OPTIMIZATION AND APPLICATION OF ENCAPSULATION TECHNOLOGY FOR CELL THERAPY IN DIABETES

por Alberto Cañibano Hernández

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Un científico en su laboratorio no es sólo un técnico, también es un niño colocado ante fenómenos naturales que lo impresionan como un cuento de hadas.

Marie Curie

ABBREVIATIONS LIST

AD-MSCs: Adipose tissue mesenchymal stem cells
ALK5: Activin-like kinase 5
ANOVA: Analysis of variance
APA: Alginate-poly-L-lysine-alginate
ATMP: Advanced therapy medicinal products
ATP: Adenosine triphosphate
AXL: AXL receptor tyrosine kinase
BCA assay: Bicinchoninic acid assay
bFGF: Basic fibroblast growth factor
BM-MSCs: Bone marrow mesenchymal stem cells
BMP4: Bone morphogenetic protein 4
BSA: Bovine serum albumin
CAM: Cell adhesion molecules
CBMPs: Cell-based medicinal products
CCK-8: Cell counting kit-8
cDNA: Complementary deoxyribonucleic acid
CFU: Colony forming units
CTT: Cell and tissue-based therapeutic
CYC: Cyclopamine
D1-MSC: D1-mesenchymal stem cells
D1-MSCs-Epo: D1-MSCs engineered to release EPODCs: Dendritic cells
DE: Definitive endoderm
DMEM: Dulbecco´s modified Eagle´s medium
DMSO: Dimethyl sulfoxide
DNA: Deoxyribonucleic acid
dNTPs: Deoxynucleotides
DPBS: Dulbecco-s phosphate buffered saline
DTZ: Dithizone
EBs: Embryoid bodies
ECM: Extracellular matrix
EGF: Epidermal growth factor
ELISA: Enzyme-linked Immunosorbent Assay
EMA: European medicines agency
EPO: Erythropoietin

EPP: Endocrine pancreatic precursors
ESCs: Embryonic stem cells
ET: Endocrine tissue
FBR: Foreign body reaction
FBS: Fetal bovine serum
FDA: Food and drug administration
FGF2: Fibroblast growth factor-2
FITC: Fluorescein-5-isothiocyanate
GDM: Gestational diabetes mellitus
GLP1: Glucagon-like peptide-1
GMP: Good manufacturing practice
GSIS: Glucose-stimulated insulin secretion
GTMPs: Gene therapy medicinal products
GTP: Good tissue practice
HA: Hyaluronic Acid
HGF: Hepatocyte growth factor
HIF: Hypoxia inducible factor
HLA: Human leukocyte antigens
IDE 1/2: Inducer of definitive endoderm 1/2
IFN-γ: Interferon gamma
IGF1: Islet growth factor-1
IgG: Immunoglobulin G
Il-1β: Interleukin 1 beta
IPCs: Insulin producing cells
iPSCs: Induced pluripotent stem cells
ISCT: International society for cellular therapy
ITS: Insulin-transferrin-selenium
KGF: Keratinocyte growth factor
LDH: Lactic dehydrogenase
MACF: Methacrylamide chitosan fluorinated
MSCs: Mesenchymal stem cells
MW: Molecular weight
NCys: N-acetylcysteine
NK: Natural killer cells
PAR1: Protease-activated receptor 1
PBS: Phosphate buffered saline

PCR: Polymerase chain reaction
PDGF: Platelet-derived growth factor
PDL: Poly-D-lysine
PDMS: Polydimethylsiloxane
PE: Pancreatic endoderm
PF: Posterior foregut
PHS: Public health services
PI3K: Phosphoinositide 3-kinase
PKC: Protein kinase C
PLG: Polylactide-co-glycolide
PLGA: Poly lactic-co-glycolic acid
PlGF: Placental growth factor
PLL: Poly-L-lysine
PLO: Poly-L-ornithine
PP: Pancreatic progenitors
PTG: Primitive gut tube
PVP: Polyvinyl pyrrolidone
RA: Retinoic acid
RNA: Ribonucleic acid
RPMI: Roswell park memorial institute medium
SD: Standard deviation
SEM: Scanning electron microscopy
SHH: Sonic hedgehog
T1DM: Type I diabetes mellitus
T2DM: Type II diabetes mellitus
T3: thyroid hormone 3
TGF-β: Transforming growth factor beta
TNF-α: Tumor necrosis factor alpha
UCB-MSCs: Umbilical cord blood mesenchymal stem cells
VEGF: Vascular endothelial growth factor
WJ-MSCs: Wharton's jelly mesenchymal stem cells

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Chapter 1

Introduction: Current advanced therapy cell-based medicinal products for type-1 diabetes treatment

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Current advanced therapy cell-based medicinal products for type-1 diabetes treatment

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ABSTRACT

In the XXI century diabetes mellitus has become one of the main threats to human health with a higher incidence in regions such as Europe and North America. Type 1 diabetes mellitus (T1DM) occurs as a consequence of the immune-mediated destruction of insulin-producing β-cells located in the endocrine part of the pancreas, the islets of Langerhans. The administration of exogenous insulin through daily injections is the most prominent treatment for T1DM but its administration is frequently associated with failure in glucose metabolism control, finally leading to hyperglycemia episodes. Other approaches have been developed in the past decades, such as whole pancreas and islet allotransplantation, but they are restricted to patients who exhibit frequent episodes of hypoglycemia or renal failure because of the lack of donors and islet survival. Moreover, patients transplanted with either whole pancreas or islets require of immune suppression to avoid the rejection of the transplant. Currently, advanced therapy medicinal products (ATMP), such as implantable devices, have been developed in order to reduce immune rejection response while increasing cell survival. To overcome these issues, ATMPs must promote vascularization, guaranteeing the nutritional contribution, while providing O2 until vasculature can surround the device. Moreover, it should help in the immune-

protection to avoid acute and chronic rejection. The transplanted cells or islets should be embedded within biomaterials with tunable properties like injectability, stiffness, and porosity mimicking natural ECM structural characteristics. And finally, an infinitive cell source that solves the donor scarcity should be found such as insulin-producing cells derived from mesenchymal stem cells (MSCs), embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). Several companies have registered their ATMPs and future studies envision new prototypes. In this review, we will discuss the mechanisms and etiology of diabetes, comparing the clinical trials in the last decades in order to define the main characteristics of future ATMPs.

1. Introduction

Nearly 350 million people worldwide are affected by Diabetes mellitus (DM), a chronic disease that has become as one of the major diseases in the XXI century. Diabetes is classified by the American Diabetes Association as type I diabetes mellitus (T1DM), type II diabetes mellitus (T2DM), gestational diabetes mellitus (GDM) and other minor types grouped as type III diabetes mellitus. Type 1 diabetes mellitus (T1DM), where we will focus on this review, is characterized by an autoimmune destruction of pancreatic β-cells resulting in severe insulin deficiency, after an asymptomatic period over years. It develops mostly in young people accounting for 5-10% of the diabetic subjects (1). T1DM patients have shown that β-cells from the islets of Langerhans are destroyed by infiltration of dendritic cells (DCs), macrophages and T lymphocytes (both CD4+ and CD8+). Immune reaction is specific against insulin-producing β-cells, not affecting other cells in the islets of Langerhans, such as α-cells (glucagon-producing cells) or δ-cells (somatostatin-producing cells) (2). Type II diabetes mellitus is also known as insulin-independent diabetes because patients present insulin resistance and deficiency, without the need of insulin treatment to survive. The specific etiology of T2DM is not completely clarified and there are probably different causes, including obesity and genetic predisposition (3). Gestational diabetes mellitus can be defined as a deficiency in glucose metabolism control identified during pregnancy, which normally is reverted post-partum (4).

To define diabetes, it is necessary to analyze the progression of symptoms in the disease. Attending to changes in the cells mass, phenotype and cell functionality five stages can be defined in the progression of diabetes (5) (Figure 1). The regular stage of β-cells corresponds to blood glucose levels of 4.5 mmol/l (80mg/dl) while the first stage of diabetes is characterized by an insulin secretion increase to maintain the regular glucose levels, because of insulin resistance caused by obesity, physical inactivity, and genetic predisposition. During this stage, it has been described an increase of β-cell mass, probably due to an increase of β-cell number and β-cell

hypertrophy (6). In stage 2, the blood glucose levels overcome 5.0-6.5 mmol/l (89-116 mg/dl) and, normal glucose levels from stage 0 cannot be long maintained. Despite people in stage 2 usually evade progression to type II diabetes for years by adhering to a diet and exercise regimen (7), people with T1DM experience a fast increase of β-cell mass destruction. Next, T1DM evolves to a decompensated stage 3, when glucose levels rise rapidly over 7.3 mmol/l (130 mg/dl), probably determined by glucose toxicity effects on β-cells, leading to β-cell mass reduction and less efficient insulin secretion (8). In stage 4, the increment of β-cells destruction displays blood glucose values higher than 15 mmol (280 mg/dl) which induces a progression to ketoacidosis. This stage lasts mostly the lifetime of T2DM patients, while the rapid progressive autoimmune destruction of β-cells inT1DM, often leads to stage 5 relatively quickly (9). In stage 5, there is a fast β-cell mass reduction enhancing the glucose levels up to 22 mmol/l (350 mg/dl). At this stage, the progression to ketosis and insulin dependence is unavoidable. Once β-cell destruction is completed at stage 5, there is no possibility to return across the stages. Stage 5 is common in T1DM, while rarely occurs in T2DM.

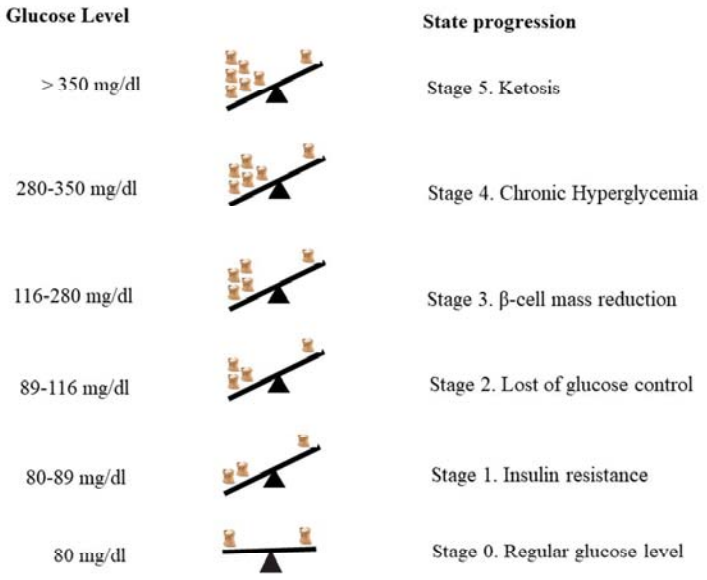

Figure 1.-Five stages of the progression of diabetes.

The treatment of T1DM usually depends on the stage of progression. The ideal goal of a future treatment for T1DM would be to reverse the β-cell destruction, restore the glucose metabolic control and prevent the onset and progression of autoimmunity. The most prominent treatment is the insulin replacement by exogenous administration through daily injections or an insulin pump. To avoid the issues related with insulin daily injections, other research groups have focused on healing T1DM with β-pancreatic cell replenishment, either by whole vascularized pancreas transplantation or by islet transplantation. However, whole pancreas transplantation requires complex surgical techniques and immunosuppression for life. Currently, pancreatic islets transplantation represents the best option for a T1DM cure, even with limitations such as donor scarcity, requiring new administration routes.

2. Characteristics of an optimal advanced therapy medicinal product

Nowadays, new technologies are investigated to heal T1DM, trying to overcome those failures of T1DM classical treatments. The advanced therapy medicinal products (ATMP) are one of these technologies applied to diabetes treatment which, can be defined as a combination of a wide variety of medicines or therapeutic products in a complex device. ATMPs consist of Cell-Based Medicinal Products (CBMPs) and Gene Therapy Medicinal Products (GTMPs) but, in this review, we will focus on the application of CBMPs to T1DM treatment. ATMPs containing cells growing inside should gather some characteristics for cell survival. The regular oxygen and nutrient supply, as well as the ability of the device to generate its own vascular network, are very important for cell survival in the ATMP. Besides, the device needs to avoid immune rejection by biocompatible materials protection. Also, it would be desirable the possibility of injecting the embedded cell without the need of surgery. Cell sources enclosed inside the ATMP are another characteristic to be tuned, being preferred solutions based on the incorporation of new cell sources to solve the problem of donor scarcity of pancreatic islets. Finally, the ATMPs should be subjected to a series of safety and quality regulations. We will focus on the minimal

characteristics gathered by the ideal advanced therapy medicinal product applied to diabetes treatment (Figure 2).

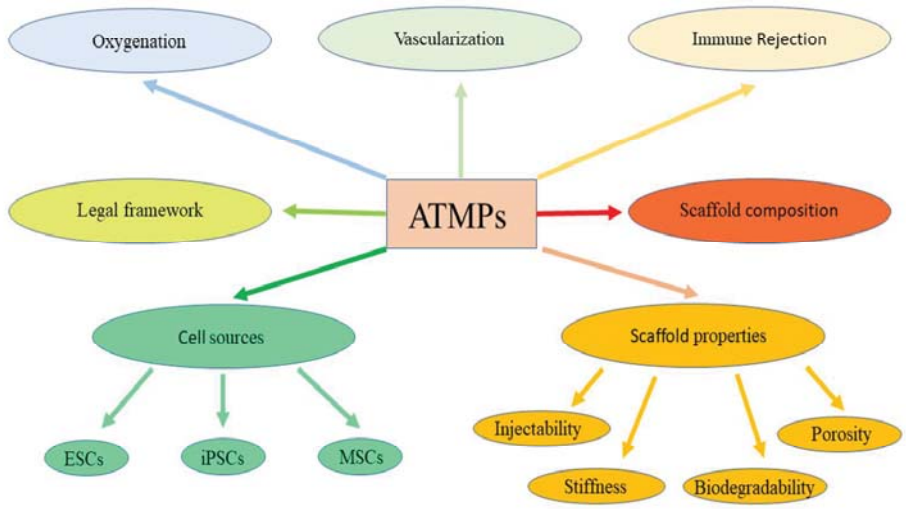

Figure 2.-Influence factors of an ideal ATMP. ATMPs (Advanced Therapy Medicinal Product), ESC (Embryonic Stem Cell), iPSC (induced Pluripotent Stem Cell), MSC (Mesenchymal Stem Cell)..

2.1. Oxygen supply

Pancreatic cells are highly oxygen dependent, consuming approximately 15% of the oxygen contained in blood flow (10), and susceptible to functional impairment at moderate oxygen tensions (11). The natural microvasculature of the islets of Langerhans is lost during isolation, inducing a defective oxygenation. This lack of oxygen induces the expression of hypoxia-inducible factor (HIF)-1α, among others, and the subsequent cascade reaction which activates islet apoptosis (12). Moreover, (HIF)-1α expression impairs glucose-stimulated insulin secretion (GSIS), inducing glucose intolerance (13), and the accumulated glucose also inhibits the angiogenesis of isolated pancreatic islets (14). In long term, the solution to hypoxia is re-vascularization of islets in the device but, the generation of oxygen in situ within the device is a transient solution that can improve oxygen tensions, while

revascularization occurs.

Currently, several prototypes for oxygen release are based in the breakdown of molecules (Table 1) (15). For example, CaO2 is commonly used for generating oxygen in oxygen chambers. These devices contain an oxygen release system and a cell chamber, sometimes separated by a permeable membrane that allows the oxygen flow. Thus, encapsulated polydimethylsiloxane (PDMS) is frequently used as oxygen-generating biomaterial with solid calcium peroxide (CaO2), resulting in a single disk of PDMS-CaO2 and allowing a sustained oxygen generation for 6 weeks. The coculture of PDMS-CaO2 with MIN6 murine β cells or pancreatic rat islets decrease hypoxia and cell death, limiting the activation of cell stress pathways (16). Moreover, immune-protected devices containing PDMS-CaO2 implanted in diabetic rats can increase insulin release, improving the release when islets are pretreated with growth hormones (16). Interestingly, a granulated mixture of CaO2 incorporated into collagen/ceramic composites generates elevated oxygen levels compared to compressed CaO2. Other scaffolds embedding CaO2, such as Poly Lactic-co-Glycolic Acid (PLGA)-CaO2 also contribute to oxygenation, maintaining cell viability within the construct under hypoxic conditions and mimicking the *in vivo* tissue environment (17).

Oxygenation compound	Mixture	Characteristics
Calcium peroxide (CaO_2)	CaO2-Ethyl Cellulose-Catalase-Collagen-Ceramic composite (17)	Low cytotoxicity sustainable release of oxygen
	CaO2-PLGA scaffolds (17)	*In vivo*-like environment
	CaO2-PMDS (16)	Sustainable oxygen release
Perfluorocarbons (PFC)	Alginate-PFC (23)	Oxygen sustained release for 7 days
	Fluorinated methacrylamide chitosan-PFC (24)	Long-term reloadable system
Hydrogen peroxide (H_2O_2)	H2O2-PLGA-Alginate-Catalase (26)	Low oxygen release
	H2O2-PLGA-Catalase-Alginate (27)	Low cytotoxicity
	PVP-H2O2-PLGA-Catalase (28)	Injectable properties
Modified hemoglobin solutions	Free hemoglobin (29)	Only applied to hollow fibers bioreactors

Table 1.-Oxygen generating compounds

Organic compounds with high oxygen solubility, such as perfluorocarbons or modified hemoglobin solutions, can also supply oxygen in ATMPs. Perfluorocarbonated compounds incorporated in encapsulation material are commonly used to increase oxygen availability (18). This material displays chemical

and biological inertness and is easily sterilized, recovered and recycled after used (19). Perfluorocarbons added into islet culture medium enhances islet functionality (20), improving encapsulated cell survival when combined with alginate (21). Perfluorocarbon concentration represents an important factor. Low concentrations of perfluorocarbon emulsions within alginate hydrogels show better beneficial effects on pancreatic islets than higher concentrations (22). Perfluorocarbons can also be combined with other biomaterials forming membranes improving oxygen release. Thus, perfluorocarbonated-silicone membranes enhance the oxygen diffusion to pancreatic bud explants cultured on monolayer, resulting in greater endocrine and β-cell differentiation (23). Sodium percarbonate can also release oxygen into hypoxic tissues delaying the onset of necrosis in nude mice models. Perfluorocarbons combined with methacrylamide chitosan fluorinated (MACF) hydrogels improve considerably oxygen uptake and release compared with MACF hydrogels, enhancing long-term cell survival until host neovascularization is achieved (24). The Food and Drug Administration (FDA) has approved the use of perfluorocarbons as additives for blood oxygenation during surgery (25), encouraging several enterprises into the development of artificial blood substitutes, such as Oxygent™ from Alliance Pharmaceutical Corporation or Fluosol from Green Cross Corporation (23).

The decomposition of hydrogen peroxide (H_2O_2) into oxygen and water by catalase is another oxygen-producing micro-system which increase cell survival under hypoxic conditions. Microcapsules core have been formed with H_2O_2 and poly D, L-lactide-co-glycolide (PLGA), named H_2O_2-PLGA core, covering with a second layer of alginate chemically bounded to catalase (26). In these microcapsules, H_2O_2 is released into the second layer, decomposing into oxygen and H_2O by catalase. H_2O_2-PLGA-alginate-catalase microcapsules release enough oxygen that delay the onset of necrosis of transplanted nude mice under hypoxic environments (26). However, protecting from the harmful and toxic effects of direct contact with H_2O_2 by linking the core of H_2O_2-PLGA with catalase, next coated with alginate, reduce the toxicity and increase cell viability, as described with

skeletal muscle cells (27). More gradual oxygen release is led by the stable complex formed by polyvinylpyrrolidone (PVP) and H_2O_2. The combination of PVP, H_2O_2, PLGA, and catalase within a hydrogel increase the durability of oxygen release for at least 14 days, improving, for example, the survival of the cardiosphere-derived cell in a simulated hypoxic condition (1% O_2) (28). Less known systems are the artificial oxygen carriers based on modified hemoglobin solutions. These carriers can improve the oxygen transportation, removing carbon dioxide from the medium (29). Hemoglobin-based oxygen carriers can improve oxygenation to hepatocyte cultures. However, this technology is being only applied *in vitro* within hollow fibers bioreactors, but not successfully transferred to *in vivo*.

2.2. Vascularization generation

Vascularization of islet implants is a finely tuned and complex process, whose molecular mechanism remains elusive, because of the high number of factors implicated in the revascularization process (30). Some pro-angiogenic cells and factors have been used to obtain a competent and functional vascular network after implantation of ATMPs (Table 2). The angiogenic molecules from the vascular endothelial growth factor (VEGF) family, VEGFA, VEGFB, VEGFC, VEGFD, PlGF, are the most commonly used for the stimulation of new vessel formation surrounding the implant (31). VEGF family stimulates proliferation and migration of endothelial cells and increases vascular permeability (32). In physiological conditions, VEGF released by the liver improves islet engraftment and efficacy after transplantation. Moreover, transplanted pancreatic islets also secrete VEGF, although the expression declines 2-3 days after transplantation (33). However, although the secretion of VEGFA by cells drives the expansion of the capillary bed, the administration of recombinant VEGFA has limited ability to induce the growth of larger vessels. *In vivo*, low doses of recombinant VEGFA increase vascular permeability, while a sustained stimulation with a high VEGFA dose is required for stable vasculature formation (34). For example, the administration of 150 ng VEGFA /day to rats by

an osmotic pump, results in high vascularization, with vessels stable for at least 80 days after administration while, lower concentrations of VEGFA fails. Moreover, the theoretical determination of the optimal dose in humans is difficult because dosages differ from disease states and metabolic disorders in animal models.

Administration	Growth factor	Scaffold composition	Effects
Injection	VEGF	-	Stable vasculature formation Expansion of the capillary bed (31)
	PDGF	-	Involved in vessel maturation Recruit inflammatory cells for vessel formation (37)
	FGF	-	Endothelial and mural cells recruitment (40)
	PlGF	-	Multipotent compound for vascularization Recruit several cell types for vessel formation Promotes angiogenesis and arteriogenesis (40)
Scaffold	VEGF+PDGF	PLG	Low density of small, mature blood vessels (41)
	bFGF+HGF	Collagen microspheres	Mature vasculature formation Regular blood perfusion recovery (42)
	VEGF+FGF	Heparin-binding peptide amphiphiles	Enhance the vascularization ratios Increase glucose stimulation indices (45)
Co-encapsulation	-	MSCs and pancreatic islets	Vascularization inside/outside of the device (46)
	-	Endothelial progenitor cells	Collateral vessel formation (48)
	-	VEGF secreting-engineered cells and endothelial cells	Increase of blood vessels growth (49)

Table 2.-Approaches for pro-angiogenesis induction

Some studies have shown that VEGFA therapy may not be the ideal treatment to increase the blood perfusion to an ischemic tissue because VEGFA-driven angiogenesis does not consistently form functional and stable vasculature (35). However, the combination of VEGFA with other growth factors, such as platelet-derived growth factor (PDGF), fibroblast growth factor-2 (FGF-2) or placental growth factor (PlGF) have displayed the formation of endothelial tubules by recruiting pericytes and smooth muscle cells (36). The sequential administration of VEGFA and PDGF induces more formation of mature vessels than each factor singly (37). PlGF also promotes angiogenesis and arteriogenesis (38), displaying equally promising results than VEGFA, and increasing its effects when administered in synergy with VEGFA (39). Although VEGFA is commonly used

as a potent stimulator of endothelial cell mitogenesis, cell migration, vasodilation and a mediator of microvascular permeability, PlGF can stimulate angiogenesis and collateral vessel growth, avoiding side effects associated to VEGFA, such as edema and hypotension (36). Moreover, PlGF positions as the most multipotent compound for vascularization, since it can recruit endothelial, mural cells, and inflammatory cells, while PDGF only recruits inflammatory cells and FGF endothelial and mural cells (40).

The incorporation of angiogenic factors within porous or degradable scaffolds has been extensively studied as vascular-inductive tissue engineering matrices. When embedding angiogenic factors within hydrogels, the combination of VEGF and PDGF (41) or basic fibroblast growth factor (bFGF) and hepatocyte growth factor (HGF) (42) have shown an increase of vascularization. Thus, 2 weeks after the administration of high doses of VEGFA within a porous bi-layered poly(lactide-co-glycolide) (PLG) scaffold into severe combined immunodeficient mice, results in a high density of small, immature blood vessels. However, the sequential delivery of VEGF and PDGF within bi-layered PLG scaffolds enhance vessel size and maturity after 6 weeks (41). The simultaneous application of bFGF and HGF within a scaffold also enhance the formation of blood vessels. Thus, the sustained release of low doses of bFGF and HGF by collagen microspheres in a murine ischemic limb induce more mature vasculature formation than high doses without scaffold, with equivalent blood perfusion recovery (42). Moreover, different extracellular matrix proteins and ligands can modulate the angiogenic response (43), often through integrin activation (44) and the combination of angiogenic growth factors with appropriated matrix signals can create controlled biomimetic analogs to natural extracellular matrices. For example, the combination of heparin-binding peptide amphiphiles with VEGF and FGF enhance the vascularization ratios and glucose stimulation indices after murine islet transplantation (45).

Another approach for device vascularization is the co-encapsulation of cells that release pro-angiogenic factors, such as MSCs. The co-transplantation of

MSCs and pancreatic islets into rodent and primate models promote the vascular development inside and outside of the device (46), and therefore, the graft efficacy (47). Co-encapsulation with endothelial progenitor cells also promotes collateral vessel formation in both humans and animals, with successful engraftment and vascularization (48). Moreover, co-encapsulation of VEGF secreting-engineered cells and endothelial cells within polylactide-co-glycolide microspheres scaffolds also promote vascularization in transplanted severe combined immunodeficient mice bone tissues, with an increase of blood vessels growing into the scaffolds (49).

2.3. Immune rejection and foreign body reaction

Theoretically, the allorecognition of cells within the device can be restricted using biomaterials, which prevent the interaction of cells with immunoglobulins, avoiding immunogenic response. However, the interphase between the implanted device and the surrounding tissue induces the foreign body reaction (FBR). FBR can be defined as an altered wound healing response against the foreign body. Large devices remain in the body cannot be phagocytosed and cleared by the innate immune system (50), and therefore the host activated an immune-mediated rejection response (51), known as fibrotic cascade. Consequently, some proteins become adsorbed to the foreign body surface, inducing macrophages recruitment and their differentiation into foreign body giant cells. Next, foreign giant cells can attract and activate fibroblasts, which are able to develop the fibrous encapsulation of the foreign body, preventing the interaction with the surrounding microenvironment and obstructing the flux with the device (52).

