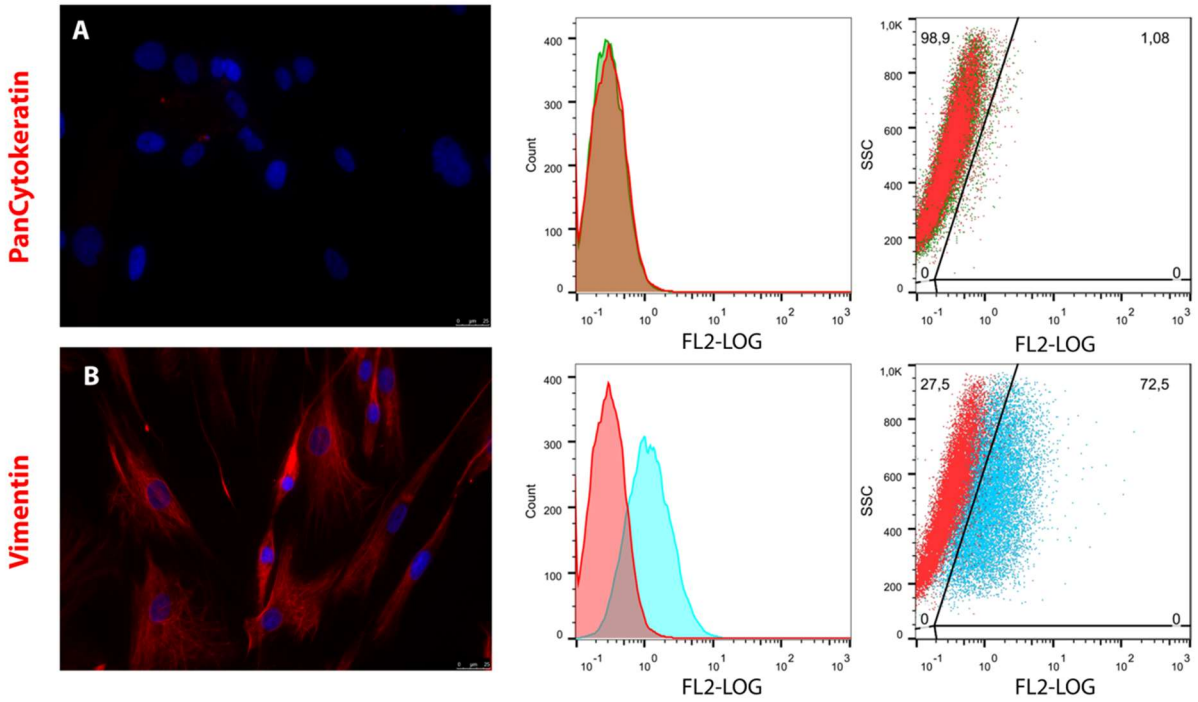


Fig. 20 - PI-MSC do not express the epithelial marker Pan Cytokeratin (A), whereas they express the mesenchymal marker vimentin (B).

In order to further confirm the mesenchymal origin of hPI-MSCs, we tested their ability to differentiate *in vitro* towards the adipogenic, osteogenic, and chondrogenic lineages.

After *in vitro* induction, Adipogenic differentiation was analyzed via Oil Red O staining, a dye that stains neutral triglycerides and lipids. A subset of PI-MSCs matured into cells containing multiple lipid-rich vacuoles in the cytoplasm, vacuoles that increased in size and number during the three weeks of induction and that were intensely stained red with Oil Red O (Fig. 21a). After two weeks of induction, osteogenic differentiation was tested with Alizarin Red staining - a staining for calcium accumulation. Intense red staining was observed in induced cells indicating extracellular mineralization. Control cells did not show Alizarin Red positive deposits (Fig. 21b). Chondrogenic differentiation was performed in a pellet culture system in order to stimulate aggregation and create a 3D structure in low oxygen tension. Fig. 21c shows the formation of a cell pellet, stained with

hematoxylin eosin, more compact and well-structured in induced cells. The chondrogenic differentiation was further documented by Alcian Blue staining (Fig. 21d). Differentiated cells appeared embedded in an abundant extracellular matrix that proved highly positive for the presence of proteoglycans, as evidenced by Alcian Blue staining in the induced cells.

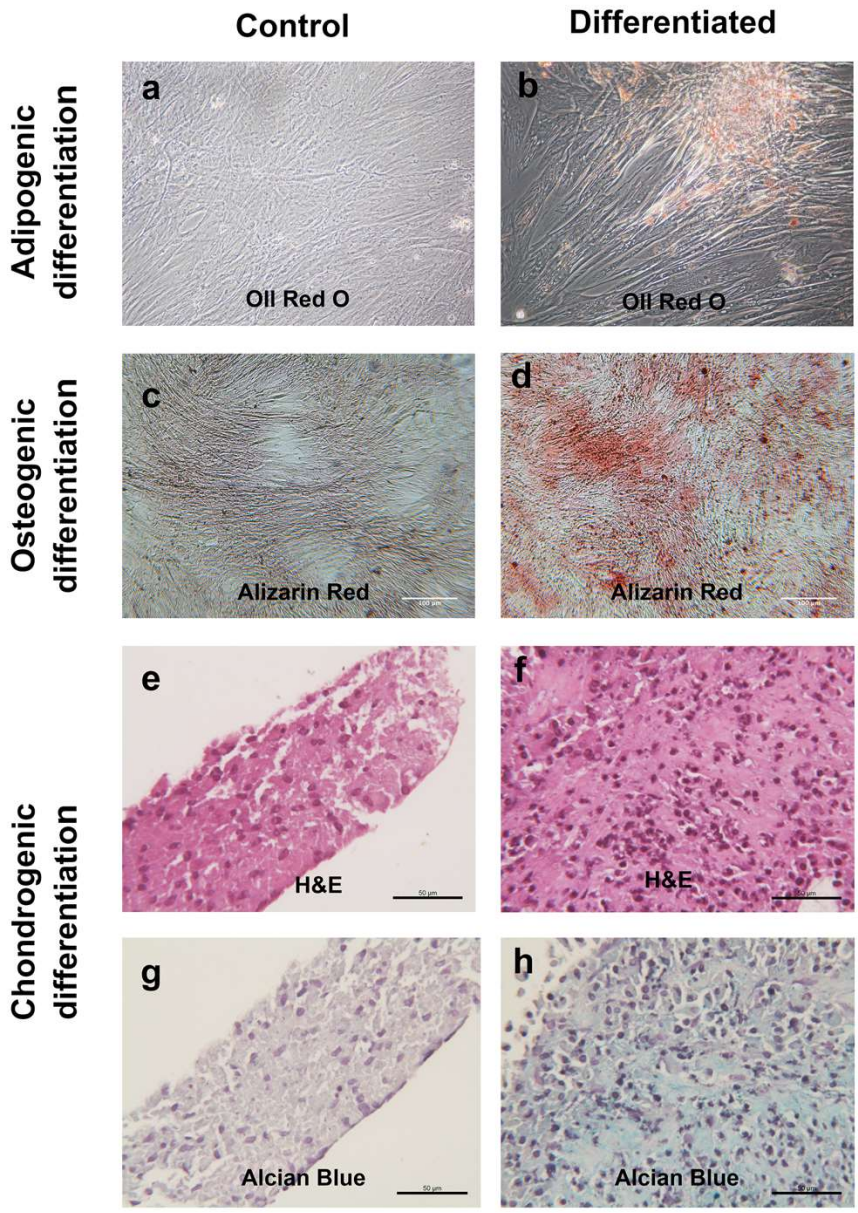


Fig. 21 - *In vitro* differentiation of PI-MSC towards multiple mesenchymal lineages. a) Oil red O staining on control and adipogenic-differentiated cells. b) Alizarin Red staining on control and osteogenic-differentiated cells. c) hematoxylin eosin staining on control and chondrogenic-differentiated cells. d) Alcian blue staining on control and chondrogenic-differentiated cells.

Pancreatic Islets are highly vascularized, with endothelial cells showing CD31 expression and pericytic cells showing NG2 expression. Pericytes form a wrap surrounding the vessel endothelium. NG2+ pericytic cells can give rise to cultures of MSCs. We observed that culture-expanded hPI-MSCs cultures present a subpopulation expressing NG2 (Fig. 22 a, b), suggesting that these cells derive from islet pericytes. Pericytes and their culture-expanded derivatives from other tissue sources exert immunomodulatory function. The observation that hPI-MSC also express the immunomodulatory molecule HLA-G (Fig. 22a) stimulated our curiosity on the analysis of their immunomodulatory activity.

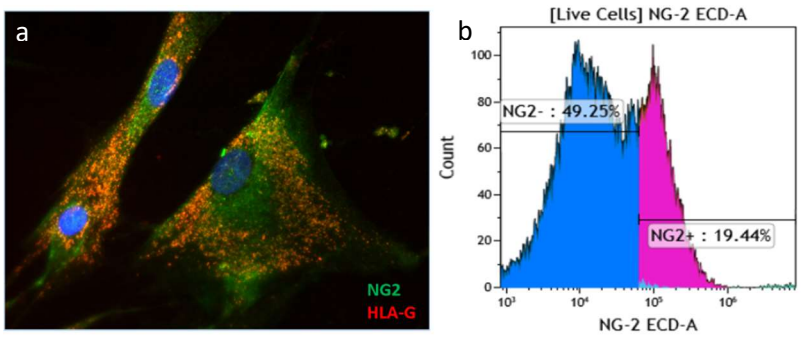


Fig 22 - Culture-expanded PI-MSC present a subpopulation of cells displaying expression of NG2, a pericytic marker, suggesting that these cells derive from pancreatic islet pericytes. These cells also express HLA-G, a molecule involved in immunomodulatory functions. (a) NG2 and HLA-G were detected via immunofluorescence staining; (b) A subpopulation of PI-MSCs express the pericyte marker NG2, as observed via flow cytometry.

hPI-MSCs inhibit the proliferation of activated PBMCs

Culture-expanded hPI-MSC presented expression of immunomodulatory molecules, hence we hypothesized that hPI-MSCs could inhibit the proliferation of Peripheral blood mononuclear cells (PBMCs). For this purpose, we performed ad *in vitro* test of immunomodulation. Figure 18a shows that after five days of co-culture, PI-MSCs are able to inhibit the proliferation of the PBMCs stimulated with PHA (phytohemagglutinin). Compared to PBMCs activated with PHA (100 ± 2% BrdU incorporation), PHA-activated PBMCs co-cultured with PI-MSCs showed a significantly lower BrdU incorporation (19,0 ± 1,7%). Morover, HLA-G expression was analyzed: HLA-G expression was found to increase, and the HLA-G+ PI-MSC subpopulation shifted from 25,8% to 83,3% after co-culture.