Since FBR is triggered by protein adsorption to the implant surface, fibrosis can be reduced by masking hydrophobic surface of the implants. Implants have been successfully coated, covering the hydrophobic surface, by natural polymers, such as chitosan (53), alginate (54), collagen (55), dextran (56), and hyaluronan (57), as well as by synthetic compounds, such as polyvinyl alcohol (58) and polyethylene glycol (59). Regarding natural polymers, the biocompatibility of

alginate capsules depends on the molecular weight of the guluronic acid, showing an overgrown of inflammatory cells after transplantation with high-guluronic alginates (54). Similarly, synthetic polymers show different immune responses depending on their composition. Thus, photopolymerizable polyethylene glycol-based hydrogels implanted subcutaneously into C57Bl/6 mice are proinflammatory, with high expression of the cytokines TNF-α and Il-1β and a robust inflammatory reaction characterized by a thick layer of macrophages at the material surface with evidence of gel degradation. However, polyethylene glycol hydrogels modified with RGD motifs attenuate this negative reaction leading to a moderate FBR. But coated implants are often used in combination with non-steroid anti-inflammatory drugs, glucocorticoids and anti-fibrotic agents to enhance the protection of devices, allowing the disruption of the cascade of inflammatory events. Non-steroid anti-inflammatory drugs inhibit the early stages of the FBR (60) and can be applied as a surface coating of the device, or through a delivery agent. The wide range of activity of glucocorticoids, including expression of anti-inflammatory cytokines, reduction of pro-inflammatory cytokines and reduction of collagen synthesis in fibroblasts, position them as ideal immune-suppressors to prevent the FBR. Besides, anti-fibrotic agents reduce fibroblast activation by macrophages, as well as collagen production, and can be used to reduce fibrosis (61). The strategies for the controlled release of these compounds include passive diffusion from coatings or polyelectrolyte layers (62) degradable coatings to release drugs by passive dissolution, (63) swelling coatings that release drugs by passive mechanisms, and hydrolyzable or enzyme-degradable linkages to release the (64). One example is dexamethasone, a synthetic glucocorticoid hormone. Dexamethasone modulates macrophage behavior and reduces the levels of numerous proinflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and interferon-γ (65). Dexamethasone-releasing coatings reduce tissue inflammation and cell activation surrounding implanted glucose biosensors (66). Another example is heparin, a highly sulfated glycosaminoglycan with strong anticoagulant activity and anti-inflammatory

properties. It is synthesized and secreted by mast cells at sites of infection and inhibits endotoxin-induced monocyte activation (67). Heparin-based coatings have reduced protein adsorption and leukocyte recruitment (68). Alpha-melanocyte-stimulating hormone has also been used as anti-inflammatory factors used with implantable devices (69), an endogenous linear peptide with potent anti-inflammatory properties, or superoxide dismutase (70), an endogenous scavenger enzyme that catalyzes its breakdown into less reactive hydrogen peroxide and oxygen. Controlled delivery of immuno-modulatory proteins through biodegradable micro- and nanoparticles are other approaches (71). Thus, IL-1 receptor antagonist or synthetic thrombin receptor (PAR1) agonist peptide within biodegradable polymeric microspheres inhibited production of pro-inflammatory cytokines and shortened the inflammatory phase and accelerated tissue healing in a rat ulcer model, respectively. Along the last decade, the development of siRNA technology to gene silencing has proven to be an effective strategy to downregulate specific endogenous inflammatory factors, creating, for example, less inflammatory macrophages (72). The siRNA can also be applied to silence genes that express inflammatory cytokines, such as COL1A1, and subsequently, down-regulate collagen production and reduce fibrosis. However, these alternatives are still far from clinical applications. Recently it has been described that *in vivo* biocompatibility of biomedical devices can be significantly improved simply by tuning their spherical dimensions (73). Moreover, new targets such as CSF1R have been postulated as new selective methods of fibrosis inhibition without the need for broad immunosuppression (74).

2.4. Scaffold composition

Islet transplants are associated with limited engraftment potential, partially attributable to the isolation process, which removes islets from their native environment. Isolation disrupts the internal vascularization and innervation of islets, changing the interactions between islet cells and macromolecules of the ECM, which are known to regulate multiple aspects of islet physiology, including survival,

proliferation, and insulin secretion. The peripheral ECM of mature human islets is mostly composed of laminin and collagen IV, with fewer amounts of fibronectin and collagen I, collagen III, collagen V and collagen VI (75). Moreover, islet cells are coupled by gap junctions, as well as cell adhesion molecules (CAMs), such as integrins, neural cell adhesion molecule (N-CAM) and E-cadherin (75). These intercellular connections are important in the transduction of signals related to processes, such as islet development, glucose sensing, and insulin secretion. Scaffold materials presenting similar composition than ECM of native islets must be carefully selected for pancreatic islets transplantation. However, some islet ECM-binding interactions have been shown to yield undesirable outcomes for transplantation (76). Short peptides, such as RGD, offer greater purity and less chance of immunogenicity than intact matrix proteins, even when is not the principal signaling ligand in mature islets. Other short adhesive sequences from collagen-IV and laminin are less known in terms of interactions with islets, but the optimal scaffold for islets should incorporate multiple matrix-based signals, including growth factors.

Several cell-secreted matrices have also been used to improve islet culture and study interactions between insulin-producing cells and matrix molecules. For example, islet survival (77) and insulin secretion (78) is improved, inducing also adult β-cell proliferation in presence of matrix secreted by bovine corneal endothelial cells (79). Matrix from rat bladder carcinoma lines acts on insulin secretion through the interaction between integrins and laminin (80). But also purified individual ECM proteins result in better islet survival and function. For example, collagen type IV (81) and laminin (82) helps to increase insulin release, while islets cultured on collagen type I-coated surfaces or treated with soluble fibronectin display less apoptosis and greater insulin secretion (83). Moreover, vitronectin, only found in fetal islet tissue (84), also enhance β-cell adhesion and migration via αv-integrin interactions (85). Thus, islet matrix interactions in three-dimensional islet cultures, such as collagen-based hydrogels (86), small intestinal submucosa (87) or Matrigel (88) improve islet survival and function, increasing, even more, the insulin secretion by the addition of

other major ECM-proteins, such as collagen type IV and laminin into collagen type I hydrogels (86). Moreover, the allogeneic transplantation of islets within hybrid hyaluronic acid/collagen hydrogels into the omentum of diabetic rats reversed long-term diabetes, prevented graft rejection and revealed viable islets and intact hydrogel in the explanted grafts without evidence of fibrotic overgrowth or cellular rejection. In conclusion, 3D culture environment more closely mimics native islet conditions than 2D culture conditions, although, these experiments are limited by the limited number of matrix proteins and the concentration ranges required for ECM gelation.

Given the architecture of islets, soluble factors and cell-cell interactions are also relevant to engraftment although their role remains undetermined. Together with ECM-cell interactions can provide the signaling for attachment prior to implantation to dissociated cells, such as purified β-cells, islet progenitor cells, or stem cells. Scaffolds may be also especially important in transplants of stem cells and progenitor cells, as many studies indicate that ECM serves as a determinant key in islet differentiation through the presentation of matrix-bound signals and regulated delivery of soluble factors (88).

2.5. Scaffold properties

An adequate cell enclosing biomaterial in diabetes should bring tunable properties in terms of injectability, stiffness, porosity and degradation rate. Tunable hydrogels would broaden the application to implantable devices for T1DM. Encapsulation of pancreatic islets for diabetes treatment requires the maintenance of 3D physical integrity with low degradation rates, avoiding low cell retrieval which can induce an immune response (75). Moreover, injectable hydrogels have gained wider appreciation, as they can be used in minimally invasive surgical procedures. Injectable gels with their ease of handling, complete filling of the defect area and good permeability have emerged as promising biomaterials. Depending on the material used for the hydrogels, several crosslinking methods are performed for injectable hydrogel systems, such as Michael addition, di-sulfide crosslinking,

photo-induced crosslinking, electrostatic crosslinking, Schiff-base crosslinking, pH/thermo-responsive crosslinking or self-assembly (89). Two kinds of biodegradable polymers are used for the preparation of injectable hydrogels: naturally derived and synthetic polymers. Naturally derived polymers are expected to have better interaction with cells along with increased cell proliferation and differentiation (90), while synthetic polymers possess tunable mechanical properties and degradation profile (91). The combination of both synthetic and naturally derived polymers has also displayed improved injectability properties (92). On this regard, the gelation time of the hydrogels is important since it regulates the ability of the hydrogel to be injected into the device. For example, the gelation time in alginate hydrogels has been tuned by combination with other compounds, such as Na2HPO4 (93) and hydrolyzed silica sol (94), tuning their physicochemical properties like stiffness and porosity while maintaining an appropriate injectability.

Stiffness and strength are required parameters of the injectable hydrogel, avoiding becoming the gel brittle and break while mimicking the stiffness of the native islets (94). The elastic modulus and yield stress from the gel should be like the pancreas since the stiffness of the gel can influence the cellular processes, such as adhesion and differentiation (95), motility (96), and even phagocytosis (97). For example, substrates approximating to the elastic moduli of pancreas (1.2 kPa) direct stem cells to differentiate into β-cells (98). The stiffness of the hydrogel can be influenced by several factors like the concentration of polymer (99), a method of preparation (91) and the degree of crosslinking (100). Hydrogels with shear thinning properties, those that start to flow with an external shear behaving solid in shear absence are gaining importance as injectable systems because of their ease handling and minimal invasiveness (101).

The interconnected porous network of hydrogels aid in the more efficient movement of solutes, nutrients, and removal of waste throughout the gel (102). The pore size or the number of pores should not affect the mechanical strength or stability of the hydrogel. Thus, an ideal hydrogel should have the desirable porosity along with

appropriate strength. Although some gels are inherently porous, other gels required to introduce porosity. For example, the addition of a larger amount of crosslinker might cause more crosslinking thereby making the pores smaller, although it might also become stiffer. Porosity can be tuned by using different techniques, such as solvent casting/solvent leaching (103), gas foaming (104) and freeze-drying (105) for macro scale pores or interconnected microvascular networks (106) or by fabrication of microchannels (107) for microscale porosity.

Finally, the rate of degradation of the hydrogel must be like the rate of native tissue regeneration, avoiding a prolonged presence of the hydrogel. Enzymatic degradation or hydrolysis are responsible of biodegradation by cleaving the 3D network of the hydrogel. The incorporation of non-toxic and non-immunogenic functional groups degradable by these processes can promote therefore the degradation process, such as lactic acid, glycolic acid, succinic acid, and glucose. Moreover, the degradation rate of polyethylene-glycol hydrogels can also be controlled by modifying polymer crosslinking (108), allowing the release of cells seeded inside the hydrogel (109).

2.6. Cell sources

Islet transplantation has used pancreatic islets derived from other species as a cell source. Pig pancreatic islets are an adequate option for xenogeneic β-cell replacement because the similarities between human and pig insulin, the ability of porcine islets to regulate glucose levels similarly than humans, the easy extraction of pig pancreatic islets, and the potential to genetically modified pigs to make their islets more suitable for human transplantation (110). In 1994, pig pancreatic islets were transplanted into humans, but effects were barely detected. The major issues detected were zoonosis and the immune rejection against galactose-alpha-1,3-galactose not present in humans or monkeys (111). Currently, pancreatic islets isolated from miniature pigs and transplanted into diabetic non-human primates with low-dose immunosuppressive therapy, have shown a good engraftment, maintaining

normoglycemia for more than 6 months (112). Despite normoglycemia cannot be maintained for periods longer than 6 months, this strategy may represent a therapeutic alternative in a close future.

In the last decade, a high debate topic is the application of stem cells for diabetes treatment to solve the pancreatic islet donor scarcity (113). Stem cells can differentiate into other specialized cells types, such as β-insulin-producing cells (IPCs), by different stimuli, such as internal chemical stimulation or physical contact. Three different stem cell types have been mainly differentiated to obtain IPCs (Figure 3): ESCs, iPSCs, and MSCs.

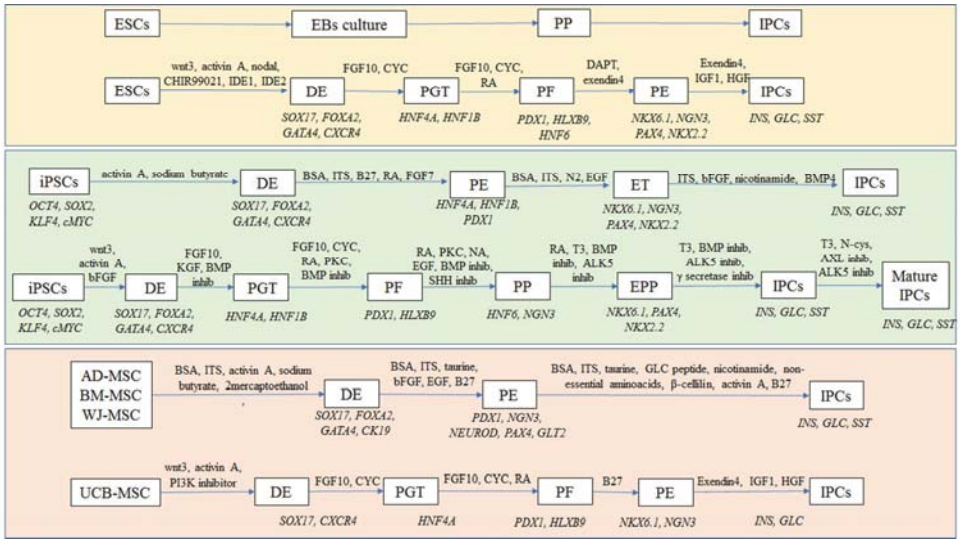

Figure 3.-Comparison between differentiation protocols of ESCs, iPSCs and MSCs. EB (embryoid body), DE (definitive endoderm), PGT (primitive gut tube), PF (posterior foregut), PE (pancreatic endoderm), ET (endocrine tissue) PP (pancreatic progenitors), EPP (endocrine pancreatic precursors). IDE1/2 (inducer of definitive endoderm), FGF (fibroblast growth factor), CYC (cyclopamine), RA (retinoic acid), IGF1 (islet growth factor 1), HGF (hepatocyte growth factor), BSA (bovine serum albumin), ITS (insulin-transferrin-selenium), EGF (epidermal growth factor), bFGF (basic fibroblast growth factor), BMP4 (bone morphogenic protein 4), KGF (keratinocyte growth factor), PKC (protein kinase C), SHH (sonic hedgehog), T3 (thyroid hormone 3), ALK5 (activin-like kinase 5), N-Cys (N-acetylcysteine), AXL (AXL receptor tyrosine kinase), PI3K (phosphoinositide 3-kinase). References for ESC differentiation:125-129. References for iPSCs differentiation: 146-149. References for MSCs differentiation: 165-168.

ESCs-based differentiation

The self-renew and differentiation potential capacities from ESCs represent a great promise for cell replacement therapies. ESCs are isolated by mechanical (114) or laser dissection (115), and immune-surgery (116) from the inner mass of the blastocyst. The pluripotency from ESCs is revealed by their capacity to form teratomas (117) and differentiate *in vitro* into the three germ layers (118), frequently through the formation of embryoid bodies (EBs) (119). The differentiation of ESCs to several lineages, such as cardiac tissue (120), neuronal tissue (121), pancreatic islet β-cells (122), hematopoietic progenitors (123), and endothelial cells (124) has widely studied.

ESCs are differentiated toward the pancreatic lineage through different procedures (Figure 3). Current protocols drive differentiation towards EBs or directly to definitive endoderm. Most of the differentiation protocols with murine ESCs (mESCs) begin with the formation of EBs, frequently by hanging drops in suspension for 5 days and followed by several differentiation steps towards IPCs (30–40 days) (125). These steps are often performed in poly-L-ornithine/laminin-coated culture dishes, adding components into the differentiation medium (126) such as N2 supplement, progesterone, putrescine, laminin, insulin, sodium selenite, nicotinamide, transferrin, fibronectin, B27 supplement or fetal bovine serum. Following these differentiation protocols, high C-peptide expression and long normoglycemic rescue of diabetic mice have been reported. Other protocols involving serum and B27-free, insulin, transferrin, selenium, and fibronectin medium with either bFGF or the PI3 kinase inhibitor LY294002 did not provide IPCs with the same results (127). However, IPCs differentiated from ESCs did not release more insulin after exposition to higher glucose concentration, suggesting that IPCs may also be malfunctioning in their glucose-sensing capacity, which was reflected in a transient correction of blood glucose levels after IPCs transplantation in streptozotocin-induced diabetic mice (128). Moreover, some teratoma formation was detected.

Human ESCs (hESCs) are differentiated towards IPCs by the direct

differentiation into definitive endoderm without the formation of EBs following the next sequence of stages: definitive endoderm, primitive gut tube, posterior foregut, pancreatic endoderm, and endocrine cells. These stages are characterized by different gene expression profiles (Figure 3). Definitive endoderm formation from ESCs is often induced by wnt3 and activin-A (129), but other molecules such as nodal, CHIR99021, IDE1 and IDE2 can also induce this differentiation (130). Primitive gut tube formation can be induced by FGF10 and cyclopamine, while posterior foregut stage can be reached by the addition of FGF10, cyclopamine and retinoic acid into the culture medium (131). The addition of DAPT to block Notch signaling, and exendin-4, induce the differentiation of pancreatic endoderm and endocrine precursors. Finally, exendin-4, islet growth factor-1 (IGF1) and HGF help in the formation of hormone-expressing cells. The yield of hormone-expressing cells following these protocols is around 7 % of insulin-positive cells of total cell population but this yield can be increased by modifying, for example, the culture medium composition (129). However, the final yield of IPCs is still very low (0.8-7.3% of the final heterogenous cell population), and the insulin secreted by these IPCs represents a small fraction compared to native β-cells (125). Despite, the manipulation of culture conditions is the most frequent procedure to differentiate ESCs into IPCs (132), the nuclear reprogramming is another important tool in generation of IPCs and has been used to induce the expression of critical transcription factors in the differentiation of ESCs into IPCs, such as the overexpression of key transcription factors like *PAX4, PDX1, NGN3, NKX6.1* and *NKX2.2* (133-136). hESCs have also been genetically modified trying to modulate the immune response, avoiding the graft rejection after their transplantation (137). However, the research with hESCs is obviously limited by ethics.

Comparing hESCs and mESCs models some differences need to be noted (138). One of the most highlighted differences is the requirement feeder layers for mESCs and its absence in human. hESCs are cultured in feeder-free cultures using conditioned media from the feeder layer or replacing serum by serum supplemented

with basic human FGF (139). Generally, population doubling time is larger in human than in mouse embryonic cells and the morphology of colonies under the microscope is also different, with flatter and more compacted hESCs colonies, easily dissociated by enzymatic dissociation. The factors that allow the efficient human and murine β-cell differentiation *in vivo* are still unknown but may include key elements that are difficult to reproduce *in vitro*, such as vascularization and interaction with adjacent tissues (140).

Low-efficiency differentiation, the inability of IPCs derived from ESCs to secrete insulin after glucose stimulation and the presence of immature IPCs are important bottlenecks to be solved before moving from the bench to the clinic with ESCs in diabetes. Although, several the transplantation of diabetic murine models with IPCs differentiated through human or murine ESCs have displayed short-term normoglycemia (141, 142), a large number of studies describe a not completely mature differentiated pancreatic progenitor. These pancreatic progenitors are not able to release insulin with glucose stimuli, which is reflected in the lack of effectivity in restoring normoglycemia. But the main obstacle to the clinical translation is their high tumorigenic risk. Currently, there are several studies related to solving the tumorigenic issues through the sorting of the pancreatic progenitors from the differentiated population, although more studies need to be done. Cell encapsulation procedures represent an interesting alternative to ensure the safety and functionality of transplanted β-cell precursors.

<u>iPSCs-based differentiation</u>

An alternative to ESCs are iPSCs, genetically reprogramed adult somatic cells that show intrinsic stem cells characteristics. Murine and human iPSCs are obtained by the forced expression of *OCT4, SOX2, KLF4* and/or *C-MYC* genes through viral (143) or non-viral methods (144). These cells are highly comparable to ESCs in terms of morphology, proliferation capacity, telomerase activity, karyotype, surface cell markers and gene expression profile (145). Human iPSCs represent a good source

for β-cell replenishment therapies because of their easy accessibility, such as skin-derived or blood cells-derived, that can enhance the development of personalized autologous replenishment therapies, circumventing the need for immunosuppression. Currently, the differentiation protocols optimized from ESCs differentiation protocols are still successfully applied to differentiate iPSCs towards IPCs (Figure 3). The first protocol completely differentiating human fibroblast-derived iPSCs into IPCs consisted in a four-stage differentiation protocol (146) through the stages of definitive endoderm, pancreatic endoderm, endocrine tissue and maturation of IPCs. Trying to increase differentiation efficiency and the expression of C-peptide and insulin, new differentiation protocols have been developed following the aforementioned developmental stages. New growth factors that activate or inhibit key signal pathways are currently studied, such as indolactam V, GLP-1, noggin, retinoic acid, BMP4 or transforming growth factor-β (TGF-β). However, non-glucose responsive IPCs can maturate *in vivo* after transplantation into immunodeficient mice, forming β-like cells with the capacity of glucose-stimulated insulin secretion (147). Moreover, high-cell density cultures and cell aggregation cultures favored differentiation from definitive endoderm stage into more functional pancreatic endoderm cells comparable to β-cells (148).

Recently, a 7 steps differentiation protocol (149) has generated 50% of IPCs from iPSCs by adding factors such as wnt3, vitamin C, protein kinase C pathway activators, TGF-β receptor inhibitors, and thyroid hormones, and identifying R428, an inhibitor of tyrosine kinase receptor AXL, as a new crucial enhancer of IPCs maturation. Cells treated with R428 along the differentiation protocol acquire responsiveness to glucose levels changes, although less potently than adult human islets. When transplanted in diabetic murine models, R428-treated cells normalize blood glucose levels after 16 days. However, further investigation is required to establish higher differentiation efficiencies and increasing insulin production in response to glucose stimuli.

The generation of iPSCs, therefore, can be envisioned as a personalized medicine, allowing the production of genuine patient-specific IPCs (150), even when using iPSCs derived from patients with genetic diseases that would require DNA-editing to correct mutations before they can be transplanted (151). However, several concerns from iPSCs need to be solved, such as the incomplete silencing and random integration of pluripotency genes that can result in genomic instability (152), the variation in the efficiency of differentiation and the tumorigenicity of iPSC-derived progeny (153). Several factors can contribute to iPSCs variability, such as the epigenetic memory of somatic cells or donor age (154). Recently, the immunogenicity of syngeneic iPSCs and their derivatives have resulted in immune rejection (155). The immune tolerance or the immune rejection of iPSCs seems to depend on the gene introduction method for the generation of the iPSC, resulting in aberrant gene expression profiles (156).

MSCs-based differentiation

MSCs could avoid the issues related to the tumorigenicity derived from ESCs or iPSCs. MSCs are multipotent stem cells characterized by self-renewal capacity, ability to differentiate into several cell types and the expression of specific cell-surface molecules, such as CD105, CD73 or CD90 (157). MSCs can differentiate into the mesodermal lineage, such as adipocytes, osteoblasts, and chondrocytes, and endodermal and ectodermal lineages, including renal tubular cells (158), lung epithelial cells (159), skin (160), neural cells (161), hepatocytes (162), and IPCs (163). Although the transplantation of MSCs promote differentiation of ductal epithelium stem cells into functional β-cells *in vivo* by secretion of cytokines (164), we will focus on the *in vitro* differentiation of MSCs towards IPCs (Figure 3), which has been described with MSCs isolated from human adipose tissue (AD-MSCs) (165), bone marrow (BM-MSCs) (166), Wharton's jelly (WJ-MSCs) (167), and umbilical cord blood (UCB-MSCs) (168).

IPCs differentiated from AD-MSCs following a three-stage protocol form

islet-like cells aggregates that release C-peptide and respond to glucose challenges *in vitro*, restoring normoglycemia in diabetic murine models, 2 weeks after transplantation of encapsulated aggregates within alginate scaffolds (165). When differentiated from BM-MSCs following a three-stage procedure (166), IPCs release *in vitro* high rates of insulin and C-peptide responding to glucose stimulation, normalizing blood glucose levels within few days after transplantation into diabetic nude mice. Transplanted IPCs differentiated through a three-stage protocol from WJ-MSCs normalize blood glucose and insulin levels 1 week after transplantation in streptozotocin-induced diabetic rats (167). However, UCB-MSCs are closer related to ESCs five-stages differentiation protocols (168), with some differences such as the presence of LY-294002 or keratinocyte growth factor. Thus, IPCs derived from UCB-MSCs release insulin and response to glucose stimuli *in vitro*, displaying a sustained normoglycemia, but with low detectable C-peptide rates, after transplantation into immune-deficient murine models.

In addition to MSCs differentiation to IPCs, MSCs possess immunomodulatory potential, an interesting property in regenerative and transplantation medicine. MSCs have shown immunomodulatory effects with T lymphocytes (T-cells), B lymphocytes (B-cells), dendritic cells (DCs) and natural killer cells (NK cells), although the mechanisms remain elusive. For example, several molecules secreted by MSCs can suppress T-cell–mediated antigen responses *in vitro* (169), such as TGF-β, HGF, prostaglandin E2, IL-10 or IL2. Thus, the secretion of interleukin IL-2 by MSCs suppress lymphocyte proliferation *in vitro*, prolonging skin graft survival *in vivo* (170). Despite chromosomal instability in long-term *in vitro* MSCs expansion without a reported malignant transformation in clinical trials (171), these intriguing properties have proposed MSCs as an alternative therapy for autoimmune disorders such as T1DM.