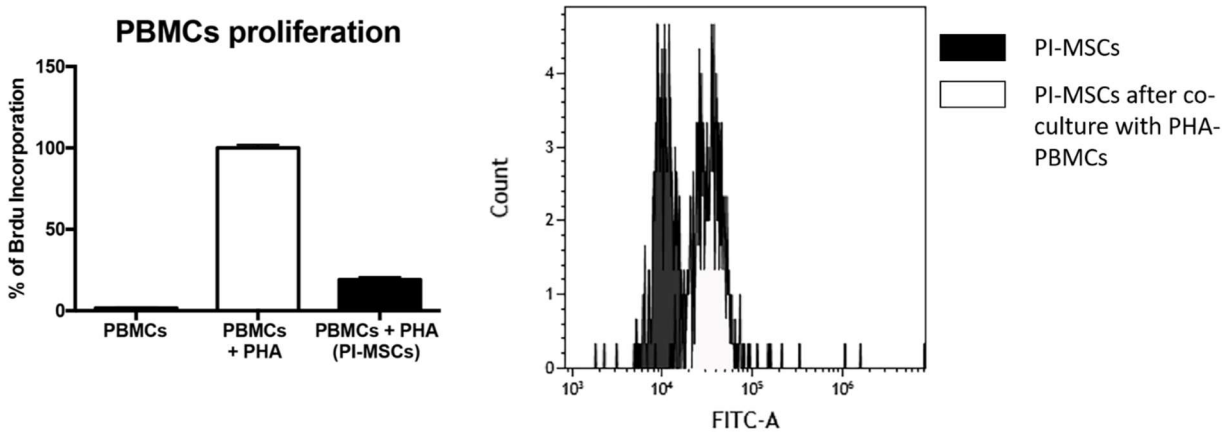


Fig. 23 - hPI-MSCs inhibit the proliferation of phytohemagglutinin-stimulated PBMCs. a) BrdU incorporation in PBMCs is high in presence of PHA stimulation (100% BrdU incorporation), but it decreases significantly in the presence of hPI-MSCs. b) HLA-G expression in PI-MSCs in basal conditions (black histogram) and after co-culture with PHA-activated PBMCs (white histogram).

hPI-MSCs stimulate the expansion of T regulatory cells (Tregs)

We focused our attention on the immunomodulatory effect of hPI-MSC on T cells. We established hPI-MSC cultures from 6 different donors and we expanded them to Passage 5 (P5), at a passaging ratio of 1:3. Separately, we stained T cells with CFSE to enable downstream analyses of their *in vitro* proliferation via flow cytometry. Subsequently, we combined T cells with monolayers of MSC and we stimulated the proliferation of T cells with anti CD3/CD28 tetrameric antibodies in the presence of IL-2 (Fig. 24).

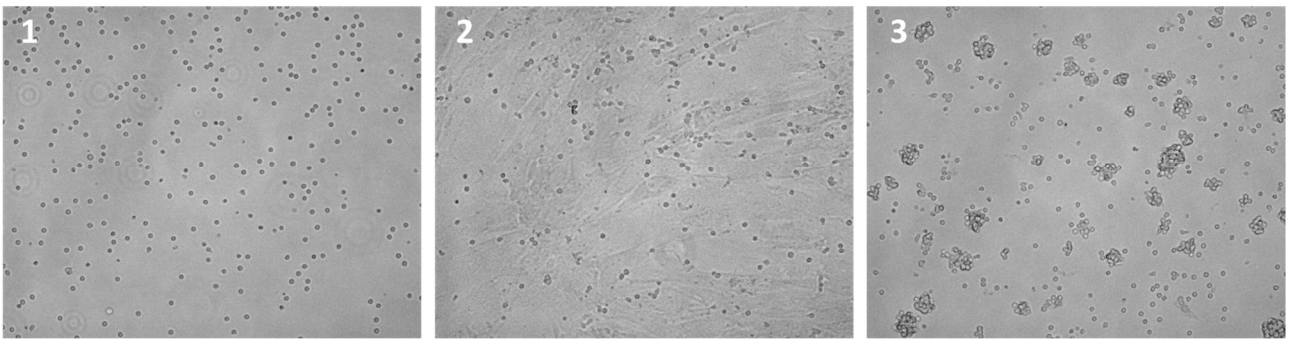


Fig. 24 - 1) Unstimulated T cells (2days 10 hours); 2) Stimulated T cells (2days 10 hours); 3) Stimulated T cells + hPI-MSCs (2days 10 hours);

After 72h of coculture, we harvested the T cells to analyze the changes occurring in the CD4+/CD25+/FoxP3+ T regulatory cell subpopulation (T regs) via Flow cytometry. We applied the

same gating strategy for all T cells, those cocultured with each of 6 cultures of hPI-MSC or those maintained in control conditions (Fig. 25). We gated for CD4+/CD25+/FoxP3+ and we observed a clear difference in the cloud of T regs related to the co-cultivation with PI-MSC (Fig. 25). Among T regs, we also observed differences in the proliferated ($CFSE^{dim}$, multiple generations) and unproliferated ($CFSE^{bright}$, fewer generations) Tregs.

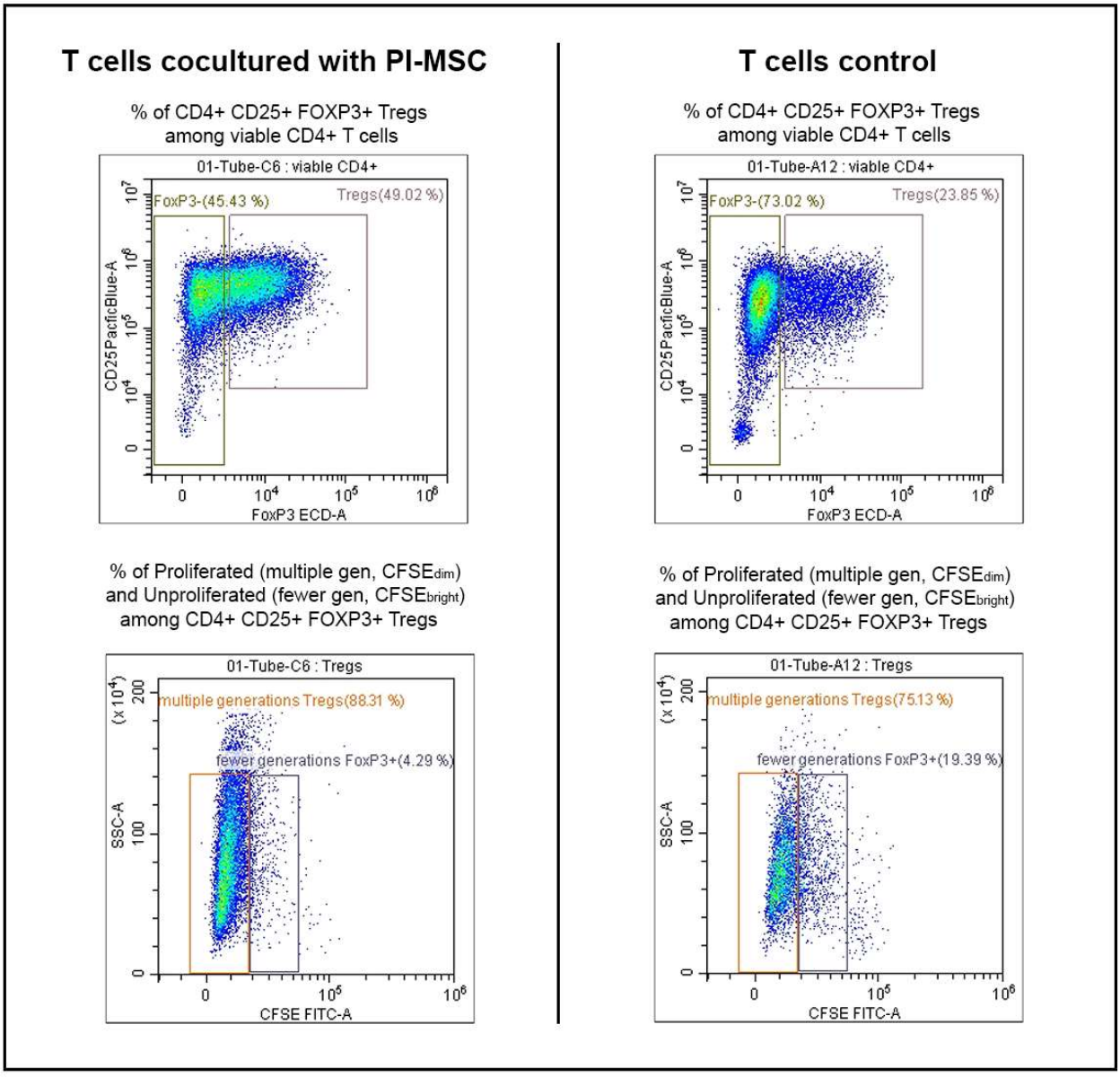


Fig. 25 - Flow cytometry dot plots and gating strategy for T reg analysis via CD4, CD25, FoxP3, and CFSE staining.