3. ATMP regulation

The development of ATMPs requires the establishment of a legal framework,

regulating the expansion and manipulation of cells *in vitro* to prevent any potential risk to public health. The concept of ATMP is only used in the European Union. In other countries, the terminology adopted for these products is cell and tissue-based therapeutic (CTT). There is not a worldwide agreement and regulation for these devices among countries, with some drug regulatory agencies cataloging these devices as drugs. However, currently, agencies have realized that the drugs regulation framework is not enough for ATMPs and they are constantly improving the legal framework of these products.

In the European Union (EU), ATMPs are divided into the regulations as three categories: somatic cell therapy medicinal product, gene therapy medicinal product and tissue engineered product. The main legal framework for ATMPs in EU is the regulation No 1394/2007 EC, which is completed by the directives 2004/23/EC and 2001/83/EC, regulating donation, manipulation, and distribution of human cells, and the regime for human medicines set out, respectively. Thus, in 2009, EU elaborated some guidelines on good clinical practice with ATMPs which includes: complete definition of ATMPs, donation, procurement and testing procedures, tissue or blood establishments and animal facilities requirements, manufacturing and importation rules, a complete list of legal responsibilities, establishment of safety reports and the considerations to be assessed by an ethical committee. The member states of the EU are the responsible on organizing inspections and control measurements to ensure the compliance with the regulations, publishing new scientific guidelines, directives and other information relevant to ATMP regulations on the European Medicines Agency (EMA) website.

In the United States, FDA evaluates the CTTs focusing on the use of safety tissues without the risk of transmitting diseases, preventing tissues damages and contaminations, ensuring the clinical safety and effectiveness. The section 351 of the Public Health Services (PHS) Act and the Food Drug and Cosmetics Act regulates those CTT that includes one of the following factors: cells or tissues minimally manipulated, used by their homologous function, combined with non-cell or non-

tissue components and used for metabolic function. CTTs not included in the above categories are regulated by section 361 of the PHS Act. In the United States legal framework, also exists other guidelines for cell and tissue therapies. They include the Good Tissue Practices (GTP) to prevent CTT contamination with infectious disease and the Good Manufacturing Practices (GMP) focused on the production of safe, pure and potent products. As in the EU, the facilities in the United States for cell, tissues and animal processing need to be registered by the competent administration. ATMP regulations are different depending on the legal framework from each region. Currently, most of the developed countries, such as Australia, Canada, Japan, South Korea or Singapore have elaborated their own legal regulation and guidelines.

4. Current advanced systems under study

At present, several international companies are involved in the development of ATMPs in T1DM. All of them gather some of the characteristics described above and are ready to respond to the legal frameworks from several countries. The most important companies currently developing ATMPs are ViaCyte, Sernova, Beta O2. Other companies such as Theracyte and Defymed have developed devices to be used by the customer as ATMP with the scaffold and cells desired (Table 3).

Company	ViaCyte	Sernova	Beta-O_2	Theracyte	Defymed
Device	VC-01	Cell Pouch	ß-Air	TheraCyte	MailPan
Implantation site	Subcutaneous	Subcutaneous	Subcutaneous	Subcutaneous	Subcutaneous
Scaffold	Polyethylene glycol (PEG)	Polymer mix	Alginate	Customized	Customized
Cells	Pancreatic progenitors from human ESCs	Pancreatic islets	Pancreatic islets	Customized	Customized
Oxygenation capacity	Per-fluorinated oxygen	Fluorinated oxygen	Externally injected	Customized	Customized
Vascularization ability	Microperforated structure	Microperforated structure	Microperforated structure	Microperforated structure	Microperforated structure
Immunosuppression	Size-exclusion	Sertoli Cells	Size-exclusion	Size-exclusion	Size-exclusion
References	172	174	15	177	No References

Table 3.-ATMPs approved by medicine agencies

4.1. ViaCyte

ViaCyte is a company based in San Diego (California) which is involved in regenerative medicine since 1999. The company has combined the differentiation technology of hESCs to pancreatic β-cell precursors and the hydrogel encapsulation, patenting the ATMP called PEC-Encap™ (also known as VC-01™). PEC-Encap™, therefore, is an ATMP designed for T1DM and T2DM composed by both patented cells (PEC-01) and cell delivery system (Encaptra). Based on developmental biologists, ViaCyte described and patented a reproducible multistep procedure for differentiating hESCs into pancreatic endoderm cells (PEC-01). They recapitulated the natural development of the human pancreas to pancreatic progenitors. Thus, ViaCyte optimized a differentiation protocol of hESCs into definitive endoderm cells as multipotent cells precursors of pancreas, liver and other cells, tissues and organs (172). Next, they differentiated definitive endoderm cells into pancreatic endoderm cells (PEC-01). PEC-01 cells can be cryopreserved and thawed when needed (173). They suppose that once implanted under the skin of a patient with diabetes, PEC-01 cells will mature into functional beta cells.

Once cells are differentiated, PEC-01 cells are embedded within PEG hydrogels, which is next introduced into the Encaptra cell delivery system, manufactured from implant-grade materials specifically selected for long-term biocompatibility. This device is indicated for subcutaneous implantation, granting the immune protection from a patient's allo-and autoimmunity, allowing the free transport of oxygen, nutrients, and proteins across the system's membrane. Moreover, the device is accessible for monitoring with common clinical imaging systems, allowing also an easy retrieval if necessary. Viacyte claims that PEC-Encap™ (VC-01™) system is completely biocompatible and biostable.

Actually, a phase I/II clinical trial has been performed to test if PEC-01 patented cells and Encaptra cell delivery system combination can be implanted subcutaneously in T1DM patients and maintained safely for two years. It will also test if the VC-01 system is an effective treatment for subjects with Type 1 Diabetes.

This study is ongoing, but not recruiting participants.

4.2. Cell Pouch System

Sernova is a Canadian enterprise from London (Ontario) which is involved, since 2006, in the development of clinical-stage products for chronical diseases treatment, such as T1DM. This company cocultures pancreatic islets with Sertoli cells that will provide immune protection to the islets, patenting this technology under the name of Sertolin™. The Sertolin™ technology has the potential to reduce or eliminate the need of expensive lifelong daily antirejection drug cocktails. Next, the therapeutic cells are combined with an implantable scalable device that contains different polymers, mimicking natural ECM environment. This patented ATMP is called Cell Pouch™. Sernova's Cell Pouch System™ combined with pancreatic islets and Sertolin™ has been proved in different small and large animal transplantation models of T1DM. Mouse transplanted into the Cell Pouch System™ displayed a restoration of glycemic control, responding to glucose challenges, and comparable to renal subcapsular islet grafts while islets transplanted subcutaneously alone failed to engraft. Also, this study demonstrated that syngeneic islets transplanted into pre-vascularized Cell Pouch System within the subcutaneous space, a function similar to islets transplanted under the kidney capsule, while islets transplanted into subcutaneous space without the Cell Pouch System fail to reverse diabetes. Despite, mouse pancreatic islets into Cell Pouch System were more glucose intolerant compared to non-diabetic mice, transplanted islets effect was enough to fully reverse diabetes at long-term (>100 days) (174).

At present, there is a Phase I/II clinical trial studying the safety and efficacy of subcutaneous implantation Cell Pouch System™ in T1DM patients. Sernova Cell Pouch™ is transplanted in T1DM patients in the subcutaneous site, two to twelve weeks after transplantation of islets into the Cell Pouch™, to determine the proportion of subjects implanted with the Cell Pouch™ and transplanted with islets into the Cell Pouch™ who achieve and maintain insulin independence after islet transplantation.

Other data of interest in the study are the efficacy of the Cell Pouch™ to maintain adequate immunological protection against both allo- and autoimmunity of islet transplant recipients, as well as the metabolic function and engraftment.

4.3. ß-Air bio-artificial pancreas

Beta-O2 Technologies Ltd. is a private company founded in 2004 and located in Rosh Ha'ayin, Israel. Recently, Beta-O2 has developed the ß-Air bio-artificial pancreas®, an ATMP intended to cure T1DM. The ß-Air device contains islets of Langerhans enclosed in alginate hydrogels. Two types of alginate are used in the manufacturing of the device: HG (high guluronic acid) is used for islet immobilization and HM (high mannuronic acid) impregnated in a Teflon membrane is used to protect from the immune system 15. The immune barrier of the device is composed of two off-the-shelf Teflon membranes. Although Teflon membrane is permeable, impregnating the membranes with high mannuronic acid (HM) alginate turns the membranes into an impermeable barrier for cells and host immune system particles, while allowing free passage of glucose and insulin. Theoretically, islets can sense the level of glucose and regulate the production of insulin and glucagon as needed, while simultaneously preventing the transfer of immunogenic substances through the membrane. Moreover, another compartment inside the device is used as oxygen storage, refilling the air in the device manually through a replenishing device. Replenishing the device to guarantee the islets survival, is performed by injecting oxygen with a dedicated injector into the oxygen port implanted under the skin (175). ß-Air bio-artificial pancreas® is subcutaneously implanted in a minimally invasive procedure in T1DM patients. Although the device is not currently commercial, Beta-O2 Technologies Ltd. has tested the β-Air device in small and large isogeneic, allogeneic, and xenogeneic animals, showing safety and long-term functionality in the non-immunosuppressed environment (176). A phase I/phase II clinical trial is ongoing to assess the safety and efficacy of the transplanted ATMP, not recruiting currently more participants.

4.4. TheraCyte system

TheraCyte Inc. is a company founded in 1999, situated in California, United States and centered on the development of cell-based therapeutic products to treat human diseases. TheraCyte system™ is the macroencapsulation device developed by the company and protected by 20 U.S. patents and multiple foreign patent filings in Europe and Japan. TheraCyte encapsulation device is a planar pouch featuring a bilaminar polytetrafluorethylene membrane system comprised of an inner semipermeable membrane, laminated to an outer membrane and covered by a loose polyester mesh (177). The outer layer promotes tissue integration, whereas an inner, cell impermeable, membrane has a 0.4 μm pore size (103). The devices are 2 cm long with an inner lumen of 4.5 μL. The durability of this encapsulation device has been exploited to sequester different kind of cell types, insulin-secreting cells among others. Its subcutaneous placement allows cells to be transplanted in a minimally invasive manner and retrieved if necessary. The device is biologically inert and, when transplanted into human patients for a year, there are no adverse effects (178). The biocompatible membrane-bounded to a polymeric inner chamber protects cells from allogeneic and autoimmune destruction allowing a long-term functionality.

The immune protection of TheraCyte™ device was evaluated in animal models by comparing the transplantation of rat islets in alloimmunized and non-immunized diabetic rats with the implantation of rat islets within the TheraCyte™ device. Results showed shorter graft survival in immunized recipients than in non-immunized recipients when non-encapsulated islets were transplanted under the kidney capsule. However, rat islets transplanted inside TheraCyte™ device maintained graft functionality for 6 months in both immunized and non-immunized rats 179. Moreover, a higher number of CD8+ T-cells producing IFN-γ were detected in immunized compared to non-immunized rats transplanted with encapsulated islets within the device, suggesting that donor-specific alloreactivity in recipient rats was sustained throughout the study period and TheraCyte™ device protects islet allografts also in immunized recipients.

4.5. MailPanβ

Defymed is a spin-off from the "Centre européen d'étude du Diabète" (European center for Diabetes studies), founded in 2011 in Strasbourg, France. The enterprise has developed an ATMP that can be used in T1DM treatment. It is called MailPan®, and it is based in the encapsulation of cells into a device with non-biodegradable, biocompatible and selective permeability membranes. This device does not require surgery for implantation and it grants the immune protection of the inner cells by the impossibility of IgGs to diffuse through the membrane, avoiding the need of immunosuppressive therapy. MailPan® was certified by the safety and quality ISO framework in 2014 and is patented for the treatment of several diseases depending on enclosed cells, T1DM among others, when insulin-producing cells are enclosed. This device is still under preclinical trial phase.

5. CONCLUSIONS

Since the insulin discovery, synthetic insulins and derivatives have been developed, increasing the quality and life expectancy of patients, but not curing definitively T1DM. β-pancreatic cell replenishment with whole pancreas transplantation or pancreatic islets have provided promising results in spite of complicated surgery, lifetime immunosuppression, and donor scarcity. The generation of new ATMPs gathering optimal environment for the host and donor cells and complying with the regulatory legal frameworks represents a promising future for the cure of T1DM. However, the accomplishment of an optimal environment is an arduous journey, requiring an initial oxygen supply, accompanied by the neo-generation of an appropriated vascular network, displaying tunable injectability, stiffness, porosity and degradation rate of the scaffold, avoiding the foreign body reaction and mimicking the ECM natural microenvironment to embed the donor cells. Moreover, the scarcity of pancreases or islets available has directed into the research new cell sources, representing the stem cells a promising alternative, even when ethical, safety and technical concerns need to be considered. Several

companies are currently performing phase I/II clinical trials with patented ATMPs and hopefully their future results will shed light in the cure of T1DM. Meanwhile, the scientific community follows investigating in the optimization of new ATMPs for T1DM treatment.

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Chapter 2 2

Objectives

OBJECTIVES

The World Health Organization declares that diabetes mellitus is the most frequent endocrine disease in industrialized regions, where diabetic patients reach 300 million. Type 1 diabetes mellitus (T1DM) is characterized by autoimmune destruction of pancreatic β-cells, after an asymptomatic period over the years, resulting in severe insulin deficiency and other significant complications such as nephropathy, renal failure, neuropathy, retinopathy, damaged vessels, and limb amputation. The insulin administration through daily injections is a regular treatment for advanced stages of T1DM, but they often produce a lack of glucose metabolism control, not resulting in a cure for the disease. Nowadays, several research groups are focused on healing T1DM with β-pancreatic cell replenishment, either by whole vascularized pancreas transplantation or by islet transplantation, but donor scarcity represents a significant limitation.

The application of stem cells for diabetes treatment has been purposed to solve this donor limitation, being the mesenchymal stem cells (MSCs) an appropriate cell source. They retain self-renewal capacity and can differentiate into IPCs avoiding the tumorigenicity associated with embryonic or induced pluripotent stem cells. Although differentiation of MSCs into β-pancreatic cells provides low and no sustainable insulin release, 3D encapsulation systems could enhance insulin secretion. However, actual 3D encapsulation matrices are not able to mimic the natural extracellular matrix (ECM) being a hot topic of study the combination of biomaterials mimicking an *in vivo* environment. Since hyaluronic acid (HA) is a major component of the natural ECM, also involved in the maintenance of islets stability and integrity, we intended to use HA as a new component in 3D differentiation of MSCs into pancreatic within alginate matrices, developing the following specific goals:

1.To select the optimal HA and alginate combination to form hybrid microcapsules suitable for cell encapsulation.

2. To determine the influence of HA within hybrid alginate microcapsules in encapsulated MSCs survival, protein release and differentiation potential.

3. To clarify if HA within hybrid alginate-HA microcapsules affects the survival and insulin release of encapsulated IPCs.

4. To implement and validate hybrid alginate-HA microcapsules as 3D support for the differentiation of MSCs toward IPCs.

Chapter 3

3

Alginate microcapsules incorporating hyaluronic acid recreate closer *in vivo* environment for mesenchymal stem cells

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Alginate microcapsules incorporating hyaluronic acid recreate closer *in vivo* environment for mesenchymal stem cells

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ABSTRACT

The potential clinical application of alginate cell microencapsulation has advanced enormously during the last decade. However, the 3D environment created by alginate beads does not mimic the natural extracellular matrix surrounding cells *in vivo,* responsible of cell survival and functionality. As one of the most frequent macromolecules present in the extracellular matrix is hyaluronic acid, we have formed hybrid beads with alginate and hyaluronic acid recreating a closer *in vivo* cell environment. Our results show that 1% alginate 0.25% hyaluronic acid microcapsules retain 1.5% alginate physicochemical properties. Moreover, mesenchymal stem cells encapsulated in these hybrid beads show enhanced viability therapeutic protein release and mesenchymal stem cells potential to differentiate into chondrogenic lineage. Although future studies with additional proteins need to be done in order to approach even more the extracellular matrix features, we have shown that hyaluronic acid protects alginate encapsulated mesenchymal stem cells by providing a niche-like environment and remaining them competent as a sustainable drug delivery system.

Keywords: alginate, microencapsulation, hyaluronic acid, mesenchymal stem cells

1. INTRODUCTION

Cell microencapsulation is a technology used for the sustainable controlled release of therapeutic proteins that has shown promising results in the future treatment of several diseases such as diabetes mellitus or Alzheimer´s disease. The outer layer of the microcapsules allows the flow of nutrients and oxygen into the core of the beads, while therapeutic proteins and waste are released from the cells outside. Moreover, the microcapsules represent a barrier to the recognition by the immune system, avoiding the entrance of immunoglobulins and, therefore, circumventing the immune rejection after encapsulated cell implantation (1, 2). Although several biocompatible materials have been involved in cell microencapsulation such as agarose (3), chitosan (4) and hyaluronic acid (5), the most common is alginate because of its mechanical properties, and the isotonic solutions used instead of cytotoxic solvents (6). Moreover, alginate microcapsules can be coated with polycations such as poly-L-Lysine (PLL), poly-D-lysine (PDL) and poly-L-ornithine (PLO) which provide higher resistance (7). Among other applications, alginate microcapsules have been extensively studied in diabetes research by enclosing pancreatic islets with promising results. For example, diabetic patients lived without daily insulin injections for more than 9 months after intraperitoneal implantation of encapsulated pancreatic islets (8). In addition, the incorporation of the chemokine CXCL12 into alginate-encapsulated islets generated a long-term (> 300 days) immune protective effect in allo- and xenogeneic transplantation, as well as a selective increase of intra-graft Treg cells (9). In Alzheimer´s disease, the implantation of microcapsules containing vascular endothelial growth factor (VEGF) secreting cells in double mutant amyloid precursor protein/presenilin mice, alleviated the symptoms for a period of three months by reducing the total brain amyloid-beta peptide load and decreasing the apoptotic cell death in the cerebral cortex (10). Moreover, nerve growth factor (NGF) secreting encapsulated cells showed no evidence of inflammation or device displacement after 12 months post-implantation (11). Our research group has previously studied the microencapsulation of genetically engineered cells to secrete erythropoietin (EPO),

showing that cell encapsulation allows the long-term survival of cells and, therefore, an EPO secretion up to 210 days, in allogeneic transplantation, and up to 98 days in xenogeneic transplantation (12-14).

Despite the promising results obtained with alginate in cell microencapsulation, this biomaterial does not provide the cell-matrix interactions required for cell adhesion and signaling, that could prolong even longer the cell survival and functionality (15, 16). Cell-matrix interactions are supported by extracellular matrix (ECM) components that supply mechanical and physiological support. Hence, different ECM molecules have been tested in alginate encapsulated cells, such as laminin, collagen I or collagen IV, confirming an enhancement in encapsulated cell viability (17). Other approaches trying to simulate the cell-matrix interactions provided by ECM are the short synthetic peptides derived from natural proteins that compose the ECM, for example, the arginine-glycine-aspartic acid (RGD) peptide derived from fibronectin. This tripeptide offers advantages over the use of the whole protein like its simplicity, cost-effectiveness, easy manipulation for functionalization and low immune response (18, 19). Our group has shown its effectiveness in enhancing the viability of several encapsulated cell types, such as myoblasts, fibroblasts or mesenchymal stem cells (20-22). Moreover, it has been described that RGD in alginate microcapsules promotes the differentiation into bone cells when compared to unmodified alginate (23). However, these short peptides do not accurately represent the ECM and do not provide the required signals for a complete reproduction of the cell environment *in vivo*. On this regard, another major component of the ECM distributed widely throughout connective, epithelial, and neural tissues is the hyaluronic acid (HA), which has been proposed for the preparation of biodegradable ECM-like constructs for tissue engineering applications (24).

Hyaluronic acid (HA) is a high molecular weight (MW) anionic non-sulfated glycosaminoglycan. It is integrated by the repetition of a disaccharide unit of an N-acetyl-glucosamine and a β-glucuronic acid and interacts with cells via the

surface receptor CD44. HA has been described for being involved in a wide variety of biological procedures like mediation of cell-signaling, regulation of cell adhesion and proliferation, and manipulation of cell differentiation (25). Hence, crosslinking of HA forms experimentally controllable hydrogels that provide a microstructure similar to native ECM (26). Therefore, embedding cells within HA permits an appropriate structural support and protection, allowing cells to interact in 3D and enhance their viability (27). Moreover, HA helps to reduce the immunogenicity of embedded cells because this biocompatible material reduces the adsorption of proteins (28), responsible of stimulating the recruitment of immune cells, such as macrophages. In terms of differentiation, HA promotes the differentiation towards murine chondrocytes when cultured either in 2D (29), or 3D, promoting the synthesis of cartilage tissue (30, 31). Other authors have combined HA with other biomaterials. For example, the combination of HA with gelatin forms a biomimetic hybrid hydrogel after photo-crosslinking gelation and mimics the ECM of native tissues, promoting the cell spreading of HUVEC cells, and improving their mechanical properties compared to their single component analogs (32). Another example of hybrid HA microcapsule is the combination with heparin crosslinked by thiolated heparin and methacrylated hyaluronic acid via visible light mediated thiol-ene reaction. These hybrid microcapsules showed better spreading, proliferation, migration, and differentiation of adipose-derived mesenchymal stem cells than their respective single component analogs (33). In summary, the combination of several biomaterials in microencapsulation technology seems to provide closer cell behavior to cells surrounded by ECM-like natural microenvironment than a single biomaterial. The optimal combination of the biomaterials that better mimic ECM still need to be determined.

In this work, we have identified the best combination of hyaluronic acid and alginate that forms hybrid microcapsules with similar physicochemical properties to alginate microcapsules, with the hypothesis that the presence of hyaluronic acid will mimic the natural ECM environment and, therefore, enhance the encapsulated

cell viability and functionality. Hence, we have selected the formulation of the hybrid microcapsule-based in the rheological behavior of an extensive number of combinations between hyaluronic acid and alginate, next studying more deeply the physicochemical characteristics of those combinations with similar rheological behavior to alginate. Finally, we have evaluated the beneficial effect of HA presence in alginate encapsulated D1 mesenchymal stem cells genetically modified to secrete erythropoietin, in terms of viability and functionality.

2. MATERIAL AND METHODS.

2.1. Materials

Ultrapure low-viscosity (20-200 mPa*s) and high guluronic (LVG) acid alginate (G/M ratio > 1.5) with MW of 75-200 kDa was purchased from FMC Biopolymer (Norway). Poly-L-Lysine hydrobromide (PLL, 15-30 kDa) was purchased from Sigma-Aldrich (St Louis, MO). Hyaluronic acid and FITC labeled hyaluronic acid with an MW 1,1 MDa were purchased from Contipro (Czech Republic).

2. 2. Cell culture

Murine D1 MSCs engineered to secrete erythropoietin (EPO) were grown with complete medium consisting of Dulbecco's modified Eagles' medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin solution (Gibco) at 37 ºC in humidified 5% CO_2 atmosphere. Cells were passaged every 4-5 days.

2.3. Cell microencapsulation

The following solutions suspended in 1% mannitol of sodium hyaluronate,1–1,25 MDa (Contipro) and alginate (FMC Biopolymer) were performed: 1% alginate 0.1% HA, 1% alginate 0.25% HA, 1% alginate 0.5% HA, 1% alginate 1% HA, 0.5% alginate 0.1% HA, 0.5% alginate 0.25% HA, 0.5% alginate

0.5% HA,0.5% alginate 1% HA. Final solutions were filtered with a 0.20 μm syringe filter (Millipore, MA, USA). All the solutions were extruded in an electrostatic atomization generator (Nisco®) and the resulting alginate beads were completely gelled by agitation for 15 min in a 55 mM $CaCl_2$. Next, beads were ionically linked with 0.05% (w/v) PLL for 5 min, followed by a second coating with 0.1% alginate for another 5 min. All the procedure was performed at room temperature, under aseptic conditions, and in complete medium.

For cell microencapsulation, cells were suspended in the solutions of sodium hyaluronate and alginate obtaining $5x10^6$ cells/mL of the solution following the aforementioned procedure for beads formation. The morphology and diameter of the microcapsules were assessed under an inverted optical microscopy (Nikon TSM).

2.4. Rheological properties

The viscosity from all the solutions (1% alginate 0.1% HA, 1% alginate 0.25% HA, 1% alginate 0.5% HA, 1% alginate 1% HA, 0.5% alginate 0.5% HA, 0.5% alginate 0.75% HA, 0.5% alginate 1% HA) were assessed on the rheometer AR1000 (TA instruments) with 40 mm flat plate geometry and compared to the viscosity of 1.5% alginate. Viscosity behavior was determined in 1 drop of 500 μL onto the rheometer platform by dynamic shear measurements in a frequency sweep range from 0,01 to 100 Hz at 20 °C. The gap between the upper plate and the sample was set up to 1000 μm. Three independent measurements were conducted for each solution.

2.5. HA content determination within hybrid microcapsules

HA FITC labeled (Contipro, 1.1 MDa) and alginate were suspended in 1% mannitol at the following solutions: 1% alginate 0.25% hyaluronic acid and 0.5%alginate 0.5% hyaluronic acid. 1.5% alginate solution was also prepared as a control. Microcapsules from the three solutions were performed as previously described, but protected from light. Next, microcapsules were imaged under a

fluorescent microscope (Olympus FV500) and their fluorescence was quantified at 488 nm excitation and 520 nm with an Infinite M200 TECAN plate reader. Three independent experiments were performed for each solution.

2.6. Scanning Electron Microscopy (SEM)

The surface of the different microcapsules was analyzed by SEM (Scanning Electron Microscopy). Samples were fixed using 2% glutaraldehyde in 0.1 M Sörenson buffer (pH 7.4), washed in iso-osmolar Sörenson/sucrose buffer and post-fixed with 1% osmium tetroxide in Sörenson buffer. Microcapsules were washed three times, dehydrated through ethanol series and washed three times in hexamethyldisilazane prior to air drying. Finally, samples were coated with gold using an Emitech K550X sputter coater. Microcapsules surface was imaged using a scanning electron microscope (Hitachi S-4800).