Data analysis indicates that hPI-MSCs stimulate an absolute (events) and relative (% of Total) expansion of T regs (figure 26). By tracking the dilution of CFSE staining after 72h of coculture, we observed a significant increase of proliferated $CFSE^{dim}$ CD4+/CD25+/FoxP3+ Tregs when the T cells

were cocultured with PI-MSCs, compared to the controls (Fig. 26). Hence, the observed T reg expansion is the result of a stimulation in T reg proliferation occurring when T cells are cocultured with hPI-MSCs.

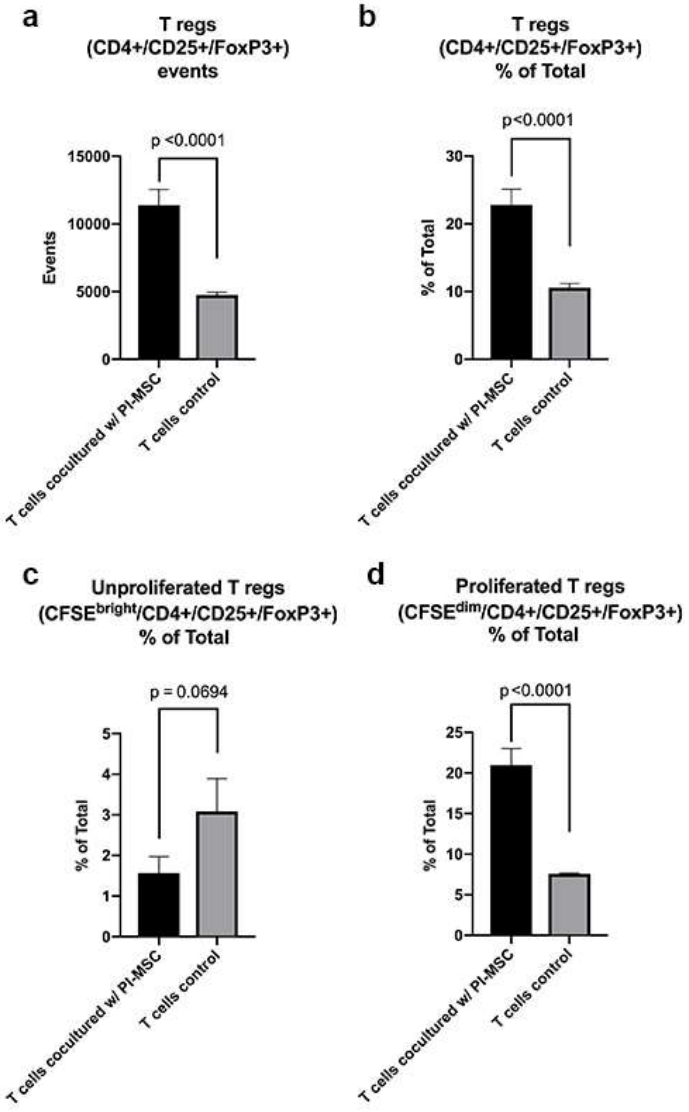


Fig 26 - Quantification of the T reg populations. Human T cells were either cocultured with hPI-MSC (from n=6 different donors, black columns) or cultured alone (T cells control, n=3, grey columns) and their proliferation was stimulated with anti-CD3/CD28 tetramers in the presence of IL-2. We observed that hPI-MSCs stimulate an (a) absolute (events) and (b) relative (% of Total) expansion of T regs (CD4+/CD25+/FoxP3+). This expansion was due to T reg proliferation (c, d), as evidenced by the significant increase in $CFSE^{dim}$/CD4+/CD25+/FoxP3+ T reg cells.

Glucose-stimulated Insulin Release (GSIR) of hPI-MSC with Islets

We tested the effect of hPI-MSC at passage 5 on the glucose-stimulated insulin release function of isolated human pancreatic islets. To investigate whether the effect is contact-dependent or mediated by soluble factors, we compared hPI-MSC in contact with islets versus hPI-MSCs separated from islets via transwell. hPI-MSCs were utilized at a ratio of 1:15 with islet cells. We utilized hPI-MSC alone and islets alone as controls.

In our experiment, we observed that pancreatic islets alone (Fig. 27, red dotted line) had minimal insulin release function, whereas the presence of hPI-MSC bolstered the glucose responsivity and absolute insulin release (Fig. 27, blue and green dotted line). The corrected Stimulation (cSI) index is calculated by dividing the amount of insulin released in high glucose conditions by the average insulin released in low glucose conditions, before and after the high-glucose stimulation. The cSI of PI-MSC+islets in Transwell was 1.5, the cSI of PI-MSC+islets in contact was 1.4, whereas the cSI of islets alone was 1.2. The supernatant of PI-MSC alone contained a negligible amount of insulin (see figure 27), and there was no glucose-responsive insulin release (cSI=1). Notably, islets that were combined with PI-MSC, both in transwell and in contact, released an amount of insulin that was significantly superior to islets alone (Fig. 27), especially in the high-glucose conditions.

We conclude that hPI-MSC can boost the glucose-responsive insulin release function of islet beta cells, and that this effect is mediated by soluble factors released by hPI-MSC.

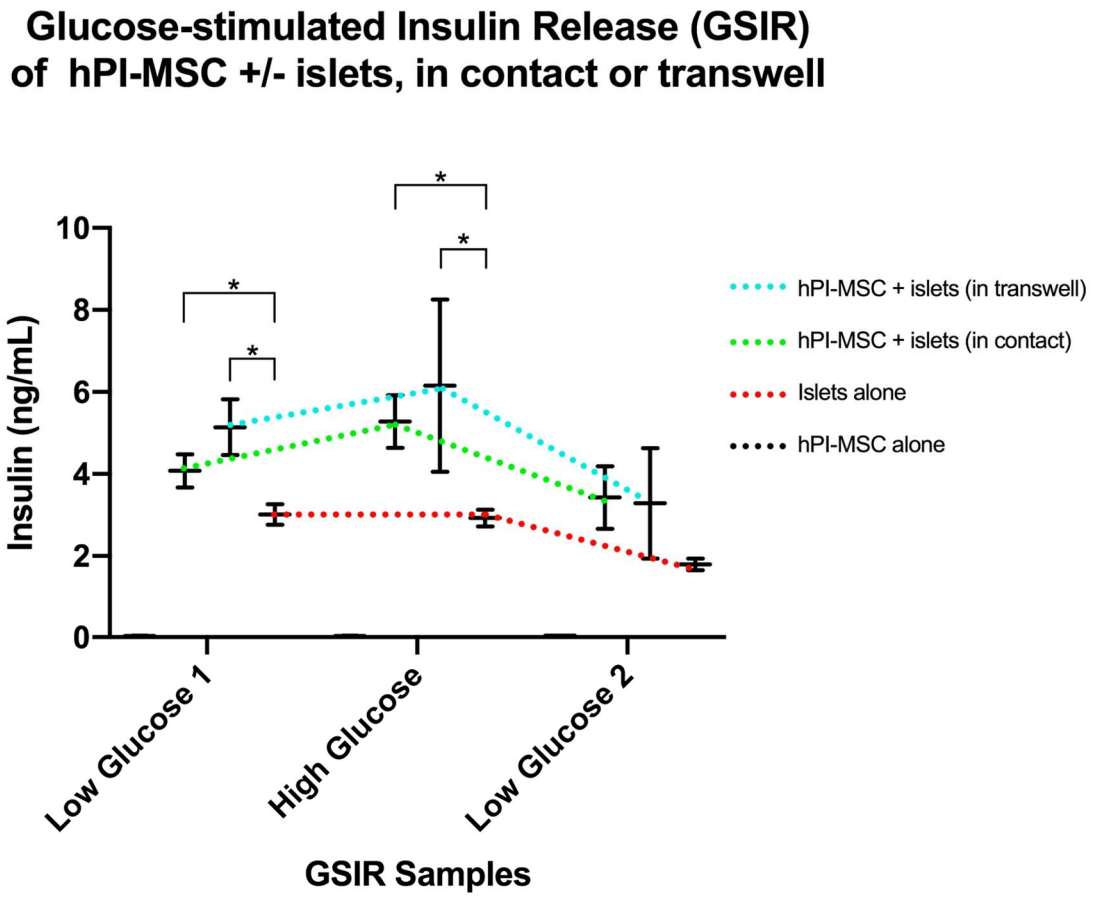


Fig. 27 - Glucose-stimulated Insulin Release of hPI-MSC +/- islets, in contact or transwell. hPI-MSC boosted the glucose-responsive insulin release function of islet beta cells, and this effect was mediated by soluble factors released by hPI-MSC, as shown by the results in the transwell.

Aggregation of MSCs and Islets

Aggregation of MSCs and islets cells was obtained by harvesting MSCs and combining them with isolated islets as it was described in Materials and Methods section. Here we show (Fig. 28) an example of the combination between MSCs and islets in Aggrewell 800 6 well plates.