2.7. Swelling properties

The osmotic resistance of alginate-HA hybrid microcapsules (1% alginate 0.25% HA; 0.5%alginate 0.5% HA) was determined by the increase of the diameter of microcapsules in a sequential treatment with 1% (w/v) sodium citrate for 6 days and compared to 1.5% alginate microcapsules. Briefly, 100 µL of microcapsules were mixed with 900 µL of PBS and placed into a 24-well plate. Plates were incubated for 1 h at 37 ºC while shaking at 500 rpm. Next, supernatants were removed from each well, 800 µL of citrate solution added and incubated for 24 hours at 37 ºC. Finally, the diameter of 20 microcapsules/sample were quantified under an inverted optical microscopy (Nikon TSM). Results were expressed as Df/Di, where Df (final diameter) is the diameter of the microcapsules after citrate treatment and Di (initial diameter) is the diameter before citrate treatment. Three independent experiments were performed for each sample.

2.8. Early apoptosis quantification

Early apoptosis of encapsulated D1 MSCs was quantified by means of Annexin-V-FITC apoptosis Detection Kit (Sigma-Aldrich). Encapsulated cells in 1.5% alginate, 1% alginate 0.25% HA and 0.5% alginate 0.5% HA were analyzed at days 1, 7 and 21 post-encapsulation. Briefly, 200 µL of microcapsules were incubated with 1mg/ml alginate lyase (Sigma Aldrich) for 30 minutes at 37 ºC. The lysate was rinsed twice with DPBS and resuspended in binding buffer consisting of 10 mM HEPES/NaOH, pH 7.5 containing 0.14 M NaCl, and 2.5 Mm $CaCl_2$. Samples were stained with annexin V-FITC and propidium iodide for exactly 10 min at room temperature and protected from light. Unstained samples or stained only with annexin V-FITC or propidium iodide were established as controls. Apoptotic cells were quantified with a BD FACS Calibur flow cytometer. At least three independent experiments were performed for each solution.

2.9. Quantification and imaging of cell viability

Cell viability was quantified by means of LIVE/DEAD viability/cytotoxicity kit (InvitrogenTM) after 1, 7 and 21 days of encapsulation. Cells were released from microcapsules by alginate lyase treatment as described above and after rinsing with DPBS, they were resuspended in culture medium with 100 nM calcein AM and 8 nM ethidium homodimer-1. Solutions were incubated for 20 min at room temperature, protected from light and dead cells were quantified with a BD FACS Calibur flow cytometer. Unstained samples or stained only with 100 nM calcein AM or 8 nM ethidium homodimer-1 were used as controls. At least three independent experiments were performed for each solution.

For microscopy imaging, a volume of 25 µL of microcapsules was rinsed twice in DPBS and resuspended in 500 µL of 0.5 µM calcein AM and 0.5 µM ethidium homodimer-1 in DPBS. Next, solutions were placed in a 96-well plate and incubated at room temperature protected from light for 45 minutes. Samples were observed under a Nikon TMS confocal microscope at the wavelength of excitation

495 nm/emission 515 nm (for calcein AM staining) and excitation 495 nm/emission 635 nm (for ethidium homodimer staining). Random images were analyzed with the Eclipse Net software, version 1.20.0.

2.10. Study of membrane integrity

Membrane integrity of encapsulated D1 MSCs was determined by the *in vitro* toxicology assay kit Lactic Dehydrogenase based (Sigma–Aldrich) at day 1 and 21 post-encapsulation following manufacturer recommendation. For the assay, 100 µL of microcapsules/sample were rinsed twice with culture medium, resuspended in 1 mL of complete medium and plated in two wells of a 24-well-plate. Wells were incubated for 90 minutes after adding 70 µL of lysis buffer to one well and 70 µL of culture medium to the other. Next, 50 µL of supernatant from each well was incubated with the kit cocktail mixture for 30 minutes, at room temperature and protected from light. The absorbance was read out on an infinite M200 TECAN microplate reader at a wavelength of 490 nm, with absorbance reading at 690 nm as background. At least three independent experiments were analyzed for each condition.

2.11. Metabolic activity assay

Metabolic activity was determined using Cell Counting Kit-8CCK-8 (Sigma–Aldrich) at day 1 and 21 after encapsulation following manufacturer recommendations. For the CCK8 assay, 25 µL of microcapsules were rinsed, resuspended with 500 µL of culture medium and plated in 5 wells in a 96-well plate. After adding 10 µL of the CCK-8 solution to each well, plates were incubated for 4 h at 37 °C inside a wet chamber. Absorbance was read out on an Infinite M200 TECAN plate reader at 450 nm with a reference wavelength at 650 nm. Three independent tests were analyzed for each condition.

2.12. EPO and VEGF quantification

The secretion of EPO and VEGF was quantified from culture supernatants at

day 1 and 21 after encapsulation. A volume of 100 µL of microcapsules was rinsed twice with culture medium, resuspended in 1mL of medium and incubated for 24 hours at 37 ºC and 5% CO_2. Then, supernatants were collected and the secretion of EPO was quantified by Quantikine IVD EPO ELISA kit (R&D Systems) while the secretion of VEGF was quantified by Human VEGF Standard ABTS ELISA Development Kit (Peprotech) following manufacturer recommendations. Three independent samples and controls for each condition were assayed. The results were expressed as D21/D1, where D21 (final value) is the amount of EPO or VEGF secretion by encapsulated cells at day 21 and D1(initial value) at day 1.

2.13. Differentiation of mesenchymal stem cells

Encapsulated D1-MSCs were differentiated into adipocytes, osteocytes, and chondrocytes. Encapsulated D1-MSCs were incubated with complete mesenchymal stem cell medium as a control in all the differentiation. For adipogenic differentiation, 200 µl of encapsulated D1-MSCs were incubated with adipogenic differentiation medium composed of DMEM-High glucose (Gibco) supplemented with 10% fetal bovine serum (Gibco), 1% penicillin/streptomycin solution (Gibco), 0.5 µM dexamethasone (Sigma-Aldrich), 0.5 µM isobutylmethylxanthine (Sigma-Aldrich) and 50 µM indomethacin (Sigma-Aldrich). Encapsulated cells were incubated for 3 weeks, changing medium every 3 days, at 37 ºC with 5% CO_2 atmosphere. Cells attached to the plate were fixed with 10% formalin (Sigma-Aldrich) for 1 hour and stained with oil-red-C (Sigma-Aldrich) for 5 minutes at room temperature.

For osteogenic differentiation, 200 µl of encapsulated D1-MSCs were incubated with osteogenic differentiation medium composed of DMEM-High glucose (Gibco) and supplemented with 10% fetal bovine serum (Gibco), 1% penicillin/ streptomycin solution (Gibco), 100 nM dexamethasone (Sigma-Aldrich), 20 nm β-glycerophosphate (Sigma-Aldrich) and 0.5 µM L-ascorbic acid (Sigma-Aldrich). and 50 µM indomethacin (Sigma-Aldrich). Encapsulated cells were incubated for 3 weeks, changing medium every 3 days. Attached cells were fixed with 10% formalin

(Sigma-Aldrich) for 1 hour and stained with alizarin red-S (Sigma-Aldrich) for 5 minutes at room temperature.

Chondrogenic differentiation was tested incubating 200 µl of encapsulated D1-MSCs with chondrogenic differentiation medium composed of DMEM-High glucose (Gibco) supplemented with 10% fetal bovine serum (Gibco), 1% penicillin/streptomycin solution (Gibco), 10 ng/mL TGF-β1 (Sigma-Aldrich), 50 nM L-ascorbic acid (Sigma-Aldrich) and 6.25 µg/ml bovine insulin (Sigma-Aldrich). After changing medium every 3 days during 21 days, attached cells were fixed with 10% formalin (Sigma-Aldrich) for 1 hour and stained with alcian blue (Sigma-Aldrich) for 30 minutes at room temperature.

2.14. Statistics

Statistical analysis was performed with SPSS software, version 21.00.1. Data were expressed as means ± standard deviation and differences were considered significant for comparison of groups using ANOVA, Tukey's Post Hoc Test when $p < 0.05$.

3. RESULTS

3.1. Characterization of the biomaterial

First, we tried to search several hyaluronic acid/alginate composites that could show the same viscosity than 1.5% alginate, the standard concentration for cell encapsulation in our research group. On this regard, the rheological behavior of different hyaluronic acid and alginate mixtures at the following proportions were compared to 1.5% alginate: 1% alginate 0.1% HA, 1% alginate 0.25% HA, 1% alginate 0.5% HA, 1% alginate 1% HA, 0.5% alginate 0.1% HA, 0.5% alginate 0.25% HA, 0.5% alginate 0.5% HA and 0.5% alginate 1% HA. Thereby, 1% alginate 0.25% HA showed the most similar rheological behavior to 1.5% alginate and, therefore, similar viscosity (Fig. 1A). Moreover, close similarities to 1.5% alginate were detected with 1% alginate 0.1 %HA and 0.5% alginate 0.5% HA, while 1%

alginate 0.5% HA, 1% alginate 1% HA, 0.5% alginate 0.1% HA, 0.5% alginate 0.25% HA, and 0.5% alginate 1% HA displayed differences with 1.5% alginate (Fig1.A).

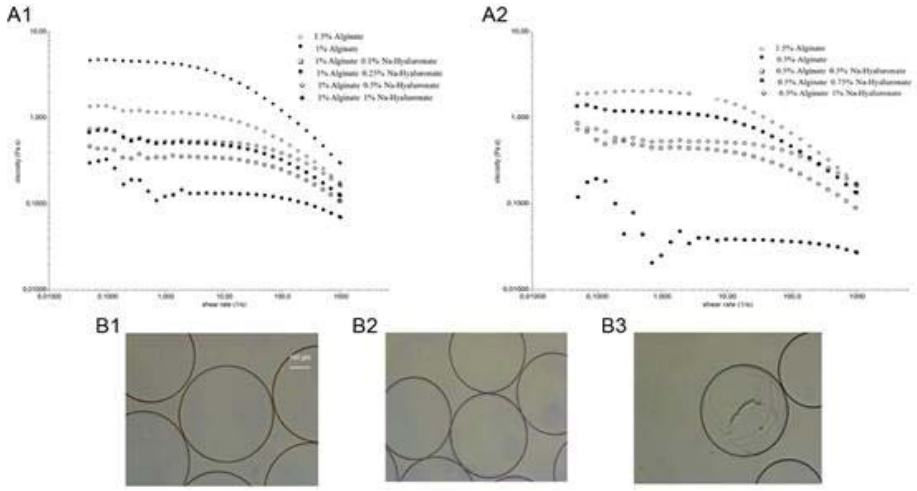

Figure 1.- (A) Rheological behavior comparison to 1.5% alginate of: (1)1% alginate 0.1% HA, 1% alginate 0.25% HA, 1% alginate 0.5% HA, 1% alginate 1% HA and (2) 0.5% alginate 0.1% HA, 0.5% alginate 0.25% HA, 0.5% alginate 0.5% HA and 0.5% alginate 1% HA. (B) Micrographs of microcapsules at the following compositions: (1) 1% alginate 0.1% HA, (2) 1% alginate 0.25% HA and (3) 0.5% alginate 0.5% HA. Note: Scale bar represents 100 μm.

1% alginate 0.25% HA,1% alginate 0.1 %HA and 0.5% alginate 0.5% HA were selected as the compositions to be tested for encapsulation by means of an electrostatic atomization generator (Nisco ®). Spherical homogenous microcapsules were formed with all the compositions providing smooth surfaces similar to 1.5% alginate, with some wrinkled surfaces in microcapsules formed by 0.5% alginate 0.5% HA (Fig. 1B). The diameters shown at all the microcapsules independently of the composition was of 450 μm. We decided to discard 1% alginate 0.1% HA mixture for the following assays since it contains a low concentration of HA and any effect detected with this mixture should be enhanced in the 1% alginate 0.25% HA composition.

With the selected compositions, 1% alginate 0.25% HA and 0.5% alginate 0.5% HA, it resulted imperative to determine the content of HA within the

microcapsules. So, we next proceeded to carefully analyze and quantify the content of HA inside them. On this regard, we elaborated microcapsules with FITC-labelled hyaluronic acid at the same molecular weight (1.1 MDa) than the aforementioned HA. FITC-labeled hyaluronic acid afforded us to quantify the HA content by measuring the fluorescence intensity and to observe the fluorescence under a confocal microscope. Thereby, we quantified double fluorescence intensity of 0.5% alginate 0.5% HA-FITC compared to 1% alginate 0.25% HA-FITC with no fluorescence intensity in their respective controls without FITC and in 1.5% alginate (Fig. 2A). The fluorescence of these composites was confirmed by means of confocal microscopy (Fig. 2B, C). No fluorescence was detected in their respective controls (data not shown). However, the higher intensity in 0.5% alginate 0.5% HA-FITC compared to 1% alginate 0.25% HA-FITC due to a higher presence of HA-FITC was not noticeable with this technology.

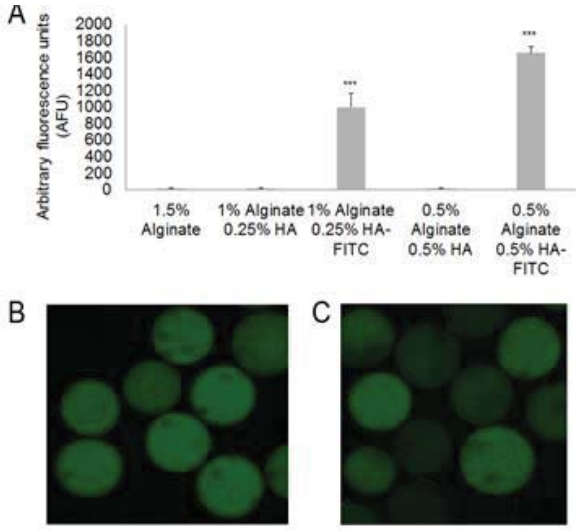

Figure 2.-Determination of HA content within microcapsules. (A) HA-FITC content quantification of 1.5 % alginate, 1% alginate 0.25% HA, 1% alginate 0.25% HA-FITC, 0.5% alginate 0.5% HA and 0.5% alginate 0.5% HA-FITC. Micrographs by means of confocal microscopy of (B) 1% alginate 0.25% HA-FITC microcapsules and (C) 0.5% alginate 0.5% HA-FITC microcapsules. Note: Values represent mean ± SD and *** represents

After determining the presence of HA within the microcapsules, we imaged the surface of the microcapsules by scanning electron microscopy (SEM) since smoother surfaces on the alginate microcapsules have shown better biocompatibility

in vivo. Micrographs from SEM showed differences in the surface smoothness of the microcapsules indicating that the presence of HA induces to differences in the microcapsules surface (Fig. 3), even when rheological behavior is similar.

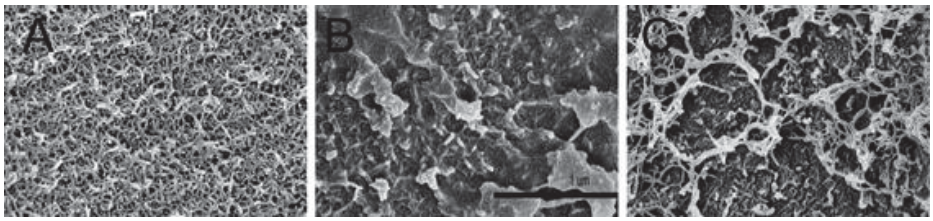

Figure 3.-Microcapsules surface micrographs by Scanning Electron Microscopy.(A) 1.5% alginate, (B) 1% alginate 0.25% HA and (C) 0.5% alginate 0.5% HA. Note: Scale bar represents 1 μm.

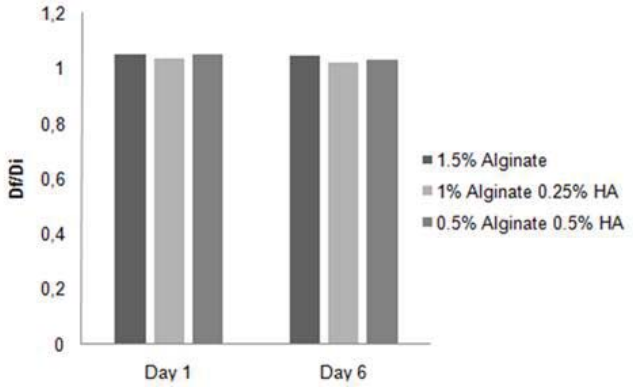

Figure 4.-Results from swelling assay of 1.5% alginate, 1% alginate 0.25% HA and 0.5% alginate 0.5% HA microcapsules expressed as Df/Di: final diameter/initial diameter were Di corresponds to day 0 and Df is indicated in the abscises ax.

So, we decided to study if these differences could be reflected on the swelling behavior of the microcapsules. No significant differences were detected when these studies were performed 6 days along. After placing microcapsules into a monovalent ion solution like sodium citrate, 1% alginate 0.25% HA and 0.5% alginate 0.5% HA showed similar expansion of the core diameter than 1.5% alginate microcapsules (Fig. 4). These results indicate that the presence of HA in the microcapsules does not affect the conversion of gel into liquid caused by a monovalent ion solution, independently of the HA concentration and, therefore, the rupture ratio *in vivo* of hybrid microcapsules will be similar to alginate microcapsules.

3.2. *In vitro* studies with encapsulated cells

After observing that alginate HA hybrid microcapsules had similar rheological behavior and osmotic resistance than alginate microcapsules, we encouraged to assess the impact in the viability and bioactivity of encapsulated cells by the presence of HA within alginate microcapsules. We proceeded to encapsulate cells with the selected alginate-HA hybrid biomaterials. We chose to study D1-MSCs genetically modified to secrete EPO because, on the one hand, MSCs are being studied for clinical applications due to their immune tolerance properties and, on the other hand, their ability to secrete EPO allows their study as a sustainable drug delivery system. Thus, 1% alginate 0.25% HA microcapsules did not represent an issue in the encapsulation procedure of D1-MSCs-EPO while microcapsules composed by 0.5% alginate 0.5% HA agglomerated and did not display an appropriate core stability, releasing cells outside the capsule. Therefore, in the following assays, we compared 1% alginate 0.25% HA to 1.5% alginate excluding the hybrid biomaterial composed of 0.5% alginate 0.5% HA from our futures studies.

First, we quantified the percentage of early apoptotic cells within the microcapsules for 21 days after cell encapsulation, by means of annexin V/propidium iodide staining and subsequent quantification by flow cytometry. Apoptotic cells percentage was lower in 1% alginate 0.25% HA than in 1.5% alginate the next day after encapsulation without statistical significance, showing the same percentage of apoptotic cells 7 days after encapsulation in both types of microcapsules (Fig. 5A). However, a significant decrease of apoptotic cells ($p<0.05$) was detected in 1% alginate 0.25% HA encapsulated cells 21 days after encapsulation compared to 1.5% alginate (Fig. 5A), indicating that the presence of HA within the alginate microcapsules influences apoptotic processes of encapsulated D1-MSC cells over time. Next, we quantified if the percentage of dead cells was also modified by the HA presence within the alginate microcapsules, by means of calcein/ethidium staining and subsequent flow cytometry. In correlation with apoptotic cell percentage, no significant differences were quantified in the percentage of dead cells the next day

after encapsulation (Fig. 5B). However, 7 days after encapsulation, the presence of HA in alginate microcapsules reduced significantly ($p<0.001$) the number of dead cells, even when apoptotic cell percentage did not show differences (Fig. 5B). This result was also reflected at day 21 after encapsulation when the presence of HA significantly ($p<0.001$) reduced the number of dead cells similarly to apoptotic cell percentage at this time point (Fig. 5B). To verify these results, we stained encapsulated D1-MSC-EPO in 1% alginate 0.25% HA and 1.5% alginate with calcein/ethidium and observed them under a fluorescent microscope. Micrographs obtained after staining confirmed the data displaying a higher number of live cells in 1% alginate 0.25% HA capsules than in 1.5% alginate, especially at day 21 after encapsulation (Fig. 5C, green staining).

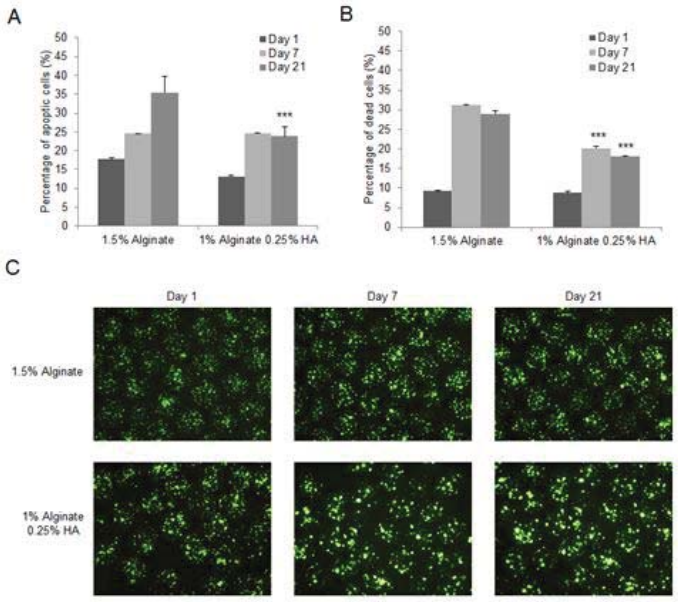

Figure 5.-Viability of D1-MSC-EPO encapsulated in 1% alginate 0.25% HA and 1.5% alginate microcapsules. (A) Early apoptotic cell quantification by means of flow cytometry after annexin/PI staining. (B) Dead cell quantification by means of flow cytometry after calcein/ethidium staining. (C) Micrographs of encapsulated cells after calcein/ethidium staining. Note: *: $p<0.05$ and ***: $p<0.001$. Scale bar represents 200 μm.

Because the main differences in viability were found at day 21 after encapsulation, we proceeded to quantify the metabolic activity at this time point. We quantified metabolic activity by the commercially available CCK8 assay studying

the progression of cell metabolic activity from the first day after encapsulation until day 21. The increment in metabolic activity was 3-fold significantly higher ($p<0.001$) in D1-MSC EPO encapsulated in 1% alginate 0.25% HA capsules than in 1.5% alginate (Fig. 6A). We also analyzed and compared the membrane integrity of the encapsulated D1-MSC EPO in both types of microcapsules determining if there was a correlation with the viability and the metabolic activity detected. By means of the commercial assay Lactic Dehydrogenase based kit, we were able to detect that the percentage of membrane damage was always significantly lower ($p<0.01$ at day1 and $p<0.05$ at day 21) in D1-MSC EPO encapsulated in 1% alginate 0.25% HA capsules than in 1.5% alginate (Fig. 6B). These analyses confirmed that the presence of HA in alginate microcapsules not only improves the viability of the encapsulated D1-MSC EPO but also improves their metabolic activity and the integrity of their membrane.

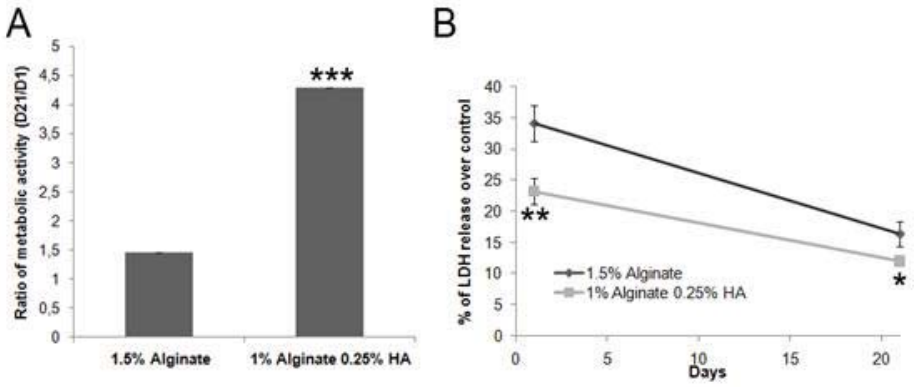

Figure 6.-Metabolic activity and membrane integrity of encapsulated D1-MSC EPO in 1% alginate 0.25% HA and 1.5% alginate microcapsules. (A) Ratio of metabolic activity between day 21 (D21) and day 1 (D1) after encapsulation. (B) Membrane damage at day 1 and 21 after encapsulation. Note: Values represent mean ± SD. *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$.

We also aimed to study the application of the hybrid biomaterial as a sustainable drug delivery system by taking advantage of the ability of D1-MSCs EPO to secrete EPO. Thus, we compared the progression of EPO release by encapsulated D1-MSCs EPO in 1.5% alginate microcapsules and 1% alginate 0.25%

HA microcapsules from the first day after encapsulation until day 21. The hybrid 1% alginate 0.25% HA microcapsules provided 2-fold significant increment ($p < 0.001$) in EPO release than 1.5% alginate microcapsules (Fig. 7A), indicating that the presence of HA, through its influence in the viability, metabolic activity, and membrane integrity, helps to improve the release of a therapeutic protein secreted by encapsulated genetically modified cells, and therefore improves the capacity of alginate microcapsules as a sustainable release system.

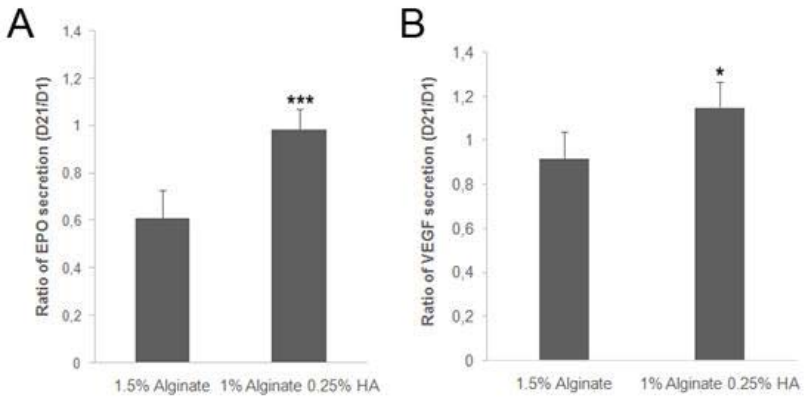

Figure 7.-Release of EPO and VEGF from encapsulated D1-MSC EPO in 1% alginate 0.25% HA and 1.5% alginate microcapsules. (A) The ratio of EPO release between day 21 (D21) and day 1 (D1) after encapsulation. (B) The ratio of VEGF release between day 21 (D21) and day 1 (D1) after encapsulation. Note: Values represent mean ± SD. *: $p < 0.05$ and ***: $p < 0.001$.