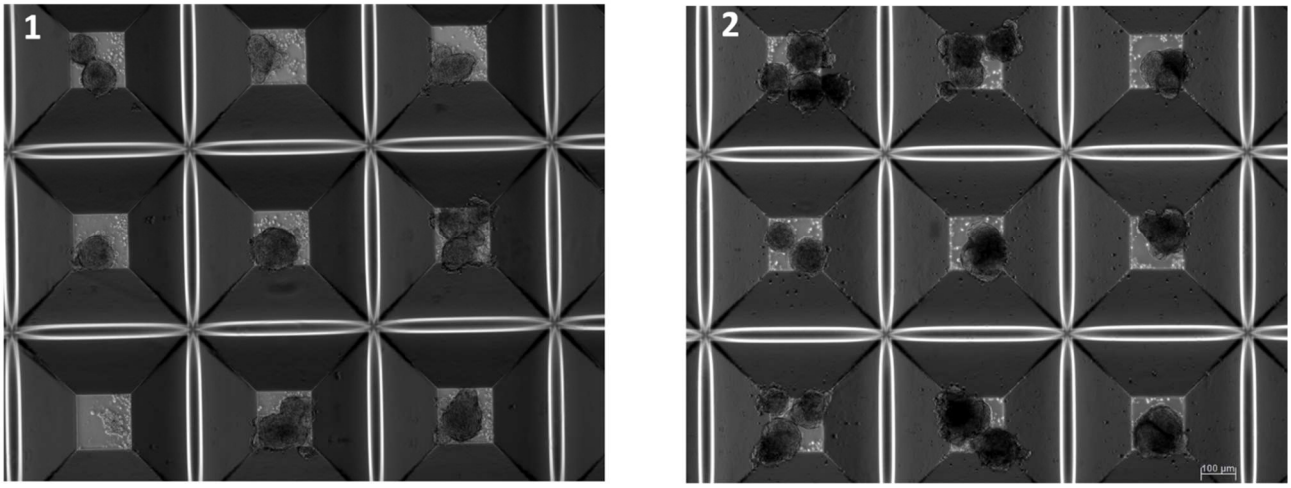
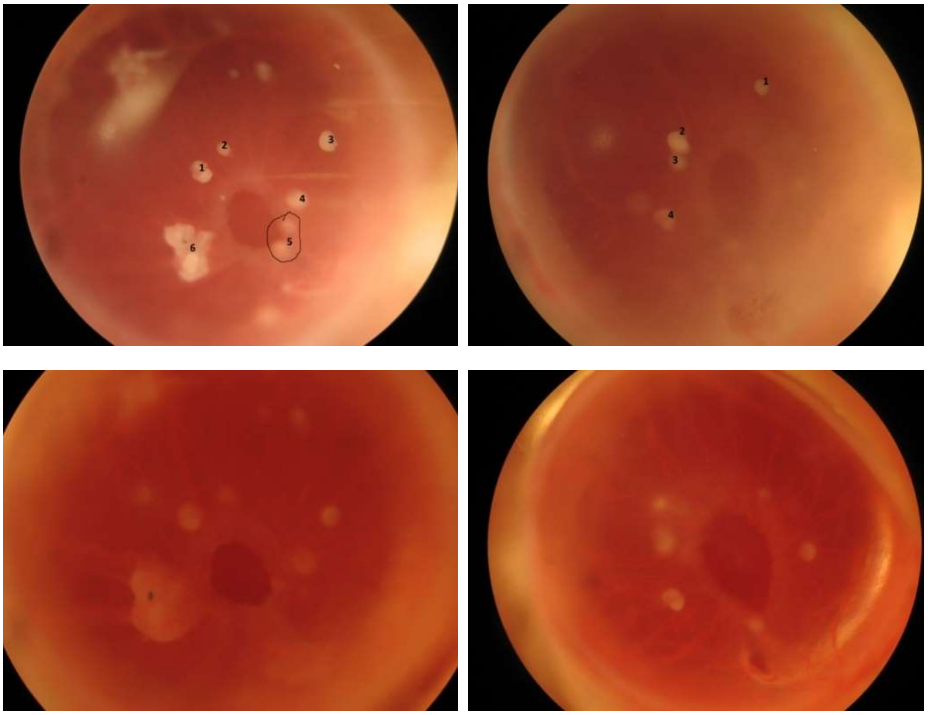


Fig. 28 -MSCs combined with islets, ready for transplantation:

1) Murine islets, 1 IEQ per well (islets alone);
2) Murine islets, 1 IEQ per well + MSCs at a ratio of 1 MSC to 15 islet cells.

In vivo imaging in the eye of NOD mice with autoimmunity and islet/MSC transplantation

We mapped the eyes of the animals and we annotated the islet structures to monitor the changes over time. We performed in vivo imaging at post-operative day (POD) 2, 7, 9. Recurrence of autoimmunity and islet loss usually occurs around POD 8.



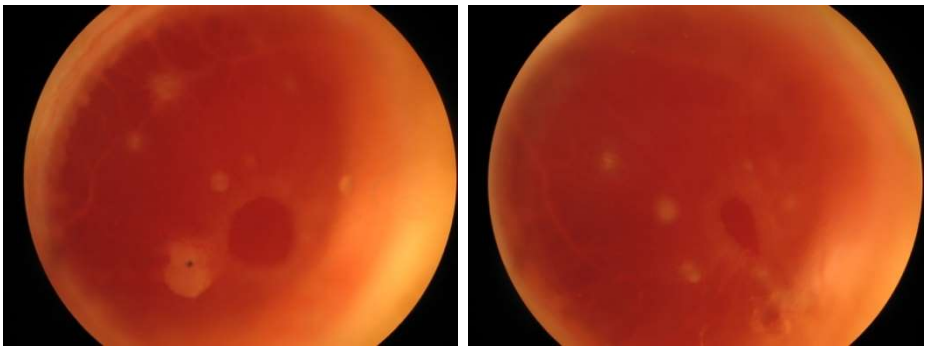


Fig. 29 - Examples of the maps of the Right eye (transplanted with NOD-GFP+ male murine Pancreatic MSC + female NOD-SCID islets) and Left eye (female NOD-SCID islets alone) of an autoimmune diabetic female NOD mouse. Post-operative day 2 (first row), 7 (second row), and 9 (third row).

On post-operative day 7, murine NOD-GFP+ Pancreatic-MSC (green) were found to reside in correspondence of the engrafted islets. MSCs appeared around and islets, creating a connective base or stalk between islets and the iris.

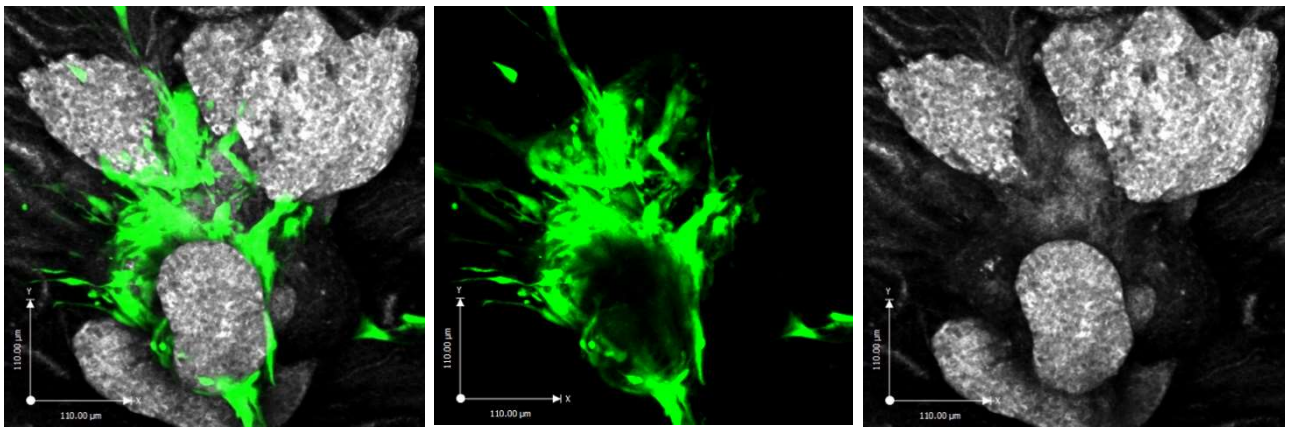


Fig. 30 - In vivo imaging in the eye at Post-operative day 7. Murine Pancreatic MSCs derived from NOD-GFP+ male mouse appear green; NOD-SCID islet cells appear bright white in the scatter channel.

After intravenous injection of red fluorescent dextran, we observed the blood vessels and capillaries in vivo. There is ongoing remodeling of the vascular structures, as highlighted by the presence of new small-caliber vessels (red) penetrating the islet structures (white). Some of the NOD-GFP+ mouse Pancreatic-MSC (green) appear aligned to and wrapping around vessels of large caliber: these cells present a phenotype consistent with that of pericytes.

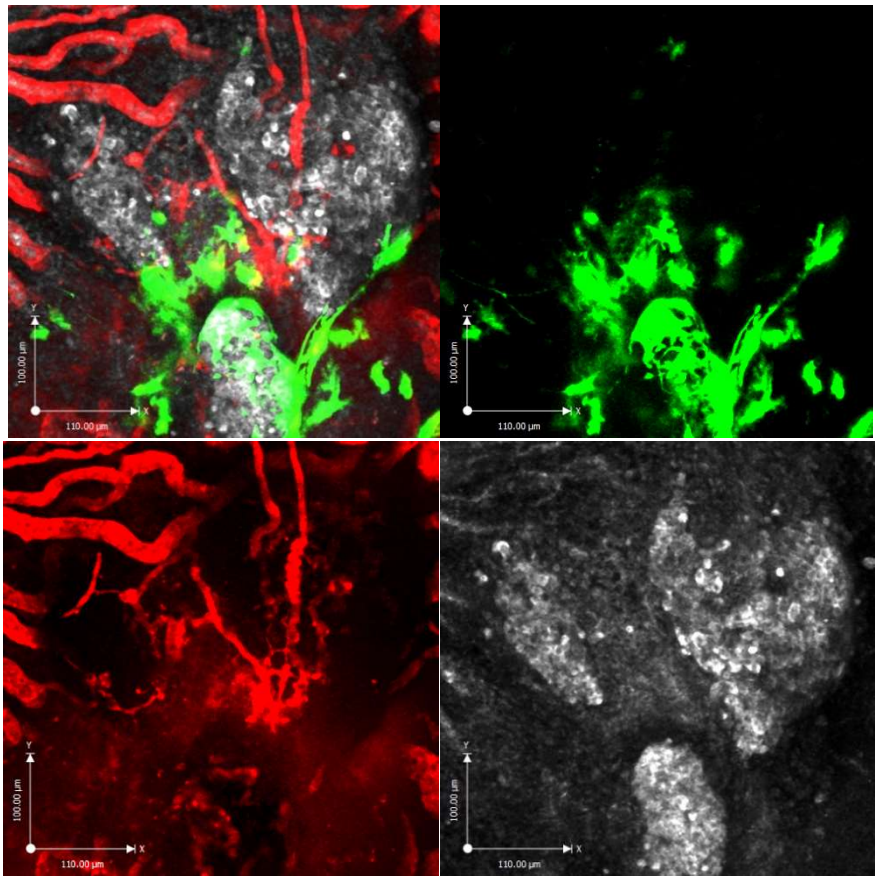


Fig. 31 - In vivo imaging in the eye at Post-operative day 7 after injection of red-fluorescent dextran. New vessels (red) of small caliber are observed, penetrating the transplanted islets. mP-MSC NOD-GFP+ appear green; NOD-SCID islet cells appear bright white.

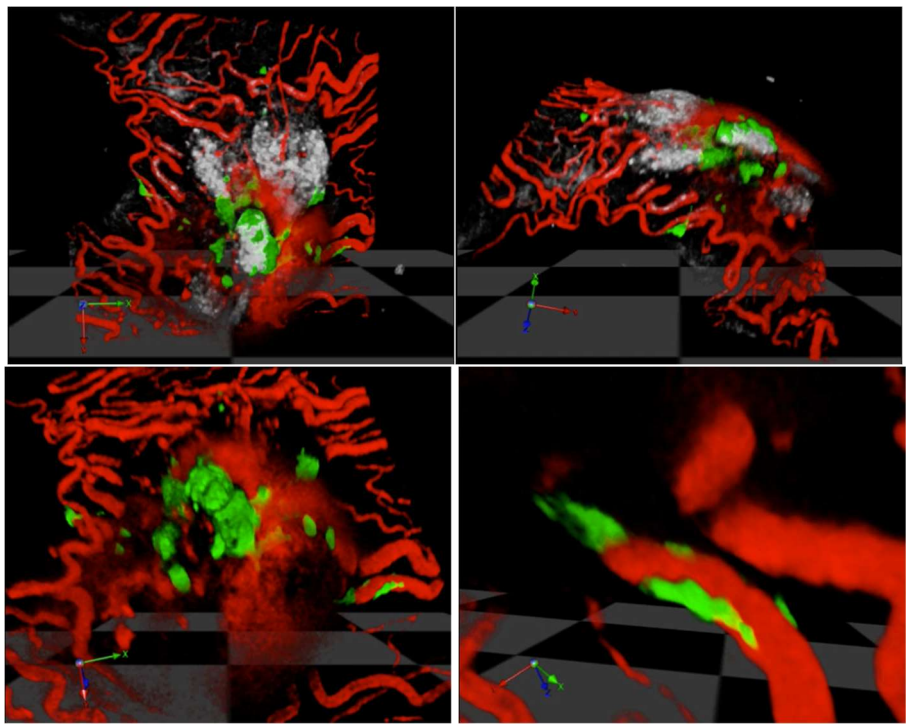


Fig. 32 - 3D reconstruction, POD7. There is ongoing remodeling of the vascular structures, as highlighted by the presence of new small-caliber vessels (red) penetrating the islet structures (white). Some of the NOD-GFP+ mouse Pancreatic-MSC (green) appear aligned to and wrapping vessels of large caliber: these cells present a phenotype consistent with that of pericytes.

Scratch Wound Assay

Patients with T1D frequently develop ulceration that do not heal easily. As of today, most studies have been conducted using the conditioned medium of MSCs derived from adipose tissue or bone marrow to accelerate wound healing *in vitro* (267).

The research we conducted seeks to determine whether the medium from human placenta-derived MSCs can accelerate the wound healing process in an *in vitro* model system, a Scratch Wound assay. After creating a scratch in a monolayer of skin fibroblast, we compared the effect of the supernatant of MSCs derived from three different Human Placenta tissues. Mesencult ACF + Supplement (animal component free) was used as a control and as supernatant diluent to test concentration-dependence. We will identify the different placenta-derived tissue sources as A, B, and C due to intellectual property rights.

The Incucyte Zoon imaging system and software enabled measurement the closed area of the scratch wound, as well as automatic calculation of the wound width (um), wound confluence (%), and relative wound density (%) at each time point.

The supernantant from different placenta-derived MSCs have differential effects on wound confluence. In particular, in comparison with the supernatant of MSCs from other sources, the (B)-MSC supernatant significantly accelerates regaining of wound confluence, and could therefore be very relevant for translational applications in wound healing and diabetic ulcers.

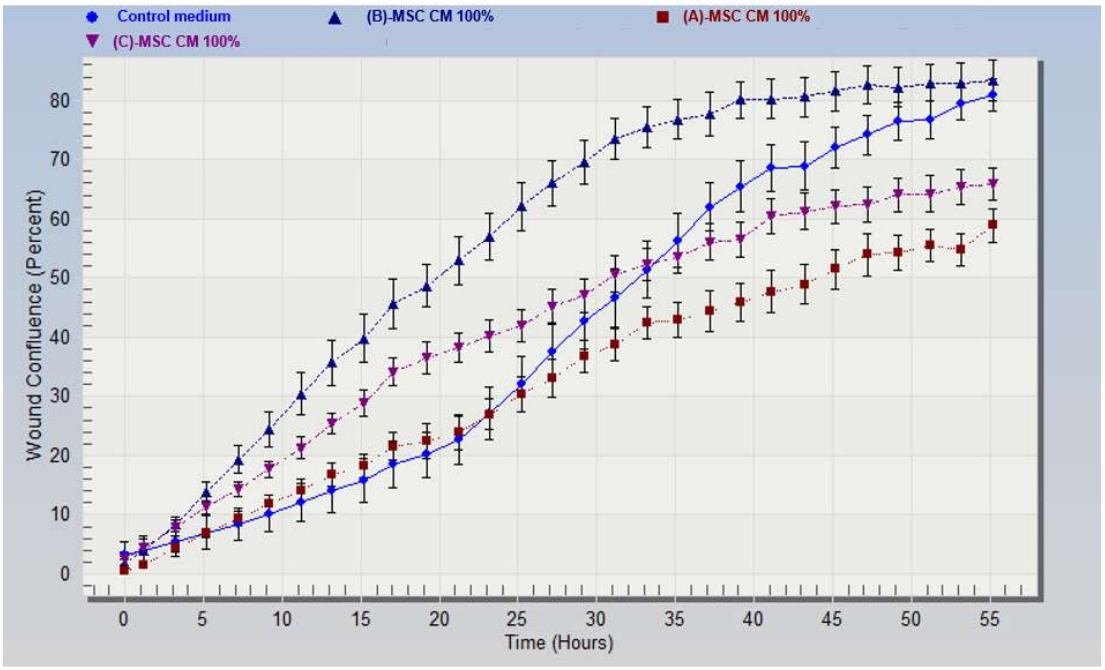


Fig. 33 - Wound Confluence: (A)-MSC – (B)-MSC – (C)-MSC supernatants (100%), Mesencult ACF + Supplement as a control. The supernatant of (B)-MSCs significantly accelerates regaining of wound confluence. Each dot shows the average, the error bars show standard deviation of tests in quadruplicate.

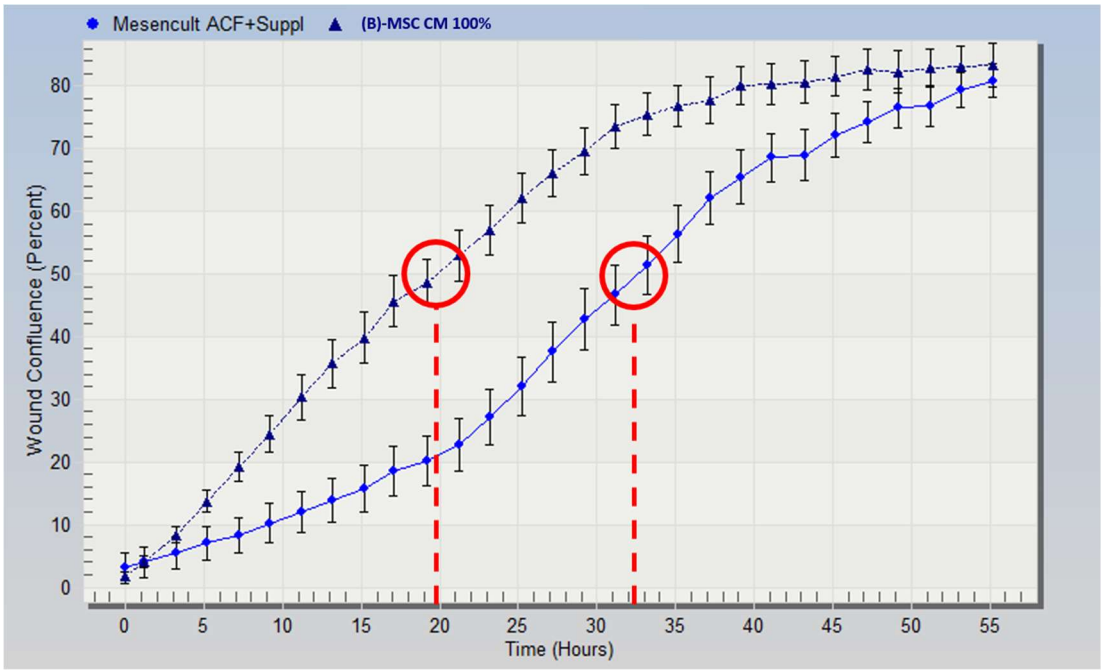


Fig. 34 - Wound Confluence: Time-to-50% confluence 39% faster with (B)-MSC CM. Each dot shows the average, the error bars show standard deviation of tests in quadruplicate.

Moreover, we have observed that this effect is concentration-dependent. In fact, we tested 33% / 66% / 100% conditioned medium (diluted in the control medium, Mesencult ACF + Supplements) and the regaining of wound confluence is accelerated with each increase in concentration of (B)-MSCs supernatant.