The presence of HA in alginate microcapsules could also be influencing the release of endogenous proteins secreted by MSCs. Hence, we compared the progression of VEGF release by encapsulated D1-MSCs EPO in 1.5% alginate microcapsules and 1% alginate 0.25% HA microcapsules from the first day after encapsulation until day 21. Hybrid 1% alginate 0.25% HA microcapsules provided higher VEGF release increment ($p < 0.05$) than 1.5% alginate microcapsules (Fig. 7B). However, this increment was lower than the increment observed in EPO, indicating that the presence of HA in alginate microcapsules influences both the release of transgenic and endogenous proteins in encapsulated MSCs, but this influence is higher on transgenic than endogenous proteins.

Finally, we compared the potential of encapsulated D1-MSCs within 1.5% alginate and 1% alginate 0.25% HA into. three mesoderm lineages: adipogenic, osteogenic and chondrogenic. After culturing encapsulated D1-MSCs in complete mesenchymal stem cell medium for 7 days, attached cells were exposed to differentiation media for 21 days and next stained. The presence of vacuoles characteristic of adipogenic differentiation was detected with no qualitative differences between D1-MSCs from 1.5% alginate and 1% alginate 0.25% HA (Fig. 8A). The calcified deposition identifying osteogenic differentiation was also detected in both types of microcapsules without qualitative differences (Fig. 8B). However, a higher amount of sulfated proteoglycan deposits, indicative of functional chondrocytes, were displayed in 1% alginate 0.25% HA than in 1.5% alginate matrix (Fig. 8C), suggesting that the incorporation of HA in alginate matrixes upgrades D1-MSCs potential for chondrogenic differentiation.

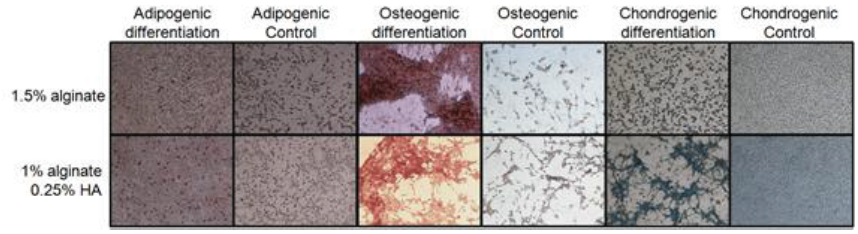

Figure 8. Differentiation potential of encapsulated D1-MSCs EPO in 1% alginate 0.25% HA and 1.5% alginate microcapsules. Microscopic images at 4× amplification 3 weeks after differentiation.

4. DISCUSSION

Cell encapsulation technology allows the continuous release of therapeutic factors avoiding the need of repeated drug administration. It has succeeded in the treatment of several pathologies showing high potential for its clinical application. However, before being translated from bench to bedside, several challenges still need to be overcome, such as the development of a matrix containing proteins from the ECM that mimics a closer natural cell environment and enhance cell survival and functionality. HA is one of the major components of the ECM (24),

representing a good candidate in microencapsulation technology in order to enhance cell signaling, regulation of cell adhesion and proliferation, as well as manipulation of cell differentiation (25). Therefore, we decided to study the incorporation of this macromolecule within alginate microcapsules since alginate has been the most commonly used biomaterial in cell encapsulation (6).

We selected HA with an MW of 1.1 MDa because, at this MW, HA-coated cell cultures show the highest cell adhesion rate, decreasing the cell adhesion and proliferation when MW is increased (25). Thus, we elaborated different solutions by mixing alginate and HA at different proportions to compared their rheological behavior with 1.5% alginate in order to find suitable mixtures that display similar viscosity properties of non-Newtonian fluid like 1.5% alginate. We chose 1% alginate 0.25% HA and 0.5% alginate 0.5% HA due to, on the one hand, their similar viscosity behavior to 1.5% alginate along the frequency range studied and, on the other hand, their ability to form microcapsules by an electrostatic atomization generator. High HA concentrations increased viscosity over 1.5% alginate ratios when mixed with either 1% or 0.5% alginate, maybe due to a higher presence of hydrogen bonding between hydroxyl groups along the chains (34). However, low HA concentrations were not able to increase solutions viscosity to 1.5% alginate values, precluding their use in cell encapsulation. In fact, when alginate increased from 0.5% to 1%, it was enough to add HA at 0.25% instead of 0.5% to reach the same viscosity than 1.5% alginate. We confirmed the presence of HA inside the selected microcapsules by imaging and by quantifying the emitted fluorescence from microcapsules after their performance with HA-FITC. The fluorescence of 0.5% alginate 0.5% HA was significantly higher than the one obtained from 1% alginate 0.25% HA but it did not exactly double, indicating that a release of HA could occur in 0.5% alginate 0.5% HA microcapsules. The breakage during the performance of 0.5% alginate 0.5% HA microcapsules could explain the release and, therefore, a lower HA-FITC intensity than expected.

We detected differences in the uniformity and the cross-linking among

the surface of both HA-alginate combinations and 1.5% alginate after forming the microcapsules. In spite of the observed differences, all samples showed a smooth surface, which has shown to provide better biocompatibility than rough surfaces (35). We next confirmed that surface differences were not reflected in the swelling behavior, indicating that the osmotic resistance of all the microcapsules was enough to preserve them in an *in vivo* environment, avoiding a cell exposure to the host (36). The similarities between the macromolecular structure of alginate and hyaluronate, allowing the formation of an alginate network where hyaluronic maybe accommodated, might explain the same swelling behavior among all microcapsules (37).

After our extensive physicochemical evaluation of the new hybrid microcapsules, we proceeded to encapsulate D1-MSCs EPO to determine the beneficial impact of the presence of HA within alginate microcapsules. The first bottleneck we met was the plugging up of the electrostatic atomization generator when trying to encapsulate D1-MSCs EPO in 0.5% alginate 0.5% HA, that when overcome, generated microcapsules that agglomerated and released cells outside the capsules, precluding future studies. However, 1% alginate 0.25% HA encapsulated MSCs displayed homogenous round microcapsules with a smooth shape. The presence of HA in the aforementioned microcapsules reduced the percentage of apoptotic MSCs overtime compared to 1.5% alginate, which could be mediated by the endocytic internalization of HA through the surfaced receptor CD44 expressed in MSCs, and subsequent protection of DNA from damage (38). In fact, it has been proven that the pretreatment of chondrocytes with hyaluronic acid decreases mitochondrial DNA damage while enhancing DNA repair capacity, cell viability, preservation of ATP levels and amelioration of apoptosis (39). This beneficial effect seems to be mediated by the CD44 receptor, since the anti-CD44 antibody at saturating concentrations abolishes the protective effects of hyaluronan, suggesting that CD44 mediates this mechanism (40). The reduction in apoptotic cells was also reflected in a reduction in cell death in the hybrid microcapsules, showing

a higher number of live cells. HA has shown to directly influence the activation of cell proliferation. High concentration of HA (> 2mg/ml) causes the release of endogenous growth factors, stimulating cell-cell interactions, that results in faster cell proliferation *in vitro* (41). Thus, the growth rate of adipose-derived MSCs shortly supplemented with HA is increased in the culture at early passages, contributing to their lifespan extension, with a marked reduction of cellular senescence and a prolonged differentiation potential (42). Moreover, the presence of HA in the hybrid microcapsules increased the metabolic activity of encapsulated MSCs, similarly to tendon-derived cells exposed to HA (43), while reducing the membrane damage suffered by MSCs the next day after encapsulation (44). All together afford us to conclude that HA protects encapsulated MSCs from the high stress derived from the encapsulation process.

We also aimed to evaluate the controlled released of a therapeutic protein from our hybrid microcapsules, thanks to the ability of the genetically modified MSCs to secrete EPO. Thus, we could quantify that MSCs increase EPO secretion for 21 days when allocated in microcapsules containing HA, similarly to hydrogels formed by 100% HA (45). This increase of transgenic protein secretion was also correlated with an endogenous protein increment, reflecting that the secretion enhancement might be related with the boost of metabolic activity.

Finally, we assessed the MSCs potential to differentiate into adipogenic, osteogenic and chondrogenic lineages shown in 2D (46). It has been previously described the alginate encapsulated MSCs are able to differentiate into the above-mentioned lineages (47) but we have demonstrated that the presence of HA into an alginate matrix promotes the differentiation of MSCs into chondrocytes may be due to an induction of aggrecan and proteoglycan accumulation, nodule formation, and inhibition of TNF-alpha induced inhibition of chondrogenic differentiation (29).

5. CONCLUSIONS

Our work has shown that it is possible to produce hybrid microcapsules of 1% alginate 0.25% HA containing MSCs that retain 1.5% alginate physicochemical

properties while mimicking a natural ECM environment, which helps to enhance the viability and functionality of encapsulated cells. However, future studies need to be performed with the inclusion of other proteins derived from ECM in order to improve improve these beneficial effects, while maintaining the physicochemical properties of the microcapsules.

We can conclude that HA protects MSCs when encapsulated within alginate, providing a niche-like environment and improving the beneficial effects of alginate microcapsules after encapsulated MSCs implantation. Encapsulated MSCs into such bio-artificial niches are protected and remain competent in terms of cell delivery or sustained drug release systems.

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Chapter 4

Hyaluronic acid enhances cell survival of encapsulated insulin-producing cells in alginate-based microcapsules

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Hyaluronic acid enhances cell survival of encapsulated insulin-producing cells in alginate-based microcapsules

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ABSTRACT

Pancreatic islet transplantation has proved to be a promising therapy for T1DM, in spite of the chronic immunosuppression required. Although cell microencapsulation technology represents an alternative to circumvent the immune system rejection of transplanted pancreatic islets, the environment provided by classical alginate microcapsules does not mimic the natural ECM, affecting the islet survival. Since hyaluronic acid, one of the major components of pancreatic ECM, is involved in cell adhesion and viability, we assessed the beneficial outcomes on encapsulated insulin-producing cells by the HA inclusion in alginate matrices. In this manuscript we describe how alginate-HA hybrid microcapsules enhance the viability of encapsulated cells, reducing early apoptosis percentage and decreasing membrane damage. A stable insulin production was maintained in encapsulated cells, not altering the response to a glucose stimulus. Therefore, we can conclude that the inclusion of HA within alginate microcapsules is beneficial for encapsulated insulin-producing cells, representing a step forward in the clinical translation of microcapsules technology for the treatment of T1DM.

Keywords: T1DM; alginate; hyaluronic acid; microencapsulation; insulin-producing cells

1. INTRODUCTION

More than 350 million people in the world are currently affected by diabetes mellitus, becoming as one of the significant threats to human health. Type 1 Diabetes Mellitus (T1DM) is a disease caused by autoimmune destruction of pancreatic β-cells whose most prominent therapy is the exogenous insulin administration through daily injections. However, this treatment is associated with some complications, such as diabetic retinopathy, cardiovascular disease, diabetic nephropathy and lower limb amputations (1). An alternative therapy for T1DM is the transplantation of donor-derived pancreas or pancreatic islets able to restore the physiologic metabolic glucose control in T1DM patients. Nevertheless, islet transplantation entails some issues, such as the source of the islets or their reproducible standard preparation. Donors with more than 50 years old provide more pancreatic islets than younger donors, but with reduced capability to produce insulin (2), while high body mass donors have pancreatic islets with lower insulin secretion ratios (3). Moreover, a short cold ischemic time, the time from tissue extraction from donor to isolation of pancreatic islets, can also increase the transplantation success (4) and it should be standardized. Other issues found in islet transplantation are the low islet survival and lifelong immunosuppression to avoid immune rejection after transplantation (5). During the last decades, cell-based sustainable drug delivery systems for T1DM treatment, such as microcapsules or hydrogels containing insulin-producing cells (IPCs) have been developed as a strategy to overcome the pancreatic islets transplantation issues.

Microcapsules allow the exchange of nutrients, therapeutic factors, and gases through the outer layer, avoiding the entrance of immunoglobulins and the cells recognition by the immune system (6). At present, there are some biomaterials tested as microencapsulation matrices, such as agarose (7), chitosan (8), and hyaluronic acid (9). Among all the biomaterials, alginate is the most commonly used as a matrix for cell encapsulation due to its mechanical properties after microcapsules formation (10). Alginate microcapsules can also be coated by polycations, such as poly-L-Lysine (PLL), poly-D-lysine (PDL), and poly-L-ornithine (PLO) to provide higher

resistance (11), while still allowing the exchange of nutrients and therapeutic factors. Thanks to these properties, cell microencapsulation technology has been successfully applied in T1DM (12). Thus, the first T1DM patient transplanted in a clinical trial with encapsulated cadaveric human islets was able to discontinue all exogenous insulin for nine months (13). In later studies, four T1DM patients transplanted with microencapsulated pancreatic islets significantly reduced their exogenous insulin requirements for up to seven years (14). In a separate trial, alginate microcapsules transplanted into two patients reduced their exogenous insulin requirements but, never attained complete insulin independence (15). Although alginate scaffolds have been considered inert biomaterials to entrap cells (16), they do not provide the cell anchorage required for the survival of most cell types (17).

Currently, an approach to overcome the dying cell number inside the microcapsules is the recreation of the natural extracellular matrix (ECM). The ECM is responsible of both cell-cell and cell-matrix interactions, providing mechanical and physiological support to the cells. It is composed of several molecules including laminins, collagens, elastin or hyaluronic acid among them, with a tissue-specific composition of each component. Consequently, several ECM molecules, such as laminin, collagen I or collagen IV, have been combined with alginate to obtain new encapsulation biomaterials, promoting the viability and decreasing apoptosis of microencapsulated cells (18). Moreover, short synthetic peptides derived from natural ECM proteins, such as the arginine-glycine-aspartic acid peptide (RGD motif), are commonly used to simulate the cell-matrix interactions provided by ECM. Although RGD peptide is widely used due to its simplicity, cost-effectiveness, easy manipulation for functionalization, and low immune response (19), it does not entirely mimic natural ECM signals by providing the required stimulus for a complete reproduction of the cell *in vivo* environment. Hence, new biomaterials need to be studied to completely mimic the natural ECM stimulus within microcapsules environment, improving the encapsulated cell survival.

One biomaterial that could recreate pancreatic cells *in vivo* environment is

hyaluronic acid (HA), a major ECM component on connective, epithelial, and neural tissues, composed by repetitions of a disaccharide unit of N-acetyl-glucosamine and β-glucuronic acid. HA has been described as a mediating molecule involved in cell signaling, regulation of cell adhesion and proliferation or manipulation of cell differentiation studies (20). In pancreatic islets, HA is an abundant component of the mouse peri-islet ECM, synthesized by different islet endocrine cell types under regular conditions (21). HA participates in the maintenance of islets stability and integrity and anti-inflammatory properties (22). However, reactive oxygen and nitrogen species generated during the inflammatory response in tissue inflammation can degrade HA macromolecules, being OH radical one of the most efficient initiators of this degradation (23). The HA fragments accumulated after degradation of high-molar-mass HA, can initiate the induction of pro-inflammatory cytokines IL-6, TNF-α, and IL-1β (24). Moreover, high molecular weight HA acts as a link protein-stabilized complexes with chondroitin sulfate proteoglycans, which are essential in regulating cell processes, such as proliferation (25). HA has shown to be a promising molecule with high possibilities in cell-based therapies applied to T1DM treatment (26, 27) or wound healing (24, 27). Thus, cultured β-cells treated with exogenous high molecular weight HA increase insulin secretion and content (22), decreasing oxidative stress and neutrophil activation in a pancreatitis rat model (28). HA-based hydrogels have been extensively used in tissue engineering applications, embedding some cell types whose ECM contains HA as a major component, such as chondrocytes or cells present in connective tissue (29). Thus, HA provides a native ECM-like microstructure contributing to structural support and protection of embedded cells (30), while promoting cell viability (31). HA is also involved in immune response, reducing the immunogenicity by avoiding adsorption of proteins which recruits immune cells (32). The combination of HA with other biomaterials, such as gelatin (33) or heparin (34), have also shown an enhancement of encapsulated cell viability and functionality compared to their respective single component. Moreover, the combination of HA with PLGA to obtain hybrid biomimetic scaffolds displays an

improvement of cell adherence, increasing the cell viability and biocompatibility (26). In addition, the membranes prepared by a combination of HA, chitosan, and mitochondrial antioxidants protected and enhanced the healing of injured skin, also displaying superior healing properties in injured rabbits and rats *in vivo*. In this manuscript, we have studied for the first time the beneficial *in vitro* outcomes of Ins1E rat cells encapsulation within microcapsules composed by alginate and a high molecular weight HA commonly found in pancreatic islets ECM.

2. MATERIAL AND METHODS

2.1 Materials

Ultrapure low-viscosity (20-200 mPa*s) and high guluronic (LVG) acid alginate (G/M ratio > 1.5) with MW of 75-200 kDa was purchased from FMC Biopolymer. Poly-L-Lysine hydrobromide (PLL, 15-30 kDa) was obtained from Sigma-Aldrich. Clinical grade and free endotoxins 1.1 MDa HA was purchased from Contipro.

2.2. Cell culture

Rat Ins1E cells (35) were cultured in complete medium consisting of RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (Gibco), 1% penicillin/streptomycin/glutamine (Invitrogen), 1% sodium pyruvate 100 mM (Sigma), 1 M HEPES (Lonza), and 0.1% 2-mercaptoethanol (Sigma). Cells were maintained at 37 ºC in humidified 5% CO_2 atmosphere and passaged every 4-5 days.

2.3. Cell microencapsulation and pseudoislets formation

Alginate and alginate-HA were resuspended in 1% mannitol to obtain 1.5% alginate and 1% alginate 0.25% HA mixtures. Final solutions were filtered with a 0.22 μm syringe filter (Millipore, MA, USA). Next, cells were suspended at $5x10^6$ cells/mL in alginate and alginate-HA solutions and extruded in an electrostatic atomization generator (Nisco®). The resulting beads were completely gelled by

agitation for 15 min in a 55 mM $CaCl_2$ solution. Next, the beads were incubated with 0.05% (w/v) PLL in agitation for 5 min, followed by a second coating with 0.1% alginate for 5 min. All the procedures were performed at room temperature, under aseptic conditions, and using the complete medium. Finally, microcapsules were examined under an inverted optical microscopy (Nikon TSM) to monitor the microcapsules morphology and diameter.

Pseudoislets within the microcapsules were formed by incubation of microcapsules containing Ins1E single cells with 1% sodium citrate solution (Sigma-Aldrich) in agitation for 5 min to obtain a liquid core. Next, microcapsules were washed twice in DPBS (Gibco) and incubated with complete medium. Liquefied core microcapsules were maintained at 37 ºC in a humidified 5% CO_2 atmosphere incubator for 10 days until pseudoislets were formed.

2.4. Early apoptosis quantification

The quantification of early apoptosis was assessed by Annexin-V-FITC Apoptosis Detection Kit (Sigma-Aldrich). Early apoptosis of Ins1E encapsulated cells was analyzed at days 1, 7 and 14 after encapsulation. Thus, 200 µL of microcapsules were incubated with 1mg/ml alginate lyase (Sigma Aldrich) for 30 minutes at 37 ºC, centrifuging and rinsing twice with DPBS (Gibco). Next, cells were resuspended in a binding buffer consisting of 10 mM HEPES/NaOH, pH 7.5 containing 0.14 M NaCl, and 2.5 Mm $CaCl_2$. Samples were stained with annexin V-FITC and propidium iodide for 10 min at room temperature and protected from light. Samples stained with only annexin V-FITC, only propidium iodide and unstained were used as controls. Apoptosis was quantified with a BD FACS Calibur flow cytometer and data analyzed with FlowJo LLC software. At least three independent experiments were performed for each solution.

2.5. Quantification and imaging of cell viability

Cell viability of encapsulated Ins1E cells was quantified using LIVE/DEAD

viability/cytotoxicity kit (Invitrogen TM) after 1, 7 and 14 days of encapsulation. Encapsulated cells in 1.5% alginate, 1% alginate 0.25% HA were released from microcapsules by alginate lyase treatment as described above. Cell suspensions were rinsed twice in DPBS (Gibco) and suspended in culture medium with 100 nM calcein AM and 8 nM ethidium homodimer-1. After an incubation of 20 min at room temperature and protected from light, cell viability was quantified with a BD FACS Calibur flow cytometer. Unstained samples or stained only with 100 nM calcein AM or 8 nM ethidium homodimer-1 were used as controls. Data were analyzed with FlowJo LLC software. At least three independent experiments were performed for each solution.

Cell viability of Ins1E microencapsulated cells was also monitored by microscopy imaging. Thus, 25 µL of microcapsules were rinsed twice in DPBS and resuspended in 500 µL of staining solution composed of DPBS supplemented with 0.5 µM calcein AM and 0.5 µM ethidium homodimer-1. Next, the samples were incubated for 45 min at room temperature and protected from light. Finally, samples were imaged under a Nikon TMS fluorescence microscope at the wavelength of excitation 495 nm/emission 515 nm (for calcein AM staining) and excitation 495 nm/emission 635 nm (for ethidium homodimer staining). Random images were analyzed with the Eclipse Net software, version 1.20.0.

2.6. Study of membrane integrity

Membrane integrity of Ins1E microencapsulated cells was determined by the *in vitro* toxicology assay kit Lactic Dehydrogenase based (Sigma-Aldrich) at days 1, 7 and 14 after encapsulation. Thus, 100 µL of microcapsules per sample were rinsed twice with culture medium and resuspended in 1 mL of complete medium. Each sample was plated in two wells of a 24-well-plate and incubated for 90 minutes with 70 µL of lysis buffer in one well and 70 µL of culture medium in the other. Next, 50 µL of supernatant from each well was incubated with the kit cocktail mixture for 30 minutes, at room temperature and protected from light. The color development was

quantified on an infinite M200 TECAN microplate reader at a wavelength of 490 nm, with absorbance reading at 690 nm as background. At least three independent experiments were analyzed for each condition.

2.7. Insulin quantification

Insulin secretion of Ins1E encapsulated cells was quantified from culture supernatants at days 1, 7 and 14 post-encapsulations. Briefly, 200 µL of microcapsules were rinsed twice with culture medium and suspended in 1mL of complete medium. After, a 24 hours incubation at 37 ºC and in a humidified 5% CO_2 atmosphere, supernatants of microcapsules cultures were collected and stored at -80 ºC. The glucose-stimulated insulin secretion (GSIS) was also tested for all the conditions. Thus, 200 µL of microcapsules were rinsed twice in DPBS with calcium and magnesium. Cells were equilibrated for 2 hours in Krebs-Ringer buffer, composed by 129 mM NaCl (Sigma-Aldrich), 5 mM $NaHCO_3$ (Sigma-Aldrich), 4.8 mM KCl (Sigma-Aldrich), 2.5 mM $CaCl_2$ (Sigma-Aldrich), 1.2 mM $MgSO_4$ (Sigma-Aldrich), 1.2 mM KH_2PO_4 (Sigma-Aldrich), 10 mM HEPES (Gibco), and 0.5% w/v bovine serum albumin (Sigma-Aldrich). Next, samples were incubated with Krebs-Ringer buffer supplemented with 3.3 mM glucose for 2 hours, collecting and storing the supernatants at -80 ºC. Then, microcapsules were placed in Krebs-Ringer buffer supplemented with 16.7 mM glucose and, after 2 hours of incubation, supernatants were collected and stored at -80 ºC. The insulin content of supernatants was quantified by Mercodia High Range Rat Insulin ELISA (Mercodia). Three independent samples and controls for each condition were assayed.

2.8. Statistics

Statistical analysis was performed with SPSS software, version 21.00.1. Data were expressed as means ± standard deviation and differences were considered significant for comparison of groups using ANOVA, Tukey's Post Hoc Test when $p < 0.05$. Normality test was performed to confirm a normal distribution.

3. RESULTS AND DISCUSSION

We began encapsulating Ins1E cells within alginate and alginate-HA hybrid microcapsules using electrostatic atomization. We chose a density of 5x106 cells/ mL, a current density used in cell encapsulation. The microcapsules generator was not clogged with both biomaterial compositions, providing spherical microcapsules when observed under an inverted optical microscope. We were able to form beads with a mean diameter of 450 ± 10 μm and a smooth homogeneous surface (Fig 1), below the 600 μm diameter microcapsules have shown unfavorable molecular diffusion kinetics in therapeutic factors release (36). The formed capsules also showed a smooth surface, an important factor since it reduces the foreign body reaction cells recruitment (37), providing higher biocompatibility compared to rough surface microcapsules. After confirming the size and microcapsules surface, we proceeded to monitor the viability of the encapsulated cells analyzing the influence of alginate-HA microcapsules on IPCs apoptosis and viability.

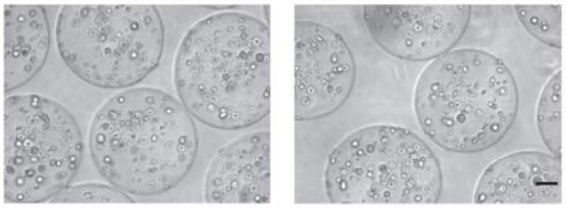

Figure 1.- Microcapsules morphology. Brightfield microscopy micrographs of Ins1E encapsulated cells. Scale bar: 100 μm.

3.1 Hybrid alginate-HA microcapsules enhance encapsulated insulin-producing cells survival

After embedding Ins1E cells into alginate and alginate-HA microcapsules, we quantified the early apoptotic cells percentage inside the microcapsules at days 1, 7 and 14 after encapsulation. The percentage of apoptotic encapsulated Ins1E cells displayed a statistically significant reduction in alginate-HA compared to alginate microcapsules ($p<0.05$) at days 1 and 7 after encapsulation, with a more pronounced

decrease at day 14 ($p<0.01$) (Fig 2A). Previous studies with β-cells cultured on laminin-5 enriched ECM have already shown protection against apoptosis, with a reduction of caspase-8 activity, enhancement of focal adhesion kinase, protein kinase B and extracellular signal-regulated kinase phosphorylation, suggesting that ECM plays an essential role on apoptosis of IPCs (22). Since HA is an extensive component of the ECM from pancreatic islets, it is not surprising that the addition of exogenous HA within the microcapsule's matrix can decrease the apoptosis percentages of the encapsulated cells.