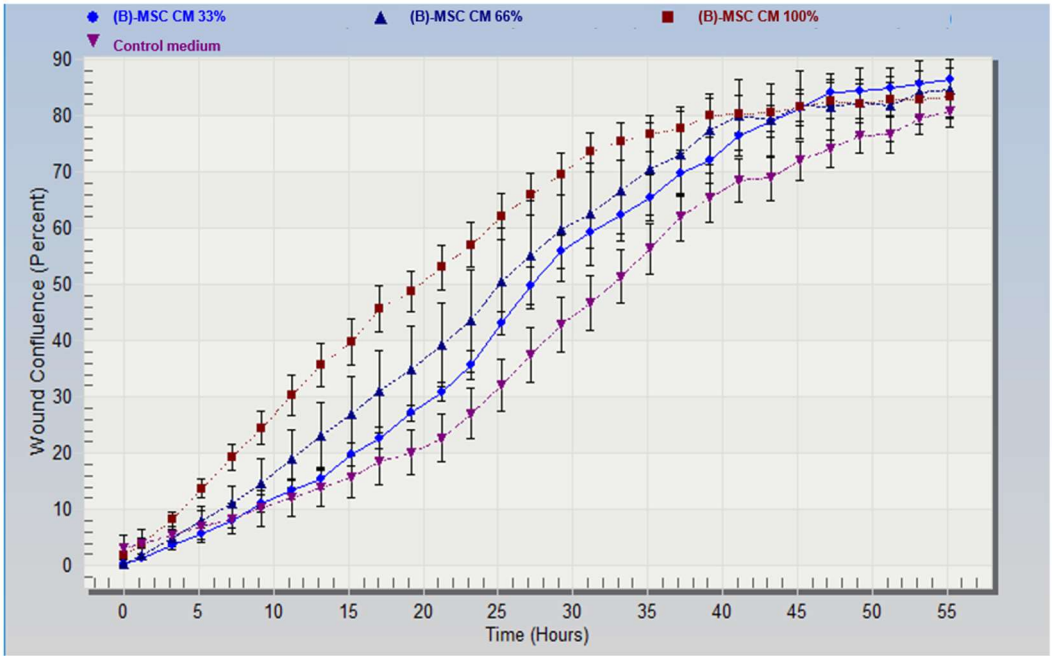


Fig. 35 - Re-acquisition of Wound Confluence is dependent on the concentration of (B)-MSC conditioned medium: (B)-MSC supernatants (100%, 66%, and 33% concentration - diluted in control medium); Mesencult ACF + Supplements was used as a control. Each dot shows the average, the error bars show standard deviation of tests in quadruplicate.

With the Essen IncuCyte ZOOM it is also possible to automatically collect time-lapse images and generate videos. This allows to appreciate how (B)-MSCs accelerate scratch wound healing (Fig. 36).

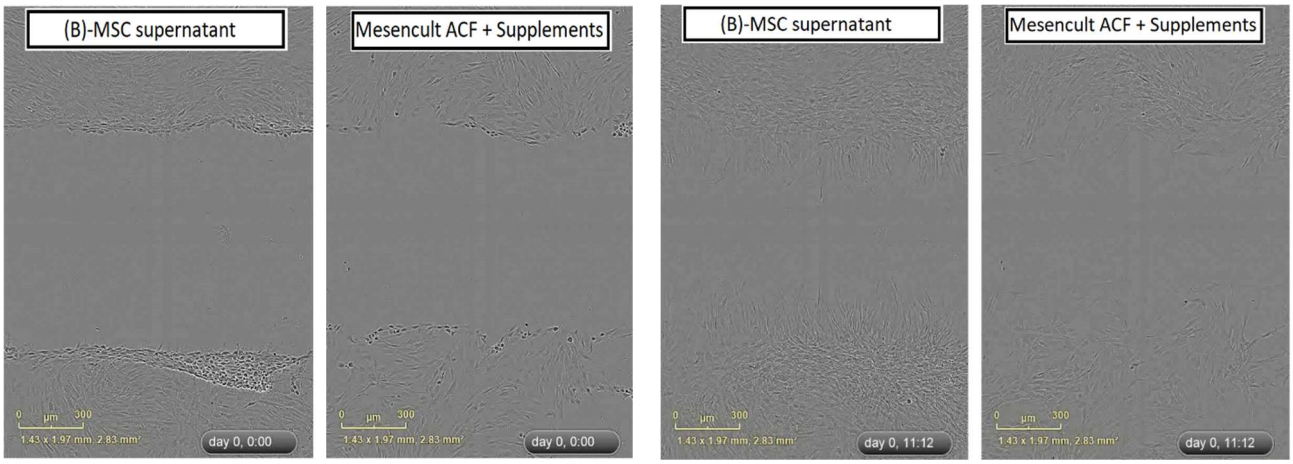


Fig. 36 - Time-lapse images in the same area (Magnification 10x) show how the supernatant from (B)-MSC accelerates wound closure in our *in vitro* Scratch Wound Assay. (B)-MSC conditioned medium appears promising for wound healing.

Similar observations were made by analyzing different metrics. The supernatant of (B)-MSC was found to accelerate regaining of Relative Wound Density (RWD) and was found to be superior to that of (A)-MSC, (C)-MSC, and control medium.

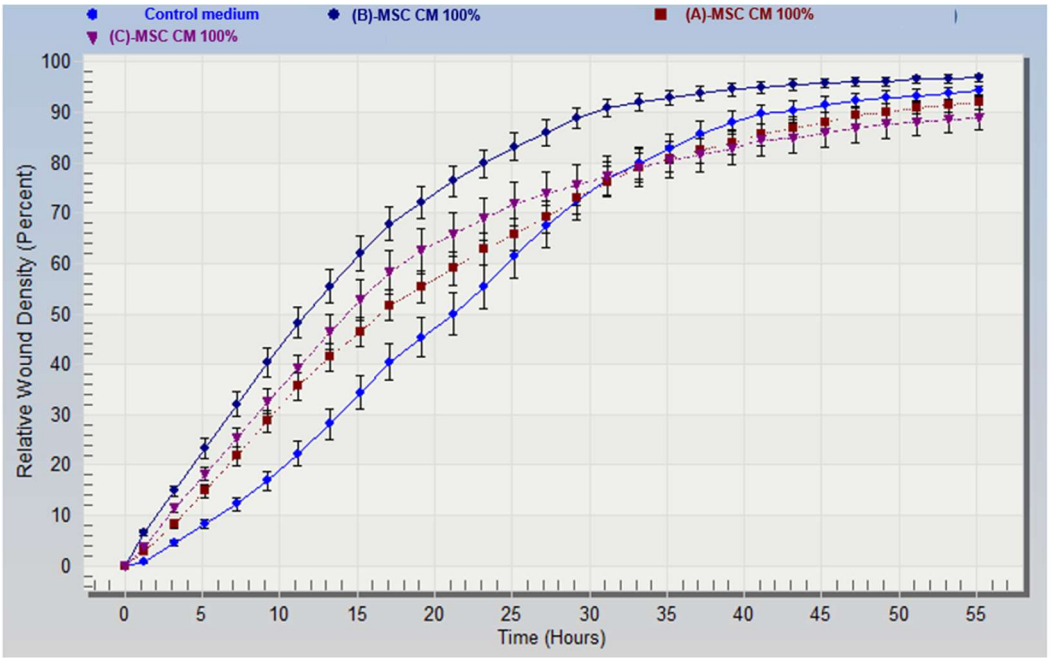


Fig. 37 - Relative Wound Density: (A)-MSC – (B)-MSC – (C)-MSC supernatants (100%), Mesencult ACF + Supplement as a control. (B)-MSC supernatant accelerates re-acquisition of relative wound density. Each dot shows the average, the error bars show standard deviation of tests in quadruplicate.

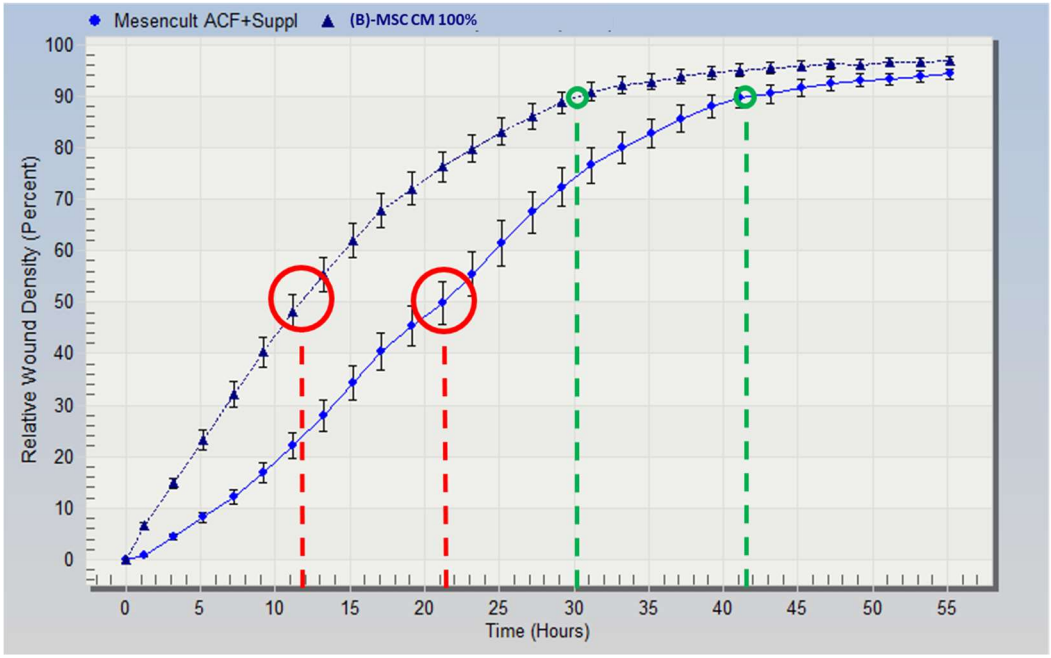


Fig. 38 - Relative Wound Density: Time-to-50% Relative Wound Density resulted 54% faster with (B)-MSC conditioned medium (in red); Time-to-90% Relative Wound Density resulted 28% faster with (B)-MSC CM (in green). Each dot shows the average, the error bars show standard deviation of tests in quadruplicate.

The positive effect of (B)-MSC supernatant on Relative Wound Density is concentration-dependent.

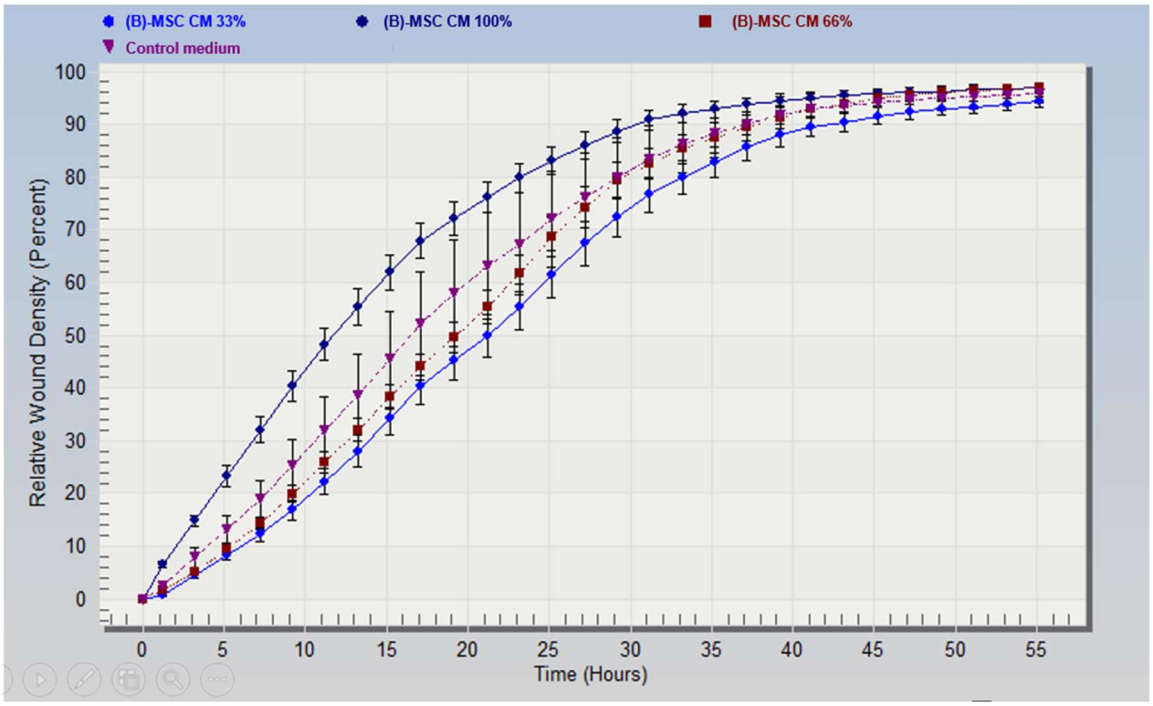


Fig. 39 - The rate of increase of relative wound density (%) is dependent on the concentration of (B)-MSC conditioned medium: (B)-MSC supernatants (100%, 66%, and 33% concentration - diluted in control medium); Mesencult ACF + Supplements was used as control. Each dot shows the average, the error bars show standard deviation of tests in quadruplicate.

The effect of (B)-MSC supernatant on the closure of wound width is superior to that of (A)-MSC, (C)-MSC, and control medium.

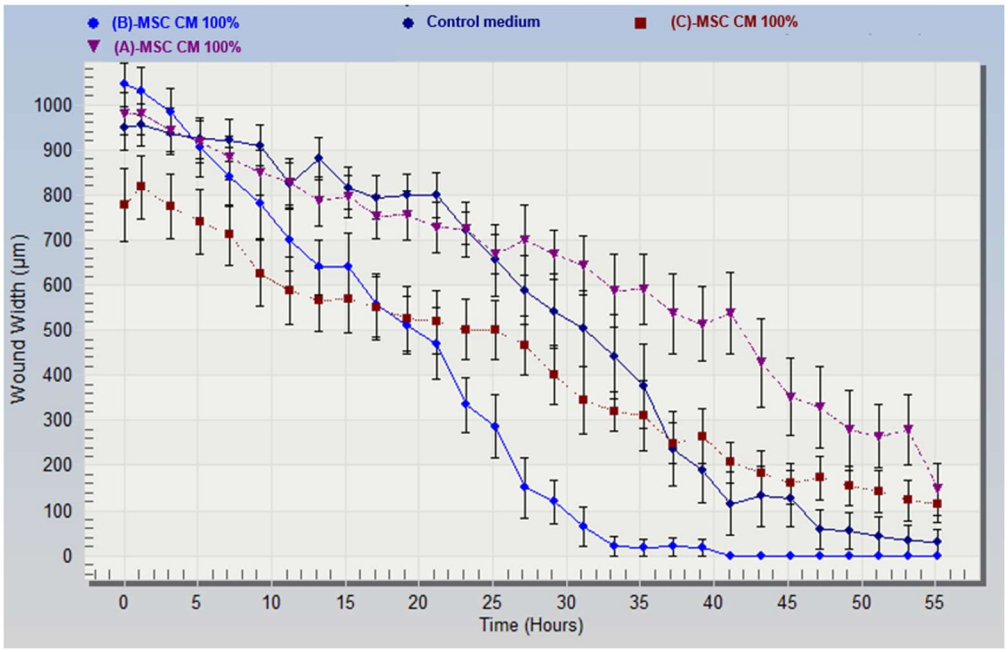


Fig. 40 - Wound Width: (A)-MSC – (B)-MSC – (C)-MSC supernatants (100%), Mesencult ACF + Supplement was used as control. Each dot shows the average, the error bars show standard deviation of tests in quadruplicate.

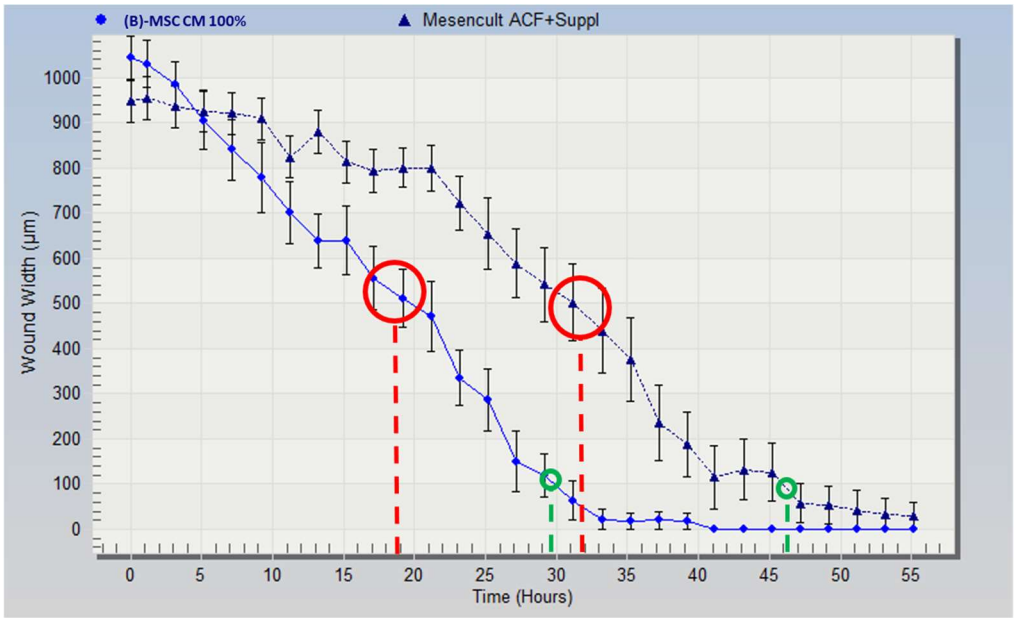


Fig. 41 - Wound Width: Time-to-50% Wound closure resulted 59% faster with (B)-MSC CM (in red), Time-to-90% Wound closure resulted 64% faster with (B)-MSC CM (in green). Each dot shows the average, the error bars show standard deviation of tests in quadruplicate.

The positive effect of (B)-MSC SN on wound closure (measured as wound width) is concentration-dependent.