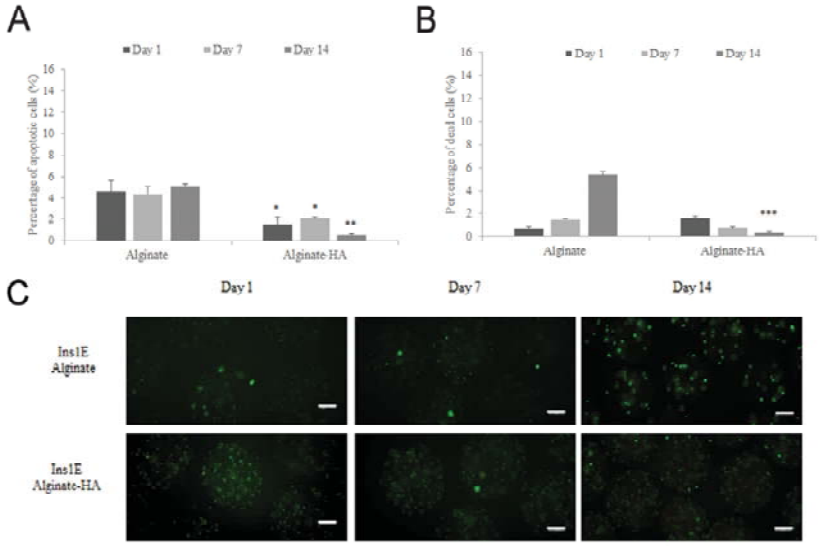

Figure 2.- Viability of Ins1E encapsulated cells. (A) Early apoptotic cell percentage quantification of Ins1E cells by means of flow cytometry after annexin/PI staining. **(B)** Cell death percentage quantification of Ins1E cells by means of flow cytometry after calcein/ethidium staining. **(C)** Fluorescence microscopy micrographs of encapsulated cells after calcein/ethidium staining. Note: Values represent mean ± SD. *: $p<0.05$; **: $p<0.01$ and ***: $p<0.001$. Scale bar: 100 µm.

We also quantified the live/dead cell percentage by flow cytometry to confirm apoptosis results. The next day after encapsulation, encapsulated Ins1E cells displayed no differences of cell death percentage between alginate and alginate-HA microencapsulated cells (Fig 2B). Similarly, no differences of cell death were detected at day 7 between alginate and alginate-HA microcapsules. However, 14 days after encapsulation, encapsulated Ins1E cell death was significantly reduced ($p<0.001$) in

alginate-HA compared to alginate microcapsules, confirming the results observed in the apoptosis quantification at this time point. These results were also corroborated by micrographs obtained after the staining of microencapsulated Ins1E cells at days 1, 7 and 14 after encapsulation (Fig 2C). The improvement observed with encapsulated IPCs viability through the inclusion of HA within alginate microcapsules is closely related to previous studies describing that pancreatic islets or single β-cells exposure to whole ECM or individual ECM components improve β-cell survival (38). These data suggest that specific ECM components support β-cell function and viability. In fact, pancreatic islets embedded within hybrid HA-collagen hydrogels significantly displays a viability enhancement compared to collagen embedded islets or unembedded islets, retaining their morphology and insulin secretion ability, showing also better immunoprotection than alginate hydrogels (39). This beneficial effect of high molecular weight HA on cell viability and apoptosis is mainly mediated by CD44 receptor, since saturating concentrations of anti-CD44 antibody abolish the protective effects of hyaluronan (40). However, it is essential to remark that depending on the molecular weight of HA, the outcomes on cell survival can change. In spite of, under regular conditions, high molecular weight HA displays anti-inflammatory effects, under stress conditions, high molecular weight HA become fragmented, acting as proinflammatory, reducing cell viability, and enhancing cell apoptosis (21).

We also quantified the membrane integrity of encapsulated Ins1E cells in both alginate and alginate-HA microcapsules to provide more useful evidence of HA inclusion within alginate microcapsules. A progressive reduction of membrane damage was quantified from days 1 to 14 post-encapsulation in both microencapsulation matrices, with a statistically significant membrane damage reduction ($p<0.05$) at the three studied time points comparing alginate-HA and alginate microcapsules (Fig 3). These data verified the apoptosis and viability results described above, similarly to previous studies showing better viability and morphological integrity of neonatal rat islets within cuprophane hollow fibers containing HA (41). In fact, the reduction of

membrane damage is widely influenced by the immobilization efficiency provided by HA and its similar dynamic viscosity to natural soft tissues, leading to an enhancement of cell membrane integrity in HA-containing solutions (42). Therefore, we can conclude that HA-containing microcapsules protect encapsulated Ins1E from the high stress derived from the encapsulation process, enhancing cell viability and decreasing cell apoptosis and membrane damage.

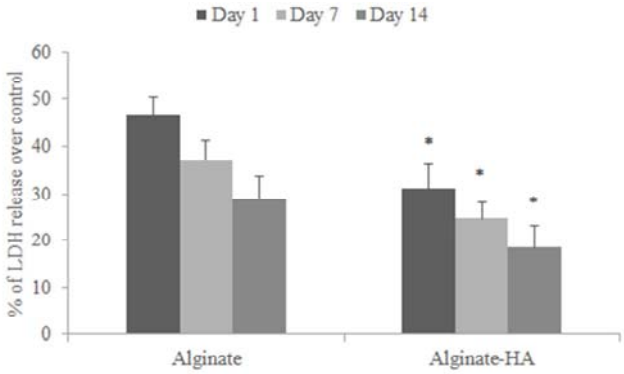

Figure 3.- Membrane integrity of Ins1E encapsulated cells. Cell damage percentage quantification of Ins1E cells by means of the Lactic Dehydrogenase *in vitro* toxicology kit. Note: Values represent mean ± SD. *: $p<0.05$.

3.2. HA does not affect the insulin secretion ability of encapsulated insulin-producing cells

We also tested the ability of encapsulated Ins1E cells to secrete insulin during the same periods studied above when embedded within both biomaterials. Encapsulated Ins1E cells progressively upregulated the secretion of insulin during the considered time points, without statistically significant insulin secretion differences between both biomaterials (Fig 4). It was surprising the lack of differences in insulin release after the different viabilities quantified at both matrices along the studied timepoints, but the presence of HA in the matrices could influence, not only the cell viability but also the release of insulin, may be exerting a compensative effect. Currently, there are controversial data regarding the role of HA in the insulin secretion by pancreatic islets. On the one hand, some authors describe that high molecular weight HA increases the insulin secretion. For example, coating culture

well-plates with high molecular weight HA increases insulin secretion from HIT-T15 cells through the enhancement of connexin 43-mediated gap-junctional intercellular communications (22). On the other hand, other authors describe that the accumulation of HA in autoimmune diabetes leads to fewer compact islets than healthy islets, affecting the insulin production of the mechanosensitive islets (43). In fact, some ECM component interactions, such as integrin-laminin, have shown to be important in regulating insulin release from β-cells, underscoring the importance of ECM components in regulating β-cell function (44), while the role in islet structure and function of other components, such as proteoglycans and hyaluronan, remains unknown. We consider that more exhaustive studies should be performed to clarify the role of HA in the functionality of IPCs, considering factors such as HA concentration or molecular weight.

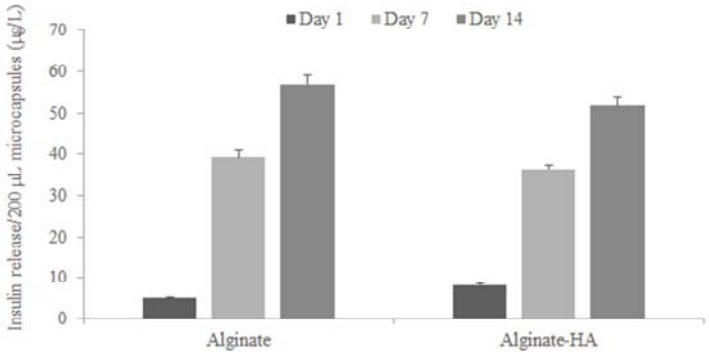

Figure 4.- Insulin release of Ins1E encapsulated cells. Insulin release of Ins1E cells determined by ELISA after 24 hours of complete medium incubation. Note: Values represent mean ± SD.

Finally, we quantified and compared the insulin secretion responding to glucose concentration from Ins1E encapsulated cells in alginate and HA-alginate matrices. Encapsulated cells responded to glucose stimulus by secreting insulin at all the studied time points, without statistically significant differences between both microencapsulation matrices (Fig 5), reflecting again that the inclusion of HA does not affect the functionality of IPCs. Other hybrid biomaterials with HA, such as HA-collagen hydrogels, have also shown to be able to improve *in vitro* viability of

embedded rat islets retaining their glucose sensitivity for 28 days. These encapsulated rat islets administered to the omentum of outbred rats reversed long-term diabetes and prevented graft rejection in all animals for more than 80 weeks without fibrotic overgrowth or cellular rejection (39).

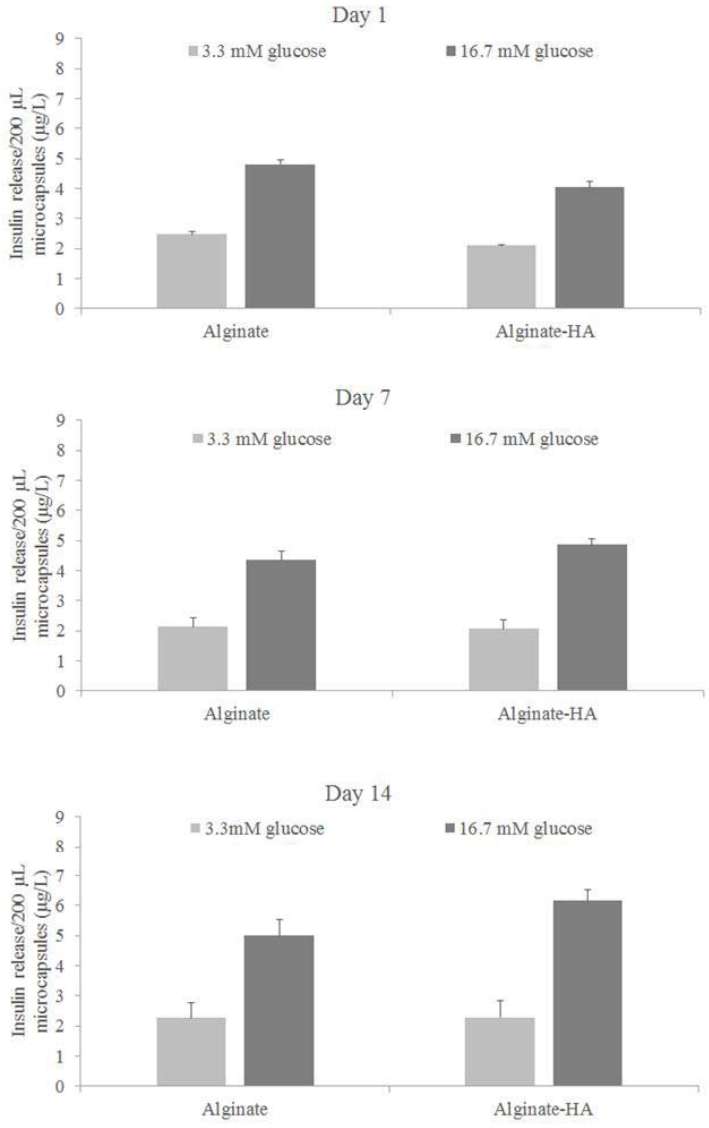

Figure 5.- Insulin release after glucose stimulation of Ins1E encapsulated cells. Insulin release of Ins1E cells determined by ELISA after 2 hours of incubation with 3.3 mM glucose Krebs-Ringer Bicarbonate Buffer and 2 hours of incubation with 16.7 mM glucose Krebs-Ringer Bicarbonate Buffer after 1, 7 and 14 days of encapsulation. Values represent mean ± SD. **: $p<0.01$ and ***: $p < 0.001$.

3.3. Cell viability of encapsulated cell clusters is also enhanced by HA

To confirm the results obtained with single cells on cell cultures with a similar environment than pancreatic islets, we prepared a new batch of microcapsules with a liquefied core to test encapsulated Ins1E cell clusters within alginate-HA microcapsules (Fig 6A). Similar to non-liquefied microcapsules, Ins1E alginate and alginate-HA liquefied microcapsules displayed no statistically significant differences at day 1 and 7 after encapsulation (Fig 6B). However, at day 14, we quantified a statistically significant reduction ($p<0.01$) of alginate-HA encapsulated Ins1E cells compared to alginate microcapsules, showing HA as a specific ECM component to support β-cell viability.

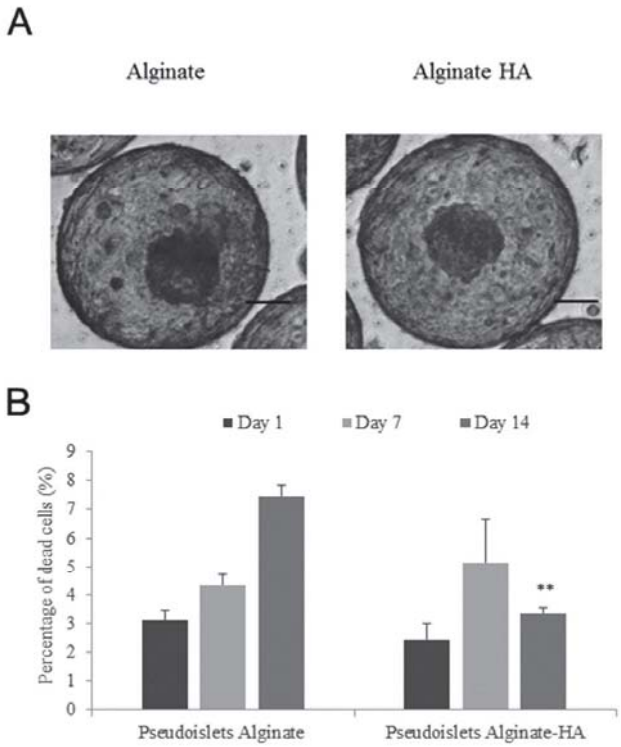

Figure 6.- Viability of Ins1E pseudo-islets formed within liquefied microcapsules. (A) Brightfield micrographs of pseudo-islets formed with Ins1E cells after 14 days. **(B)** Cell death percentage quantification of liquefied microencapsulated Ins1E cells by means of flow cytometry after calcein/ethidium staining. Note: Values represent mean ± SD. **: $p<0.01$ and ***: $p<0.001$. Scale bar: 100 μm.

Likewise, other ECM molecules, such as collagen, have shown beneficial effects on islet cells survival, while reducing necrosis and apoptosis. In fact, the combination of alginate with some proteins, such as collagen IV, fibronectin, and laminin, reestablishes cell-matrix interactions lost during cell isolation, resulting in a cell viability enhancement and postulating those combinations of alginate and ECM molecules as an encapsulation platform for islet cell delivery (45). Moreover, the encapsulation of immunoisolated pancreatic islets with collagen type IV and the laminin sequences RGD and PDSGR, reduces the release of danger-associated molecules and nitric oxide from islets, enhancing the survival of pancreatic islets embedded within alginate-collagen-laminin biomaterials, compared to alginate microcapsules without ECM molecules (46). Poly-lactide-co-glycolide scaffolds modified with collagen IV also improve mouse islet survival, decreasing early-stage apoptosis in islet cells while reducing the restoring time to euglycemia from 17 to 3 days after transplantation in a syngeneic mouse model (47).

4. CONCLUSIONS

Based on the results described in the present manuscript, we can conclude that the inclusion of HA in alginate matrices forming microcapsules provides beneficial effects regarding viability increment, apoptosis reduction, and lower membrane damage to encapsulated insulin-producing cells, while maintaining their insulin secretion ability and glucose responsiveness. Since HA represents one of the major components of the pancreatic ECM, we conclude that mimicking the natural pancreatic ECM can improve islets survival, one of the main current bottlenecks in the cell replenishment therapies proposed for the treatment of T1DM. However, more detailed studies with other pancreatic ECM molecules, such as collagen IV or laminin, should be performed to approach closer bio-artificial matrices to the *in vivo* islet microenvironment.

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Chapter 5

Hyaluronic acid promotes differentiation of mesenchymal stem cells from different sources towards pancreatic progenitors within 3D alginate matrices

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Hyaluronic acid promotes differentiation of mesenchymal stem cells from different sources towards pancreatic progenitors within 3D alginate matrices

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ABSTRACT

Islet transplantation has shown to be a successful alternative in Type 1 Diabetes treatment, but donor scarcity precludes its worldwide clinical translation. Stem cells are an unlimited source that could circumvent the lack of donors if complete differentiation into insulin-producing cells (IPCs) would be solved. We have performed the differentiation of mesenchymal stem cells (MSCs) from different sources into IPCs within 3D alginate matrices. We quantified an increased insulin release at the final stage of differentiation compared to undifferentiated MSCs, more pronounced in IPCs differentiated from pancreatic-derived MSCs tissues. Moreover, the addition of hyaluronic acid (HA) in alginate microcapsules enhanced, even more, the insulin release from the final IPCs, independently of the MSCs source. We can conclude that MSCs can be differentiated into IPCs within alginate microcapsules, enhancing insulin release when HA is present in the 3D alginate matrices.

Keywords: Alginate; microencapsulation; hyaluronic acid; mesenchymal stem cells; T1DM; cell differentiation

1. INTRODUCTION

In the last decades, type 1 diabetes mellitus (T1DM) has become one of the main threats to human health. T1DM is characterized by autoimmune destruction of pancreatic β-cells, resulting in severe insulin deficiency after an asymptomatic period over the years (1). Nowadays, the daily insulin injections are the most commonly used treatment for T1DM but, there are still other significant complications associated with failures in glucose metabolism control, such as nephropathy, renal failure, neuropathy, retinopathy, damaged vessels and limb amputation (2). All these complications related to the hyperglycemia episodes in diabetic patients, decrease the diabetic lifespan 1 to 13 years compared with the healthy population (3). In spite of insulin and its analogs administration can also attenuate diabetes and increase the life expectancy of patients, they cannot be considered a cure for TIDM.

To avoid the issues related with daily insulin injections, other research groups have focused on healing T1DM with β-pancreatic cell replenishment, either by whole vascularized pancreas transplantation or by islet transplantation. Whole pancreas transplantation is a major intraabdominal surgical procedure used for pancreas replacement, including immunosuppression post-implantation for life. However, although the whole pancreas transplantation has demonstrated to be a feasible β-cell replenishment option, the exocrine tissue transplanted contributes to the risk of infection, graft thrombosis and pancreatitis (4). The transplantation of islets of Langerhans provides similar results and minor surgery than whole pancreas transplantation, decreasing the risk for the patients. In 2000, the Edmonton Protocol, one of the most relevant advances for pancreatic islets transplantation (5), displayed the highest ratio of insulin independence in T1DM patients: 5 years after pancreatic islets transplantation. T1DM patients showed insulin dependence after 3 to 5 years post-implantation, exhibiting that islet transplantation cannot maintain the insulin independence permanently. Currently, several research groups are still working to improve the parameters that will help to success islet transplantation and extended maintenance of insulin independence, but limitations such as donor scarcity remain

unresolved (6).

The differentiation of stem cells, an unlimited cell source, represent an alternative for β-pancreatic cell replenishment. On this regard, mesenchymal stem cells (MSCs) have the ability to differentiate into many cell types of mammalian organisms, insulin-producing cells (IPCs) among them, without the risk of teratoma formations characteristic of the intrinsic pluripotency from other stem cell types, such as embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). Although MSCs are commonly differentiated into three mesodermal lineages, adipocytes, osteoblasts, and chondrocytes, they can also be differentiated into some endodermal and ectodermal lineages, such as lung epithelial cells (7), hepatocytes (8) and insulin-producing cells (9). MSCs derived from different tissues, such as bone marrow, umbilical cord or fat, have been used to produce IPCs. For example, bone marrow-derived MSCs were differentiated following a 3-stages protocol during 18 days, expressing at the final stage of differentiation, Pdx1, insulin, and glucagon and secreting insulin in response to different concentrations of glucose (10). However, these IPCs differentiated from bone marrow-derived MSCs only responded to glucose challenge for 2 weeks. Following a differentiation protocol based on stepwise culture conditions described for human ESCs, umbilical cord-derived MSCs have also been differentiated into IPCs, leading to C-peptide expression in transplanted mice for 3 weeks (11). Adipose-derived MSCs have also revealed high proliferation and differentiation capacities. For example, a 21 days protocol to differentiate adipose-derived stromal cells into IPCs revealed typical islet-like cell clusters with positive DTZ staining, expressing Pdx1 and Glt2 and secreting insulin after glucose challenge (12). However, despite the promising results regarding gene profile expression and insulin production, the insulin secretion was far lower than normal human pancreatic islet cells without maintenance of a sustainable release of insulin. On this regard, IPCs differentiated from bone marrow MSCs seeded in fibrin glue 3D scaffolds, a material used in a variety of clinical applications and sustained release of factors, release 3-fold more insulin than those IPCs differentiated in monolayer.

Moreover, 3D cultures showed approximately 3.5-fold more insulin-positive cells than 2D cell cultures (13). Similarly, adipose-derived MSCs within collagen and hyaluronic acid (HA) 3D scaffolds were differentiated into IPCs, showing that the insulin release from 3D IPCs was nearly 4-fold higher than 2D cultures of adipose-derived IPCs (14). However, MSCs have not been differentiated into IPCs within alginate-HA microcapsules yet.

Cell microencapsulation represents a 3D culture system that allows the sustainable drug release of therapeutic proteins (15), such as insulin, while protecting cells inside the microcapsules core from the immune system after transplantation. Microcapsules ultra-structure allows oxygen and nutrients flow into the microcapsules core, while therapeutic proteins and waste are released outside of the microcapsules. Although the mechanical properties of alginate make them as the most commonly used microencapsulation biomaterial (16), other biocompatible materials have been used as microencapsulation biomaterials, such as agarose (17), chitosan (18), and HA (19). Microcapsules can also be covered with polycations, such as poly-L-Lysine, poly-D-lysine, and poly-L-ornithine, providing higher resistance to degradation (20). Microcapsules technology has been successfully applied to diabetes treatment, restoring normoglycemia by transplantation of alginate microencapsulated pancreatic islets (21), and could represent an excellent platform for the differentiation of MSCs into IPCs. However, in spite of alginate cell microencapsulation properties, this biomaterial does not provide an adequate environment for the cells, without mimicking the extracellular matrix (ECM) stimulus. Thus, the combination of alginate with ECM components, such as laminin, collagen I or collagen IV, have demonstrated to enhance the cell viability of encapsulated cells (22). A major component of the natural ECM is HA, an anionic non-sulfated glycosaminoglycan molecule with variable molecular weights, composed by repetitions of a disaccharide unit of an N-acetyl-glucosamine and a β-glucuronic acid. HA-based scaffolds in tissue engineering can enhance the viability of encapsulated cells (23). In previous studies, we demonstrated that hybrid alginate-HA microcapsules provide a niche-

like environment for encapsulated MSCs, increasing their viability and reducing apoptosis while remaining them competent as a sustainable drug delivery system (24). But, HA is also involved in cell differentiation, such as osteocytes, through cell-signaling or cell adhesion regulation (25). Moreover, the high molecular weight HA, typically expressed in pancreatic islets and, usually located in peri-islet and intra-islet regions adjacent to microvessels, shows anti-inflammatory properties (26) and can turn out to proinflammatory after fragmentation under injury conditions (27).

With this background in mind, we were encouraged to perform a 3D differentiation of MSCs into IPCs by following a 3-step differentiation protocol of different tissue-derived MSCs within alginate microcapsules, trying to improve the differentiation with microcapsules composed of alginate and high molecular weight HA. We postulate that hybrid alginate-HA microcapsules will promote proliferation and differentiation of MSCs into IPCs, by recreating the natural ECM environment of pancreatic islets.

2. MATERIAL AND METHODS

2.1. Materials

Ultra-pure low-viscosity (20-200 mPa*s) and high guluronic (LVG) acid alginate (G/M ratio > 1.5) with MW of 75-200 kDa was purchased from FMC Biopolymer (Norway). Poly-L-Lysine hydrobromide (PLL, 15-30 kDa) was obtained from Sigma-Aldrich (United States). Hyaluronic acid with an MW 1.1 MDa was purchased from Contipro (Czech Republic).

2.2. Cells isolation and culture

D1-MSCs were purchased from ATCC (United States). Bone and pancreatic MSCs were isolated from the femur and the pancreas of four-week-old-Balb/C mice respectively by the procedure approved by the institutional ethical committee (CEBA/272/2012/ORIVE ARROYO). Briefly, mice were euthanized by CO_2

asphyxiation, next extracting hind bones and pancreas. For bone MSCs, bone marrow was flushed with a 25G needle in complete medium consisting on Dulbecco's modified Eagles' medium (Gibco, United States) supplemented with 10% fetal bovine serum (FBS) (Gibco, United States) and 1% penicillin/streptomycin solution (Gibco, United States). Single cell suspensions were prepared by triturating the bone marrow through a 40 μm strainer. The filtered single cell suspension was next plated in culture flasks with complete medium and cultured at 37 ºC in humidified 5% CO_2 atmosphere. Regarding the pancreatic MSCs, the pancreas was digested by collagenase-P for 2 hours at 37 ºC. The enzymatic digestion was accompanied of mechanical disaggregation, obtaining a cell suspension. This cell suspension was filtered through a 40 μm strainer, culturing the single cell suspension in culture flasks with complete medium at 37 ºC in humidified 5% CO_2 atmosphere. Bone and pancreatic MSCs cultures were passaged every 4-5 days, performing all the studies described in this paper with cell cultures passaged at least three times.