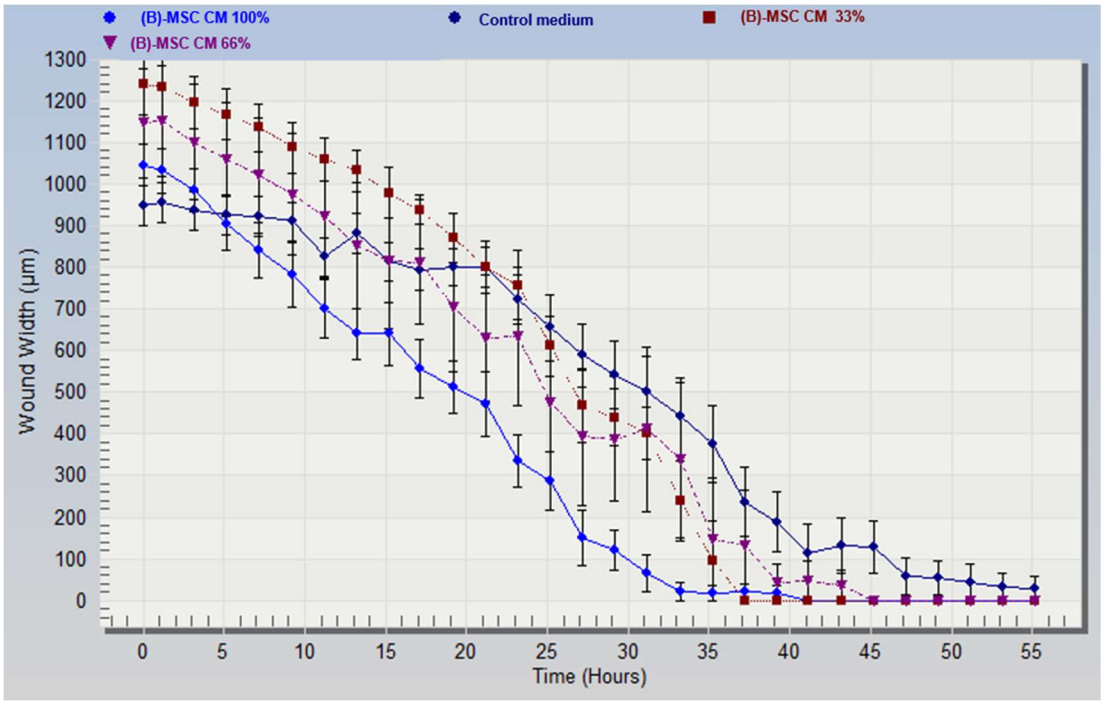


Fig. 42 - The rate of wound closure (wound width, um), is concentration-dependent: (B)-MSC supernatants 100%, 66%, and 33% concentration - diluted in control medium), Mesencult ACF + Supplements was used as control. Each dot shows the average, the error bars show standard deviation of tests in quadruplicate.

In our in vitro scratch wound assay, the effect of the conditioned medium from (B)-MSCs at Passage 5 appears superior to that of the control medium, as well as of the (A)-MSC P5, and the (C)-MSC P5 conditioned media, in all metrics. The conditioned medium significantly accelerates wound closure in our *in vitro* scratch wound assay. The acceleration of wound closure is concentration dependent. (B)-MSCs, their conditioned medium, and thus their secretome appear promising for wound healing and for the treatment of diabetic ulcers.

CONCLUSION

Pancreatic islet beta cells are endocrine cells that control glycemia by secreting insulin. In Type 1 Diabetes (T1D), pancreatic islet beta cells are lost due to a T-cell mediated autoimmune attack.

The Pancreas and its pancreatic islets represent important sources for cells for beta cell replacement and regeneration. In particular, Mesenchymal stromal/stem cells (MSCs) derived from the pancreas and pancreatic islets could play a role during the development of T1D, and could be useful in therapeutic strategies for this disease.

MSCs can inhibit autoimmune and inflammatory processes, and they have been shown to promote the function of endogenous and transplanted pancreatic islets. Moreover, MSCs have shown a good safety profile in clinical trials. MSCs harvested from different tissues have shown differences in gene expression profiles and function. Compared with MSCs from other sources, Pancreatic Islet-derived MSCs (PI-MSCs) could possess intrinsic advantages for co-transplantation with islet cells. Besides their potential for maturation/reprogramming into pancreatic islet beta cells, PI-MSCs could find application in the treatment of T1D because of their beneficial immunomodulatory and pro-angiogenic properties. We propose that Pancreas-derived (P-MSCs) and Pancreatic Islet-derived MSCs (PI-MSCs) could sustain islet-specific functions, including promotion of beta cell function and survival, regulation of beta cell regeneration, and islet immunomodulation. The goal of our studies was to modulate immune functions, stimulate islet function and stimulate pancreatic islet regeneration in the context of T1D.

We initially aimed at isolating, culturing and banking murine and human MSCs from different sources. We harvested and cultured MSCs from human placental tissues such as Umbilical Cord, Amniotic Membrane, Chorion Membrane, Pancreas, and Pancreatic Islets. We have also derived cells from murine Bone marrow, Adipose Tissue, Pancreas, and Pancreatic Islets. The cell bank, that currently counts more than 850 samples of MSCs, is now available to qualified investigators.

We have characterized in depth human Pancreatic Islet-derived MSCs, derived from 7 different donors. The immunophenotypic analysis performed on these cells confirmed their mesenchymal nature and showed that PI-MSCs are similar to MSCs from other sources. PI-MSCs in fact show a high expression of mesenchymal markers, such as CD44, CD73, CD90, and CD105. PI-MSCs are negative for HLA-DR, for hemopoietic markers CD34 and CD45, and for the epithelial marker PanCitokeratin, whereas they express the mesenchymal marker vimentin.

In order to further confirm the mesenchymal origin of hPI-MSCs, we successfully tested their ability to differentiate *in vitro* towards mesenchymal lineages – namely the adipogenic, osteogenic, and chondrogenic lineages.

We observed that culture-expanded hPI-MSCs present a sizable subpopulation expressing NG2, a marker of pericytes. This suggests that these cells derive from islet pericytes. Furthermore, we observed that hPI-MSCs also express the immunomodulatory molecule HLA-G – a fact that stimulated our curiosity on the analysis of their immunomodulatory activity.

We hypothesized that hPI-MSCs could inhibit the proliferation of Peripheral blood mononuclear cells (PBMCs), and therefore we performed ad *in vitro* test of immunomodulation. After five days of co-culture, PI-MSCs were able to inhibit the proliferation of the PBMCs stimulated with phytohemagglutinin. Moreover, PI-MSCs appear to be responsive to an inflammatory microenvironment, and they responded to such condition by increasing their expression of HLA-G: the HLA-G+ PI-MSCs subpopulation shifted from 25,8% to 83,3% after co-culture with activated PBMCs.

Afterwards, we focused our attention on the immunomodulatory effect of hPI-MSCs on T cells. We combined T cells with monolayers of hPI-MSC and we stimulated the proliferation of T cells with anti CD3/CD28 tetrameric antibodies in the presence of IL-2. After 72h of coculture, we harvested the T cells to analyze the changes occurring in the CD4+/CD25+/FoxP3+ T regulatory cell subpopulation (T regs) via Flow cytometry. We gated for CD4+/CD25+/FoxP3+ and we observed an increase of the T regs subpopulation, resulting from the co-cultivation with PI-MSCs.

Data shows that hPI-MSCs (passage 5) stimulate an absolute and relative (% of Total) expansion of T regs. By tracking the dilution of CFSE staining after 72h of coculture, we observed a significant increase of proliferated $CFSE^{dim}$ CD4+/CD25+/FoxP3+ Tregs when the T cells were cocultured with PI-MSCs, compared to the controls. Therefore, the observed T reg expansion is the result of a stimulation in T reg proliferation occurring when T cells are cocultured with hPI-MSCs.

We tested the effect of hPI-MSC (passage 5) on the glucose-stimulated insulin release function of isolated human pancreatic islets. Remarkably, we observed that hPI-MSC can boost the glucose-responsive insulin release function of islet beta cells, and we found that this effect is mediated by soluble factors released by hPI-MSC. Islets cocultured with PI-MSCs released an amount of insulin that was significantly superior to islets alone, especially in the high-glucose conditions. PI-MSCs and, potentially, their *in vivo* counterpart - islet pericytes, release soluble beta cell-stimulatory factors in the islet microenvironment.

After having performed *in vitro* studies to test the immunomodulatory potential of MSC and the effect on islet beta cell function, we hypothesized that locally delivered pancreatic MSCs could modulate immunity, re-acquire a pericytic phenotype, inhibit recurrence of beta cell autoimmunity, and stimulate beta cell survival.

To test this hypothesis, we studied the effect of Pancreatic MSC co-transplantation with islets in a murine model of autoimmune diabetes.

Studies of human MSC transplantation in non-immunodeficient mice showed that an immune response against xenogenic cells can occur, and this could be a confounding factor in subsequent interpretations. Therefore, we decided to focus our attention on the effect of murine pancreatic MSC. Murine male NOD-GFP+ Pancreatic MSCs were tested for their ability to boost survival of co-transplanted NOD-SCID female islets in a stringent model of transplantation in the presence of autoimmune diabetes: the cells were transplanted in female NOD mice with recent spontaneous onset of autoimmune diabetes. The diabetic NOD mice were utilized as recipient of transplantation in the anterior chamber of the eye. The transplantation in this site allows *in vivo imaging* over time of the transplanted cells, as well as the monitoring of revascularization and immune cell trafficking. To minimize the risk of transplanting functional passenger immune cells with isolated islets, NOD-SCID animals were utilized as islet donors. NOD autoimmunity targeting beta cells is expected to recur at approximately 8 days post transplantation of NOD-SCID islets in autoimmune-diabetic NOD mice. On post-operative day 7 and 9, murine NOD-GFP+ Pancreatic-MSC were still present around the islets. We observed an ongoing remodeling of the vascular structures, as highlighted by the presence of new small-caliber vessels penetrating the islet structures. Intriguingly, some of the NOD-GFP+ mouse Pancreatic-MSC appear aligned to and wrapping around vessels of large caliber, confirming a phenotype consistent with that of pericytes. The *in vivo* experiments, still ongoing, are currently focused on long-term analyses of islet survival and immune cell trafficking.

In a separate project, relevant to the field of diabetic wound healing, we analyzed the effect of the secretome of placenta-derived MSCs in an *in vitro* Scratch Wound assay. Our research seeks to determine whether the conditioned medium can accelerate the wound healing process.

We expanded MSCs from 3 different tissues of the human term placenta. We defined these tissues as A, B, and C due to intellectual property rights. After creating a scratch in a monolayer of cultured skin fibroblast, we compared the effect of the secretome of MSCs (at Passage 5) derived from these three different Human Placenta tissues, with the appropriate control.

The supernatant from different placenta-derived MSCs yielded differential and concentration-dependent effects on wound confluence. In particular, in comparison with the supernatant of MSCs from other sources, the (B)-MSC supernatant significantly accelerated regaining of Wound Confluence, Relative Wound Density, and closure of the Wound Width. Therefore, this MSC cell source and its secretome have potential for translational applications in wound healing and diabetic ulcers.

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