2.3. MSCs characterization

The phenotype of MSCs was characterized by flow cytometry. Briefly, single cell suspension from each cell culture was stained with anti-CD16/32 antibody to block Fc-γ II/III receptors. After blockage, cells were stained for 15 minutes at 4 ºC with the following antibodies: Alexa 488 conjugated anti-CD105, PE conjugated anti-CD73, APC-Cy7 conjugated anti-CD90, APC conjugated anti-CD34, PE-Cy7 conjugated anti-CD45, Alexa 488 conjugated anti-I-A/I-E, APC conjugated anti-CD11b, PE conjugated anti-CD19, Alexa 700 anti-CD44, PE-Cy7 conjugated anti-CD146, and APC conjugated anti-SCAI (Biolegend, United States). For isotype controls, cells were stained for 15 minutes at 4 ºC with the following antibodies: Alexa Fluor 488 Rat IgG2a, κ Isotype Ctrl (RTK2758), PE Rat IgG1, κ Isotype Ctrl (RTK2071), APC/Cy7 Rat IgG2b, κ Isotype Ctrl (RTK4530), APC Armenian Hamster IgG Isotype Ctrl (HTK888), PE/Cy7 Rat IgG2b, κ Isotype Ctrl (RTK4530), APC Rat IgG2b, κ Isotype Ctrl (RTK4530), PE Rat IgG2a, κ Isotype Ctrl (RTK2758),

Alexa Fluor® 700 Rat IgG2b, κ Isotype Ctrl (RTK4530), PE/Cy7 Rat IgG2a, κ Isotype Ctrl (RTK2758), and APC Rat IgG2a, κ Isotype Ctrl (RTK2758) (Biolegend, United States). Next, cells were rinsed 3 times with PBS and resuspended in culture medium with DAPI at 6 µM. 10^6 cells were stained for analysis. Minus one-isotype controls were used for each fluorochrome were used to determine the appropriate gating. Unstained cells were used to evaluate cell autofluorescence. Flow cytometer was controlled by Macs Quant Analyzer 10 software (Miltenyi Biotec, Germany), compensating the experiments by single staining of the samples with the appropriate antibody-fluorochrome combination. At least, three independent samples were analyzed by flow cytometry. The analysis was further performed with FlowJo LLC software.

The ability of MSCs to differentiate into osteocytes and adipocytes was assessed by seeding 150.000 cells into 6-well plates with complete medium, allowing to reach 80% of confluence. For differentiation into adipocytes, complete medium was replaced with the following medium: high glucose-DMEM (Gibco, United States) supplemented with 10% FBS (Gibco, United States), 1% penicillin/streptomycin solution (Gibco, United States), 0.5 µM dexamethasone (Sigma-Aldrich, United States), 0.5 µM isobutyl-methylxanthine (Sigma-Aldrich, United States), and 50 µM indomethacin (Sigma-Aldrich, United States). For differentiation into osteocytes, complete medium was replaced with the following medium: high glucose-DMEM (Gibco, United States) supplemented with 10% FBS (Gibco, United States), 1% penicillin/streptomycin solution (Gibco, United States), 100 nM dexamethasone (Sigma-Aldrich, United States), 20 nm β-glycerophosphate (Sigma-Aldrich, United States) and 0.5 µM L-ascorbic acid (Sigma-Aldrich, United States), and 50 µM indomethacin (Sigma-Aldrich, United States). Both differentiations were incubated for 21 days at 37 ºC in 5% CO_2 humidified atmosphere, changing differentiation media every 3 days. Cultured cells in complete medium were also incubated for 21 days as undifferentiated controls. After 21 days adipocytes and osteocytes, as well as controls, were fixed with 10% formalin (Sigma-Aldrich, United

States) and next stained for 1 hour with oil red-C (Sigma-Aldrich, United States) or alizarin red-S (Sigma-Aldrich, United States) respectively. All the staining was rinsed with deionized water until no background was detected. For differentiation to chondrocytes, 400.000 cells were spin down at the bottom of a 15 mL conical tube and cultured at 37 ºC in 5% CO_2 humidified atmosphere for 21 days with the following medium: high glucose-DMEM (Gibco, United States) supplemented with 10% FBS (Gibco, United States), 1% penicillin/streptomycin solution (Gibco, United States), 10 ng/mL TGF-β1 (Sigma-Aldrich, United States), 50 nM L-ascorbic acid (Sigma-Aldrich, United States), and 6.25 µg/ml bovine insulin (Sigma-Aldrich, United States). The medium was changed every 3 days, culturing undifferentiated controls with complete medium. After 21 days of culture, pellets were stained with alcian blue (Sigma-Aldrich, United States) for 30 minutes, and next, rinsed with 0.1% hydrochloric acid until no background was detected. Representative micrographs from each differentiation were acquired with Nikon Eclipse TE2000-S confocal microscope. Differentiations were performed from three independent samples.

For colony forming unit (CFU) analysis, 100 cells were seeded into 60 mm Petri plates (Corning, United States), incubating with complete medium for 14 days at 37 ºC in 5% CO_2 humidified atmosphere. After 14 days, colonies were stained with 3% violet crystal dissolved in methanol for 10 minutes. Next, plates were rinsed with water until only colonies were visualized. The number of colonies was counted, and the percentage of CFU was calculated with the following equation:

$$\frac{Number\ of\ colonies}{Number\ of\ seeded\ cells} \times 100 = \%\ CFU$$

2.4. Cell microencapsulation

Cells suspended in 1.5% alginate (FMC Biopolymer, Norway) or 1% alginate 0.25% HA (1–1.25 MDa, Contipro, Czech Republic) solutions at a density of $5x10^6$ cells/mL were extruded in an electrostatic atomization generator (Nisco®, Switzerland) and, the resulting microcapsules were completely gelled by agitation

for 10 min in a 55 mM $CaCl_2$ solution. Next, microcapsules were ionically linked with 0.05% (w/v) PLL for 5 min, followed by a second coating with 0.1% alginate for another 5 min. All the procedure was performed at room temperature, under aseptic conditions and using the complete medium. The morphology and diameter of the microcapsules were assessed under an inverted optical microscope (Nikon TSM, Japan).

2.5. Differentiation of MSCs into IPCs

Next day after encapsulation, the complete medium from microencapsulated MSCs cultures was removed, and replaced by stage 1 differentiation medium, consisting on 4.5 mg/mL glucose DMEM (Gibco, United States) supplemented with 10% FBS (Gibco, United States), and 1 μM retinoic acid (Sigma-Aldrich, United States). Microcapsules were incubated at 37 ºC in 5% CO_2 humidified atmosphere for 3 days. Next, microcapsules were rinsed with DPBS containing calcium and magnesium, finally adding stage 2 differentiation medium, composed by 1 mg/mL glucose DMEM (Gibco, United States) supplemented with 1% N2 (Gibco, United States), 1% B27 (Gibco, United States), 10 mM nicotinamide (Sigma-Aldrich, United States), 10 ng/mL epidermal growth factor (Sigma-Aldrich, United States), and 2 nM activin A (Sigma-Aldrich, United States). This medium was changed every two days, replacing with stage 3 differentiation medium after 6 days. Microcapsules were cultured during 4 days in stage 3 differentiation medium, consisted on 1 mg/mL glucose DMEM (Gibco, United States) supplemented with 1% N2 supplement (Gibco, United States), 1% B27 supplement (Gibco, United States), 10 mM nicotinamide (Sigma-Aldrich, United States), 10 ng/mL epidermal growth factor (Sigma-Aldrich, United States), 2 nM activin A (Sigma-Aldrich, United States), and 10 nM exendin-4 (Sigma-Aldrich, United States). This medium was changed every two days.

2.6. Metabolic activity

The metabolic activity was determined by using Cell Counting Kit-8 (CCK-8) (Sigma-Aldrich, United States) at day 13 of differentiation protocol and following manufacturer recommendations. For the CCK8 assay, 25 µL of microcapsules were rinsed, resuspended with 500 µL of culture medium and plated in 5 wells of a 96-well plate. Next, 10 µL of CCK-8 reagent was added to each well, and plates were incubated for 4 hours at 37 °C. Absorbance was read out on an Infinite M200 TECAN plate reader at 450 nm and corrected at 650 nm. Three independent tests were analyzed for each condition.

2.7. Cell viability

The cell viability was assessed by microscopy imaging of microcapsules at end-point of differentiation protocol. For microscopy, 25 µL of microcapsules were dosed and rinsed twice in DPBS (Gibco, United States), resuspending in 500 µL of 0.5 µM calcein-AM (Life Technologies, United States) and 0.5 µM ethidium homodimer-1 (Life Technologies, United States) diluted in DPBS. Solutions were placed in a 96-well plate and incubated at room temperature protected from light for 45 minutes. Samples from three independent experiments were observed under a Nikon Eclipse TE2000-S confocal microscope at the wavelength of excitation 495 nm / emission 515 nm (for calcein AM staining) and excitation 495 nm / emission 635 nm (for ethidium homodimer staining). Random images were analyzed with the NIS Elements AR software, version 4.51.00.

2.8. Semiquantitative gene expression analysis

Samples were collected at the end of each stage of differentiation. Cells were de-encapsulated by incubating 500 µL of microcapsules with 1 mg/ml alginate lyase (Sigma Aldrich, United States) for 30 minutes at 37 ºC. The lysates were spun down, rinsed twice with DPBS and resuspended in 1% sodium citrate for 15 minutes at room temperature. After completed lysis of microcapsules, they were spun down

for 5 minutes, and pellets were resuspended in the RLT buffer from the RNeasy Mini Kit (Qiagen, Germany), storing them at -80 ºC until RNA extraction. RNA was extracted following the manufacturer recommendations from the RNeasy Mini Kit (Qiagen, Germany). After extraction, total extracted RNA was quantified with a SimpliNano nanodrop (GE Healthcare Life Sciences, Iceland) and stored at -80 ºC. For cDNA synthesis, 500 ng of total RNA from each sample were incubated with 100 µM of oligo-dT18 for 5 minutes at 70 ºC, cooling the RNA mixture at 4 ºC. Subsequently, the RNA was retrotranscribed with 200 units of Linus® 55-scriptase (CMB-Bioline, Spain) in a solution containing 20 units of RNase inhibitor, 0.2 µM dNTPs and 1x Linus® 55-scriptase buffer for 60 minutes at 55 ºC, terminating by a 5 minutes incubation at 95 ºC. Negative samples without RNA and RNA positive samples extracted from the whole pancreas were run alongside each retrotranscription reaction.

All cDNAs obtained from each retrotranscription reaction were amplified with 1 unit of KAPA2G robust DNA polymerase, 1X KAPA enhancer, 1.5 mM MgCl2, 1X KAPA 2G buffer B and the specific pair of primers for the gene of interest (Table 1) at a final concentration of 1 µM/primer. PCR consisted of an initial denaturation step at 95 ºC for 30 seconds, followed by 40 cycles of amplification (30 seconds at 94 ºC, 30 seconds at 60 ºC, and 30 seconds at 72 ºC) and a final cooling step at 4 ºC. All these reactions were run on a Bio-rad T100 PCR thermocycler (BioRad, United States). The amplified cDNAs were loaded in 1.5% agarose gels and ran in parallel to a 2-log ladder by electrophoresis, confirming the expected size of the amplicons. The intensity of the bands was quantified by ChemiDoc™ MP Imaging System (BioRad, United States). All genes, including the β-tubulin housekeeping gene, were analyzed in triplicate for each sample, as well as, the negative controls. Each cDNA amplification was repeated in three independent differentiation experiments. The quotient normalized the relative semi-quantification of gene expression between the band intensity for each sample and the housekeeping control.

2.9. Insulin release quantification

Two hundred microliters of encapsulated differentiated cells at the end of stage 3, as well as, encapsulated but not differentiated cells, were rinsed twice in DPBS with calcium and magnesium. Rinsing was followed by incubation of differentiated and undifferentiated microcapsules for 2 hours in Krebs-Ringer buffer, composed by 129 mM NaCl (Sigma-Aldrich, United States), 5 mM $NaHCO_3$ (Sigma-Aldrich, United States), 4.8 mM KCl (Sigma-Aldrich, United States), 2.5 mM $CaCl_2$ (Sigma-Aldrich, United States), 1.2 mM $MgSO_4$ (Sigma-Aldrich, United States), 1.2 mM KH_2PO_4 (Sigma-Aldrich, United States), 10 mM HEPES (Gibco, United States), and 0.5% w/v bovine serum albumin (Sigma-Aldrich, United States) to allow the release of stored insulin inside the cells. After the equilibration step, differentiated and undifferentiated microcapsules were incubated for 2 hours with the same culture medium. The supernatants of this incubation were stored at -80 oC for insulin quantification. After collecting supernatants, cells were de-encapsulated following the procedure above. Total protein was extracted from de-encapsulated cells with the Mammalian Cell Lysis Kit (Sigma-Aldrich, United States) following the manufacturer recommendations. Insulin was quantified from the stored supernatants with Mercodia Mouse Insulin ELISA Kit (Mercodia, Sweden), while total protein was quantified with the Pierce™ BCA Protein Assay Kit (Thermo Fisher, United States) following the manufacturer recommendations.

2.10. Statistics

Statistical analysis was performed with SPSS software, version 21.00.1. Data were expressed as means ± standard deviation and differences were considered significant for comparison of groups using ANOVA, Tukey's Post Hoc Test when $p < 0.05$. Normality tests were performed to confirm a normal distribution.

3. RESULTS AND DISCUSSION

3.1. MSCs isolation and characterization

To perform this work, we isolated MSCs from two different sources, bone marrow, and pancreas, with the goal of comparing the differentiation potential enhancement into IPCs by hybrid alginate-HA microcapsules in both cell types. After isolating bone and pancreatic cells, we characterized their phenotype by flow cytometry (28). The cell line D1-MSCs was also studied as control. Positive staining was detected for CD73 and SCAI markers in both cell types, as well as, in D1-MSCs (Fig 1). The positive staining by CD73, combined with other markers, is considered one of the minimal criteria defined by International Society for Cellular Therapy (ISCT) for MSCs (28), being SCAI another positive marker used for isolating murine MSCs (29). However, when CD105 was analyzed differences in expression were quantified among the cell types studied. While bone-derived MSCs and the cell line D1-MSCs did not express CD105, pancreas-derived MSCs resulted positive for CD105 (Fig 1). The lack of CD105 expression in bone marrow-derived MSCs and D1-MSCs is consistent since they have been isolated from the same tissue. Although ISCT has established multipotent MSCs as CD105+ (30), data suggest that CD105- cells represent a subpopulation of MSCs. In fact, when CD105- cells have been differentiated into the three mesodermal lineages, the capacity of adipogenic and osteogenic differentiation is enhanced, while chondrogenic differentiation is not affected compared to CD105+ cells (31), confirming that CD105- cells do not represent differentiated cells, but rather a distinct MSCs subpopulation.

CD44 marker was highly expressed in D1-MSCs and pancreas-derived cells, with lower expression in bone-derived cells. Although ISCT defines MSCs as CD44+, highly proliferative bone marrow-MSCs without CD44 expression have exhibited mesenchymal-like multilineage differentiation capabilities (32), confirming the mesenchymal nature of our isolated cells. In contrast, bone-derived cells expressed CD146, while D1-MSCs and pancreas-derived cells did not. CD146, usually expressed by vascular endothelium and smooth muscle cells (33), can show

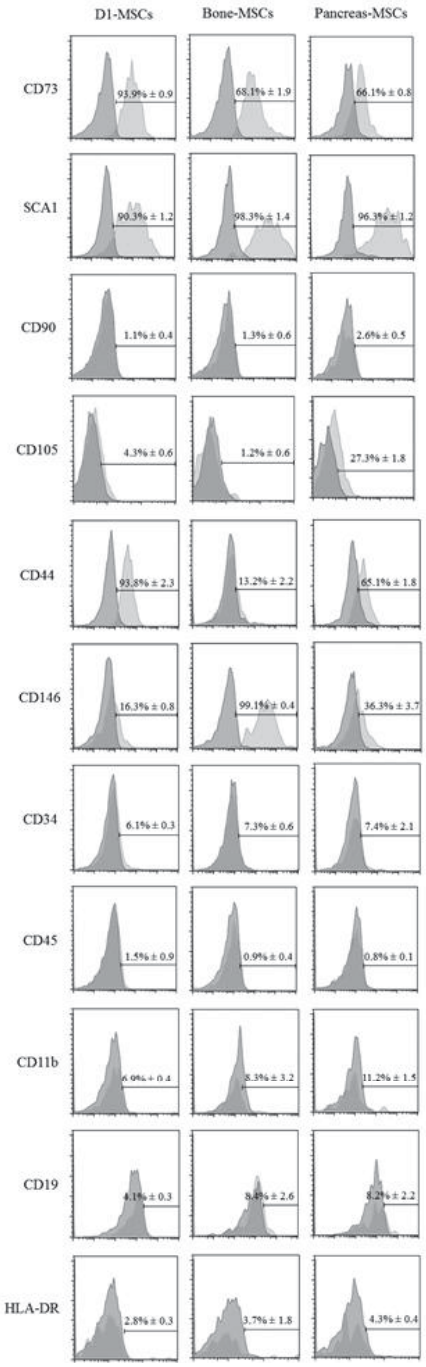

Figure 1.- Phenotype characterization of D1-MSCs, bone-derived cells, and pancreas-derived cells by flow cytometry. Dark and light histograms show isotype control and specific antibody stained sample respectively. Values represent mean ± SD for the positive stained cell population.

different expression in MSCs populations, but CD146+ or CD146- MSCs do not show differences in cell expansion, proliferative capacity, differentiation potential or CFU formation (34). Finally, and as defined by the ISCT (28), all the cell types did not express CD34, CD45, CD11b, CD19 or HLA-DR surface molecules.

ISCT also define multipotent differentiation potential as another minimum criterion for murine MSCs populations. So, we characterized both cell types, as well as the D1-MSCs control, regarding cell differentiation potential. The staining of the differentiated cells showed fat vacuoles after adipogenic differentiation in the three cell sources (Fig 2A). The presence of a calcified extracellular matrix after osteogenic differentiation was also detected for each cell type without qualitative differences among them (Fig 2A). Finally, sulfated proteoglycan deposits, indicative of functional chondrocytes, were visualized in the 3D aggregates from each cell type (Fig 2A). Altogether, the potential to differentiate into the three mesoderm lineages from both isolated cells and D1 pointed them as MSCs (34). Moreover, the adherence to plastic observed in all the cultures corroborated the third criterion proposed by ISCT to define MSCs (28).

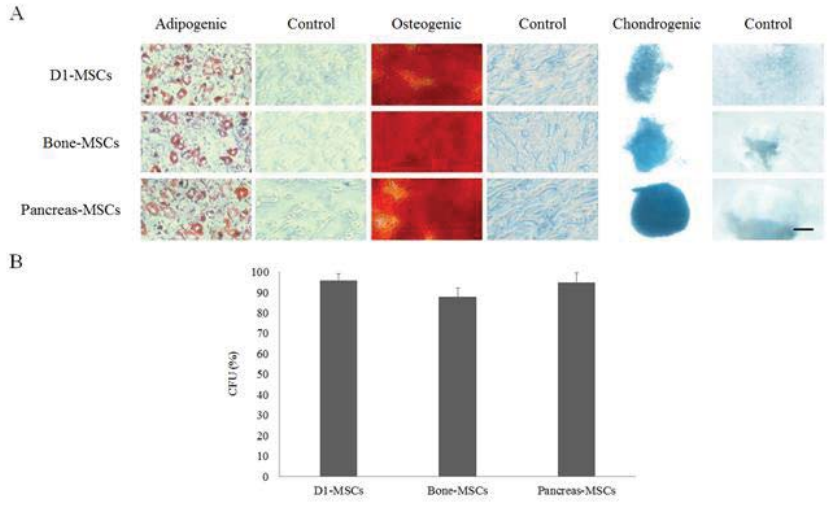

Figure 2.- Differentiation and colony forming units potential of D1-MSCs, bone-derived cells, and pancreas-derived cells. (A) Micrographs after 3 weeks of adipogenic, osteogenic and chondrogenic differentiation. **(B)** Colony forming units percentage quantification. Scale bar: 10 µm. Values represent mean ± SD.

We also tested the colony forming units (CFUs) ability from our cultures since the efficiency to form CFUs remains an essential assay for MSCs characterization (35). The three cell types displayed CFUs values upper than 95% (Fig 2B), confirming that isolated primary cells and D1 cells maintain the clonogenic ability and can be considered MSCs (36).

3.2. Hybrid alginate-HA matrix promotes the differentiation of MSCs into IPCs

After ensuring the MSCs nature of the isolated primary cells, we proceeded to evaluate their capability to differentiate into pancreatic progenitors, comparing two 3D matrices: 1.5% alginate and 1% alginate 0.25% HA. Thus, we encapsulated the different MSCs sources within both matrices, forming microcapsules with a mean diameter of 450±10 μm and a smooth, homogeneous surface, as described in our previous work related to the biomaterials characterization (37). Then, microencapsulated cells were differentiated following a described stepwise protocol for MSCs differentiation into IPCs with promising results (38).

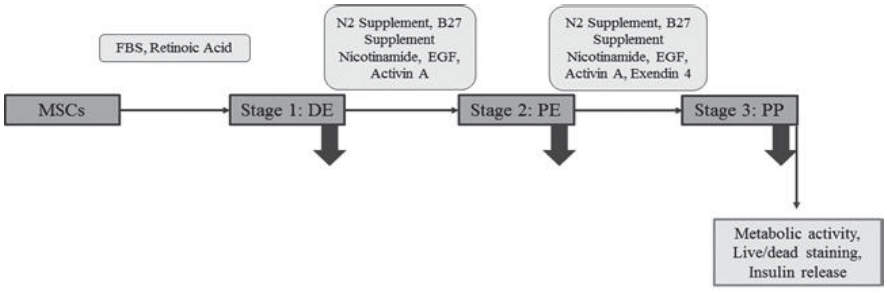

Figure 3.- Graphical abstract of differentiation protocol. MSCs: mesenchymal stem cells; DE: definitive endoderm; PE: pancreatic endoderm; PP: pancreatic progenitor. Arrows indicate that a sample aliquot was analyzed for genes expression. At the final stage a live/dead staining, metabolic activity, and insulin quantification were performed

In the first step of the protocol, retinoic acid and FBS were added to induce the differentiation into definitive endoderm (Fig 3). Next, in combination with activin A and epidermal growth factor (EGF), nicotinamide was added in the second step to effectively induce differentiation and prevent differentiated cells from dying

(39). Finally, in the third step, in combination with nicotinamide, the addition of exendin-4, a factor that potentially exhibits glucoregulatory activities and stimulates both β-cell replication and neogenesis from ductal progenitor cells while inhibiting apoptosis of β-cells (40), promoted further differentiation and maturation of cells into β-cells. The gene expression at all the steps of differentiation was monitored, also assessing the viability and metabolic activity, as well as the insulin release at the final stage of differentiation (Fig 3).

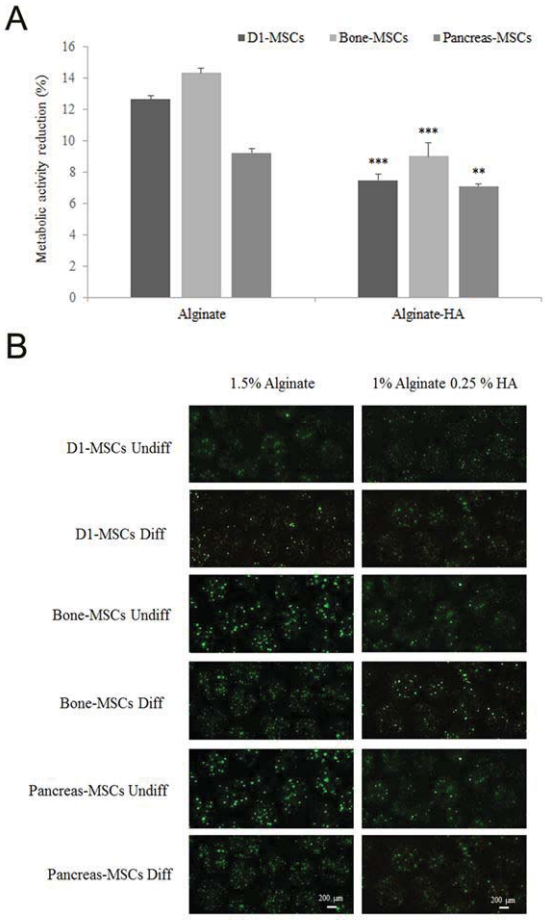

Figure 4.- Metabolic activity and viability of differentiated MSCs. (A) Metabolic activity ratio between final stage-differentiated and undifferentiated 1.5% alginate and 1% alginate 0.25% HA microcapsules cultures. Note: ***: $p<0.001$ and **: $p<0.01$ **(B)** Micrographs of differentiated cells after calcein/ethidium staining. Scale bar: 200 µm.

We quantified and compared the metabolic activity of encapsulated undifferentiated and differentiated samples. Comparing undifferentiated and differentiated samples, a reduction in metabolic activity was quantified in both 3D matrixes differentiation procedures (Fig 4A), may be caused by the dramatic cell losses during definitive endoderm induction as well as in the final differentiation into pancreatic progenitors (41). It has been described that the metabolic activity of MSCs enclosed within alginate-RGD is reduced during osteogenic differentiation (42). Although β-cells are notorious for their high metabolic activities, we could not detect this cell metabolic activity enhancement because of the dramatic reduction of cell population during differentiation. Interestingly, the reduction detected was more pronounced ($p<0.01$) in alginate microcapsules compared to HA-alginate hybrid microcapsules, indicating a protective effect of HA of encapsulated differentiated cells (Fig 4A). Micrographs after calcein/ethidium staining confirmed the reduction of viable cells in both matrixes, compared to their respective undifferentiated cultures, and independently of the MSCs source studied (Fig 4B).

Next, we monitored through the 3 stages of differentiation, the expression of the following markers related with insulin-producing cells differentiation: forkhead box protein A2 (*Foxa2*), islet 1 factor (*Isl1*), neurogenin (*Ngn3*), paired box protein (*Pax6*), pancreatic duodenum homeodomain (*Pdx1*), glucose transporter 2 (*Glt2*), pancreatic polypeptide (*Pp*), glucagon (*Gcg*), insulin 1 (*Ins1*), and insulin 2 (*Ins2*). We first quantified *Foxa2*, observing a progressive expression reduction along the 3 differentiation stages, independently of the MSCs source studied (Fig 5A). As expected, the highest *Foxa2* expression was quantified at the beginning of the differentiation procedure, next reducing its expression in all the samples studied, with a significant reduction in D1-MSCs ($p<0.01$). Interestingly, MSCs isolated from pancreas expressed *Foxa2* as previously reported (43). *Foxa2* is always found in the early endoderm layer from pancreas later arises (44), regulating the expression of other β-cell transcription factors (45). However, a minimum detectable expression in pancreatic progenitors was still quantified at the final stage of differentiation, since

this gene is always expressed at basal levels in adult foregut and hindgut endoderm-derived tissues, such as the pancreas (46), participating in the maintenance of adult β-cell function (47). Similar to *Foxa2*, *Isl1* expression was progressively and significantly reduced ($p<0.5$) along the 3 differentiation stages, independently of the MSC source studied (Fig 5B). Isl1 expression in the embryo is initiated soon after the islet cells have left the cell cycle, as well as, in the mesenchymal cells that surround the dorsal, but not ventral, evagination of the gut endoderm (48). However, *Isl1* was still expressed at the final stage of differentiation of bone marrow-derived MSCs and D1-MSCs, maybe indicating a lack of maturity of the cell at the final stage of our differentiation procedure.

Ngn3 is an essential helix-loop-helix transcription factor activated during stem cell differentiation into α- or β-pancreatic cells (49), and it was expressed by MSCs as previously reported (43). It was significantly upregulated ($p<0.01$) in the primary MSCs sources at stage 2 in both 3D cultures, downregulating the expression at stage 3 (Fig 5C). However, D1-MSCs maintained *Ngn3* expression at stage 1 of differentiation, decreasing significantly at stage 2 ($p<0.5$). This differential expression pattern associated with cell types and culture matrices can be related to temporal variations in the *Ngn3* expression described during embryogenesis. Throughout pancreatic islets development, we can observe 3 temporally separated activations of *Ngn3*, leading to the formation of glucagon-producing α-cells, pancreatic polypeptide-cells, and insulin-producing β-cells or somatostatin-producing δ-cells (50). When the paired homeobox transcription factor *Pax6*, destined predominantly for the α-cell fate (51), was quantified, a progressive reduction was registered along the different stages, reaching not detectable expression at the final stage independently of the MSCs source (Fig 5D). Finally, *Pdx1* expression, indispensable in pancreatic development and maintenance of β-cell function (52), whose lack of expression in mice embryos results in pancreas agenesis (53), was upregulated in pancreatic MSCs at stage 1 of differentiation displaying a significant downregulation ($p<0.01$) along the stages 2 and 3. However, bone marrow MSCs showed a lack

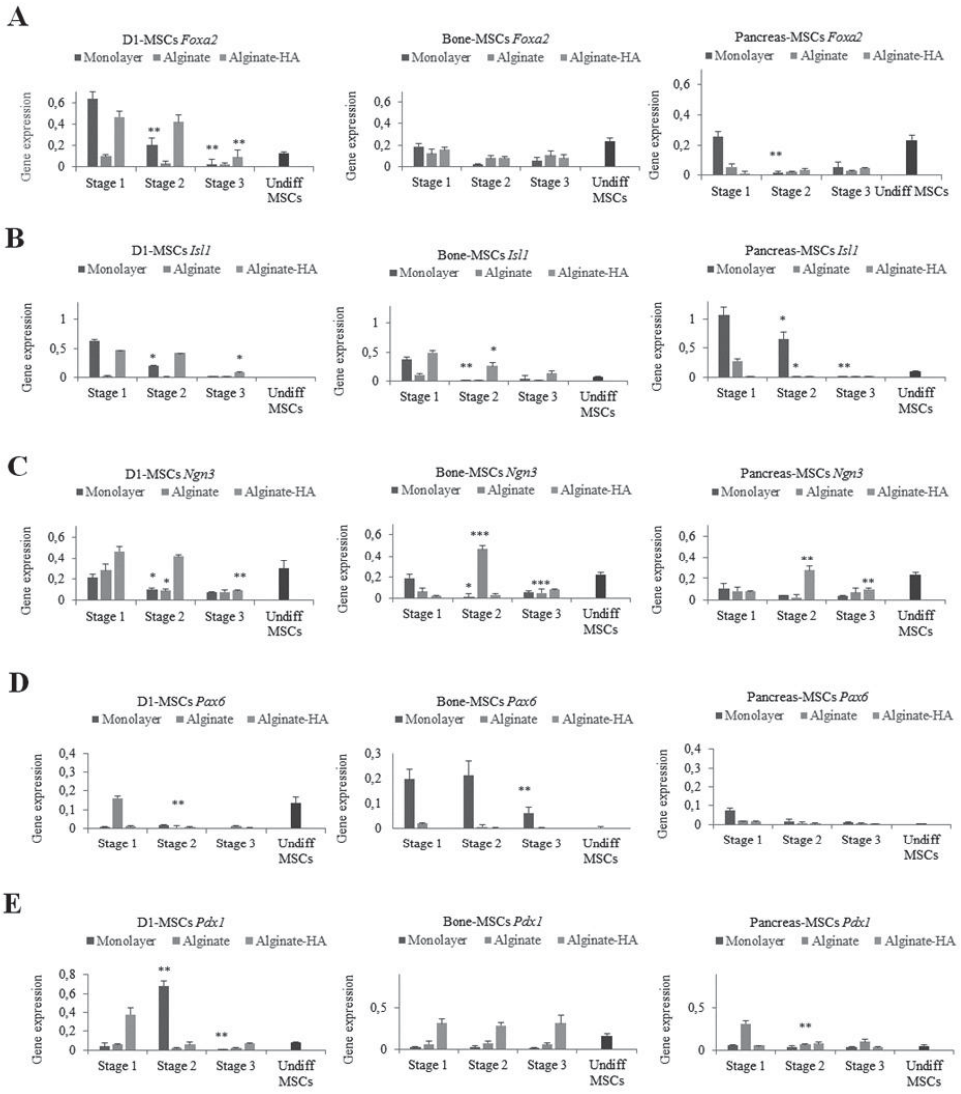

Figure 5.- Gene expression of *FoxA2, Isl1, Ngn3, Pax6*, and *Pdx1* at the 3 stages of differentiation from 1.5% alginate and 1% alginate 0.25% HA microcapsules. Data represent the fold regulation between the gene of interest and the housekeeping. Note: ***: p<0.001; **: p<0.01 and *: p<0.05

of upregulation along the differentiation protocol (Fig 5E). This divergence on results between cell types reflects the variations described in *Pdx1* expression during embryogenesis, always contributing to the specification of endocrine progenitors by regulating expression of *Ngn3* directly (54). *Pdx1* is strongly temporally upregulated during embryogenesis from day 8.5 (E8.5) to day 10.5 (E10.5) embryo (54, 55), but

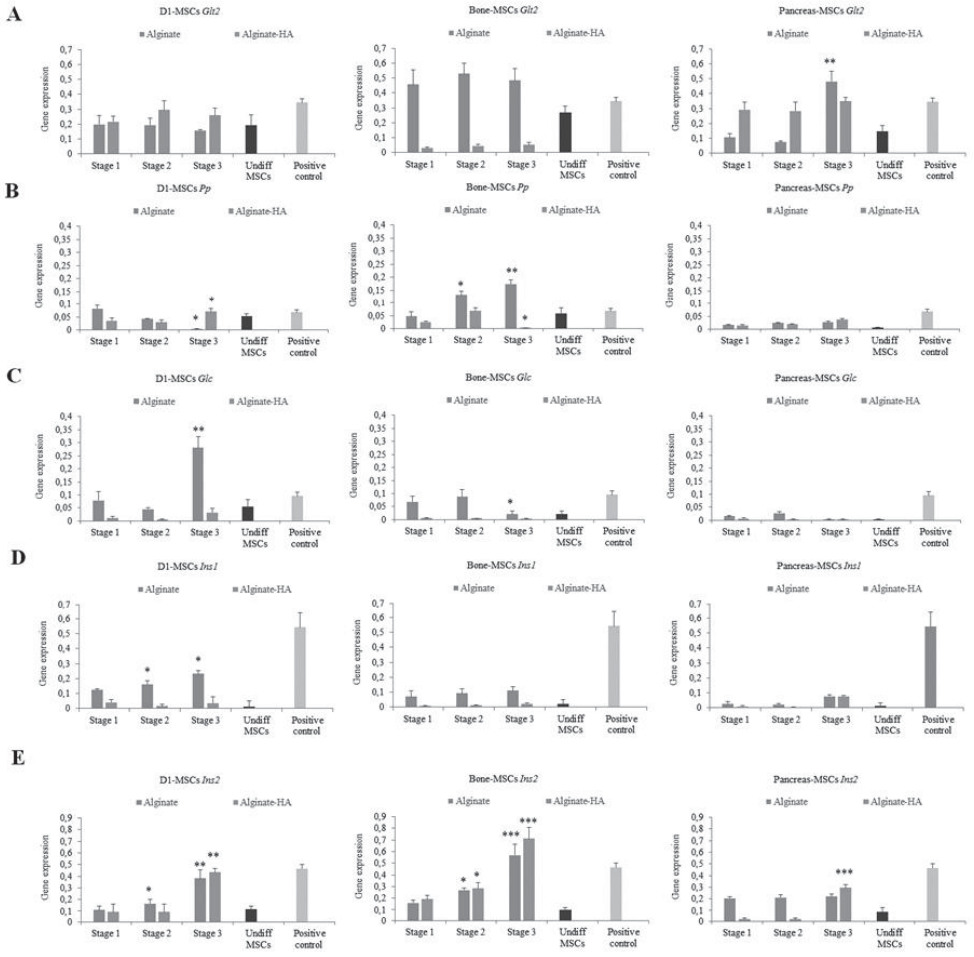

Figure 6.- Gene expression of *Glt2, Pp, Gcg Ins1,* and *Ins2* at the 3 stages of differentiation from 1.5% alginate and 1% alginate 0.25% HA microcapsules. Data represent the fold regulation between the gene of interest and the housekeeping. Note: ***: p<0.001; **: p<0.01 and *: p<0.05

also in the last stage of pancreatic beta cells differentiation (56), indicating that the obtained differentiated cells are pancreatic progenitors (57). Although this lack of maturity of pancreatic progenitors has also been reported after embryonic stem cells differentiation into insulin-producing cells procedure (58), when transplanted, were able to maturate, displaying similar *in vivo* functionality than the β-cells (59).

Next, we quantified the expression of *Glt2*, the glucose transporter expressed in the plasma membrane of β-pancreatic cells, hepatocytes, intestine, and kidney (60), which is the isoform required for the glucose sensing in β-cells

(61). As expected, no differences in expression were quantified during the 3 steps of differentiation for all the MSCs types studied (Fig 6A). In fact, β-cells arise from *Glt2* expressing epithelial cells at E11.5, and it remains upregulated from E17.5, when large aggregates of β-cells begin to form the islets of Langerhans, to adult life (62). When analyzing the expression of endocrine hormones, *Pp* was almost not detected in the differentiation of pancreatic MSCs (Fig 6B). Bone marrow-derived MSCs upregulated *Pp* expression at the final stage of differentiation, while the cell line D1-MSCs did not show differential expression among the 3 steps of the protocol (Fig 6B). These data indicate a heterogeneous population containing γ-cells in the final stage of MSCs derived from bone marrow, no detected in the differentiated cultures from pancreas-derived MSCs. Similarly, in pancreatic MSCs differentiation cultures, no *Gcg* expression was detected at any step of the procedure (Fig 6C), also quantifying a low expression of the gene in all the differentiation cultures from bone marrow MSCs. However, significant upregulation of *Gcg* expression ($p<0.01$) was quantified at the final stage of the cell line D1-MSCs (Fig 6C). Although the presence of bi-hormonal glucagon and insulin-positive cells has been described during embryogenesis (63), in insulin expressing cell lines (64) or after stem cell differentiation procedures (65), the absence of glucagon-secreting cells is desirable, and therefore, indicated that pancreatic-derived MSCs is a stronger candidate for 3D differentiation into IPCs.

Since murine and rat insulin protein is encoded in a two-gene system composed of *Ins2* and *Ins1* genes, producing preproinsulin 2 and preproinsulin 1 respectively, we quantified the expression from both genes. *Ins1* and *Ins2* were progressively and significantly upregulated ($p<0.01$) in the three MSCs sources, indicating that cells at the final stage were able to produce insulin (Fig 6D, E). Therefore, we proceeded to quantify the protein insulin release of the IPCs differentiated from the three MSCs sources. All cultures, independently of the MSCs source, increased their insulin release when differentiated into IPCs (Fig 7). Significantly higher insulin release increments ($p<0.05$) were quantified in matrices formed by alginate and HA,

compared to those containing only alginate (Fig 7). This indicates that the presence of HA, one of the major components in the pancreatic islet ECM (26), promotes the differentiation into IPCs. Moreover, 3D cultures of IPCs derived from pancreatic MSCs displayed a significantly higher increment ($p<0.01$) in insulin release after differentiation, since epigenetic memory may predispose stem cells derived from the pancreatic environment to differentiate more readily into insulin-producing cells (66). The molecular features determining the differential capacities among the stem cells sources, and predicting their differentiation potential, were already reflected in the gene expression profile quantified during the different stages of differentiation. Thus, IPCs derived from pancreatic MSCs were the only cells that did not express *Pp* or *Gcg* (Fig 6B, C) at the final stage of differentiation compared to bone marrow MSCs derived cells, indicating the absence of bi-hormonal cells, an unachieved characteristic in a wide number of stem cell differentiation procedures (65). Nevertheless, independently of the MSCs source, our data has demonstrated that MSCs can be differentiated in 3D culture systems towards IPCs, enhancing the release of insulin after differentiation in the presence of ECM components within the alginate matrix, such as HA.

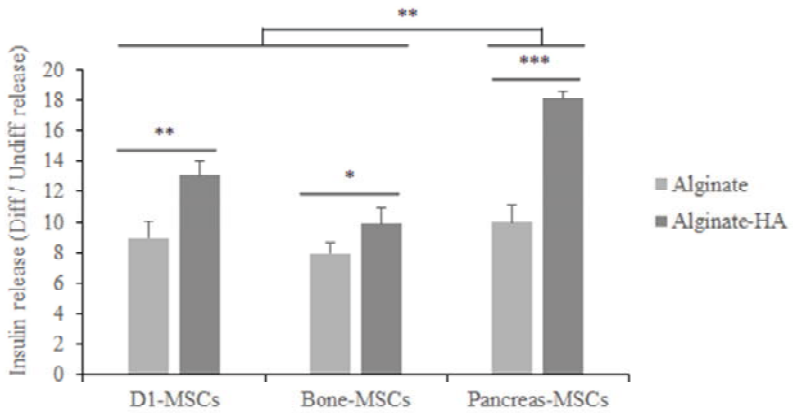

Figure 7.-Insulin release increment after differentiation of MSCs within 1.5% alginate and 1% alginate 0.25% HA microcapsules. Insulin ratios were calculated as the quotient between insulin release at differentiated and undifferentiated states. Note: Values represent mean ± SD. ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$.

4. CONCLUSIONS

We can conclude that MSCs embedded in 3D alginate matrices can differentiate into IPCs with enhanced insulin release at the final stage of differentiation, thanks to the structural support provided by microcapsules, and the protection exerted against the aggressiveness from the differentiation procedure. Moreover, the presence of HA, a major ECM component, enhances the beneficial outcomes of alginate microcapsules in MSCs differentiation towards IPCs by mimicking the natural environment of the islet of Langerhans. These results are a breakthrough in the exploration of the 3D embedded MSCs potential for T1DM treatment.

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Chapter 6

Discussion

1. The journey towards MSCs as cell source for T1DM treatment.

Nowadays, diabetes mellitus is one of the main threats to human health. Some organizations, such as the World Health Organization or the International Diabetes Federation, estimates a worldwide enhancement from 350 million to 600 million diabetic patients before 2050. Diabetes has been classified by the American Diabetes Association as type I diabetes mellitus (T1DM), type II diabetes mellitus (T2DM), gestational diabetes mellitus (GDM) and other minor types grouped as type III diabetes mellitus. Type 1 diabetes mellitus (T1DM) is characterized by autoimmune destruction of pancreatic β-cells, after an asymptomatic period over the years, resulting in severe insulin deficiency. It develops mostly in young people accounting for 5-10% of the diabetic subjects (1). T1DM patients have shown that β-cells from the islets of Langerhans are destroyed by infiltration of dendritic cells (DCs), macrophages and T lymphocytes (both CD4+ and CD8+). Immune cells action is specific against insulin-producing β-cells, not affecting other cells in the islets of Langerhans, such as α-cells (glucagon-producing cells) or δ-cells (somatostatin-producing cells) (2). Type II diabetes mellitus is also known as independent insulin diabetes because patients present insulin resistance and deficiency, without the need of insulin treatment to survive. The specific etiology of T2DM is not entirely clarified, and there are probably different causes, including obesity and genetic predisposition (3). Gestational diabetes mellitus can be defined as a deficiency in glucose metabolism control identified during pregnancy which is typically reverted post-partum (4).

To define T1DM, it is necessary to analyze the progression of symptoms in the disease, from average values of 4.5 mmol/l (80mg/dl) of blood glucose to reach higher values. Attending to changes in the cells mass, phenotype and cell functionality five stages can be defined in the progression of diabetes (Fig 1) (5). The regular stage of β-cells is normal glucose-stimulated insulin secretion with regular glucose blood levels. Then, the first stage of diabetes is characterized by an increase in the insulin secretion, to maintain the regular glucose levels, because of insulin resistance caused

by obesity, physical inactivity, and genetic predisposition. During this stage, it has been described as an increment of β-cell mass, probably due to an increase of β-cell number, although β-cell hypertrophy may also contribute (6). In the next step, the glucose levels overcome 5.0–6.5 mmol/l (89–116 mg/dl) and, normal glucose levels cannot be long maintained. Despite people in stage 2 usually evade progression to type II diabetes for years by adhering to a diet and exercise regimen (7), people with T1DM experience a fast increase of β-cell mass destruction.

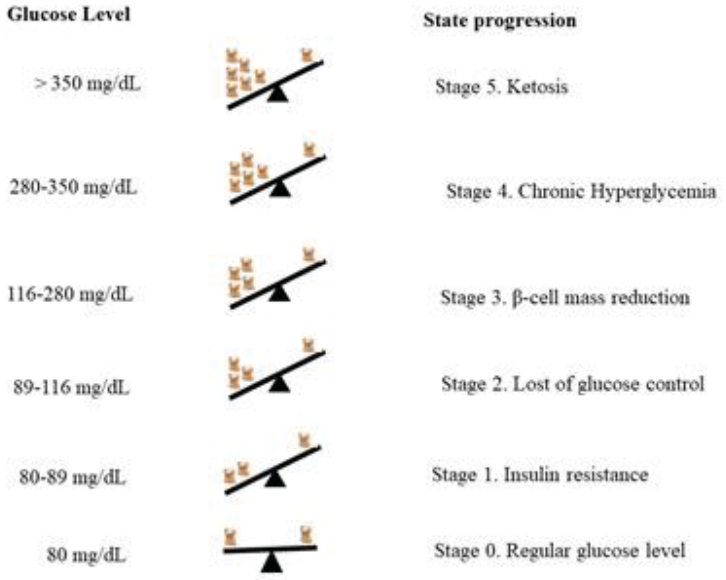

Figure 1.-Five stages of the progression of diabetes.

Next, T1DM evolves to a decompensated stage 3 when glucose levels rise rapidly over 7.3 mmol/l (130 mg/dl), probably determined by glucose toxicity effects on β-cells, leading to β-cell mass reduction and less efficient insulin secretion (8). In stage 4, an equilibrium with enough insulin secretion can be maintained, avoiding the progression to ketoacidosis. Mostly, this decompensation reduction stage lasts a lifetime for people with type II diabetes, while the rapid progressive autoimmune destruction of β-cells in type I diabetes, can lead to stage 5 relatively quickly (9). In stage 5 of T1DM, there is a fast β-cell mass reduction improving the glucose levels

which typically can range 22 mmol/l (350 mg/dl) and the progression to ketosis and truly insulin dependence for survival is unavoidable. The β-cell destruction is completed with no possibility to return across the stages, while until stage 4 it was possible. The stage 5 is typical in T1DM, and it rarely occurs in common type II diabetes.

T1DM development is determined by a genetic component, including interactions between different loci, and modulated by environmental factors. As a genetic component, one of the significant susceptibility factors to develop type 1 diabetes in children and young adults are some common allelic variants at the HLA class II loci (primarily *HLA-DRB1, HLA-DQA1*, and *HLA-DQB1* genes) located on the chromosome 6p21. This region encodes the highly polymorphic antigen-presenting proteins and explains around half of the genetic risk for T1DM (10). At present, two from the four different known haplotypes of HLA class II loci can explain 90% of the HLA genetic risk in children T1DM (11). Other five T1DM susceptibility loci have been established including the insulin locus (*INS*) VNTR on the chromosome 11p15, the cytotoxic T-lymphocyte-associated protein 4 (*CTLA4*) locus on the chromosome 2q31, the protein tyrosine phosphatase-22 (*PTPN22*) gene on the chromosome 1p13, the IL-2 receptor (*IL2RA*) locus on the chromosome 10p15 and interferon-induced with helicase C domain 1 (*IFIH1*) on the chromosome 2q24 (10). However, the interaction of the dominant genes involved in T1DM cannot wholly explain the etiology of the disease, and more genetic studies need to be performed to clarify. Moreover, there are several environmental factors which can increase the probability of T1DM from children to adults (12). For example, the exposure of children to proteins of cow milk increases the risk of T1DM (13), while human milk seems to be protective (14). The supplementation of vitamin D and the absence of medication during pregnancy also reduce the risk of T1DM (15) (16). Moreover, other lifestyle habits can influence diabetes development such as highly processed food consumption (17), high sedentarism (18), and high levels of physical stress (19).

During the past decades, several procedures were developed for T1DM diagnosis based on molecular markers. Since the autoimmune destruction of β-cells starts with the presence of autoantibodies against the three major T1DM-specific antigens in the serum (7), the major T1DM-specific antigens against β-cells are commonly used for molecular diagnosis. They are GAD65 (glutamic acid decarboxylase, 65 kDa isoform), protein IA2 (insulin autoantigen 2, an intracellular tyrosine phosphatase-like), and insulin. These markers are detectable in 90% of diagnosed pre-diabetic patients, months or years before clinical T1DM (20). Other sensitization factors, such as the proinflammatory cytokine interleukin-1b (IL-1b), tumor necrosis factor-α (TNF-α) and interferon γ (IFN-γ) or stress mediators and reactive oxygen species are involved in the β-cells destruction and can be used as indicators for T1DM diagnosis. These factors play a central role in β-cell failure and the development of diabetes. For example, the ability of β-cells to produce and release insulin is affected by prolonged exposure to proinflammatory cytokines leading, in the long term, to apoptosis or necrosis (21). Besides, other stimuli also contribute to β-cell apoptosis, such as stress mediators, hyperglycemia and reactive oxygen species which disturb the endoplasmic reticulum (ER). ER stress of β-cells and type I diabetes are related since the ER protein load is required for the proper folding of insulin within the ER (22). Moreover, a situation with large protein loads induces to protein accumulation, dysregulation of Ca2+, and disrupt of ER homeostasis (23).

Other major complications are derived from hyperglycemia episodes in T1DM such as nephropathy, renal failure, neuropathy, retinopathy, damaged vessels, and limb amputation. Diabetic nephropathy (DN) is considered one of the major complications of insulin treatment, and it is characterized by an increase of glomerular filtration rate, ending in renal failure (24). Hyperglycemia, produced by a lack of glucose control, enhance the production of reactive oxygen species (ROS), which are involved in DN development (25). Another complication derived from diabetes are neuropathies, which can be observed in almost 50% of patients (26). The damage in the peripheral nervous system of the T1DM patients along the extremities can produce numbness

or chronic pain, resulting in foot ulcers and limb amputation (26). Finally, the retinopathy, characterized by progressive retinal damage resulting in blindness, is strongly associated with T1DM and T2DM. Injuries in the retinal microvasculature lead to blindness linked to ischemia, retinal swelling, and neovascularization (27). All these complications related to the hyperglycemia episodes in diabetic patients, decrease the diabetic lifespan 1-13 years compared with the healthy population (21). Diabetes is usually treated depending on the stage progression. The ideal goal of a future treatment for T1DM would be to reverse the β-cell destruction, restore the glucose metabolic control and prevent the onset and progression of autoimmunity. Before insulin was discovered, the goal of diabetes treatment was to avoid the development of diabetic ketoacidosis and reduce mortality (8). Although, there are some current treatments and clinical trials for T1DM, the most prominent treatment is the insulin replacement by exogenous administration through daily injections or an insulin pump. In the beginning, insulin treatment was based in the administration of bovine and porcine insulin (28), increasing the life expectancy of diabetic patients. However, the impurities presented in the animal-derived insulin preparations produced highly variable efficacies among patients (29). The combination of adverse effects by animal-derived insulin, such as insulin allergy, abscesses, lipodystrophy, and insulin antibody formation (29) and its lousy control of glycemia, induced research groups to develop new approaches for the treatment of T1DM.

The development of slow-release insulin administrations allowed to reduce the incidence of hypoglycemic episodes of the animal insulin administration. The first slow-release insulin administration was Neutral protamine Hagedorn (NPH), a fish-isolated protein which reduces the solubility of insulin and zinc (8), reducing the insulin release and protecting patients from hypoglycemia. Next, the development of highly purified animal insulins allowed to alleviate some issues related to insulin administration, such as lipoatrophy and other local reactions to insulin injection (30). However, in spite of lipoatrophy was reduced, it was not eliminated since highly purified porcine and bovine insulin showed local reactions to insulin injection (31).

The next step was the discovery of synthetic insulins, potentially more effective and available. Human insulin was the first protein synthesized in vitro (32) in the 60s, involving techniques like chemical synthesis, semi-synthesis and substitution of alanine in porcine insulin with threonine (33) (34). Moreover, several studies showed controversy in the use of synthetic or animal insulin. Some studies displayed no differences regarding potency and durability of effect between synthetic and animal insulin (33). However, other publications indicated a lack of normalization of hyperglycemic episodes in patients treated using synthetic insulin (35). Finally, different approaches presented no significant differences in glucose control and absorption between synthetic and animal insulin (34).

Theoretically, data from human synthetic insulin approaches displayed higher control of glucose metabolism and, at this point, the need of large-scale production was presented. Recombinant DNA technology development supposed the possibility to biologically synthesize human insulin at a large scale, improving the availability of recombinant insulin. But insulin and recombinant insulin show some differences. Insulin is synthesized in vivo by β-pancreatic cells as proinsulin, a large polypeptide containing 3 chains (A, C and B), which are auto-processed to link chains A and B removing C-middle chain (36). Otherwise, the insulin produced in vitro requires that E. coli synthesizes both A and B chains separately, next chemically bonded (37). Some pharmaceutical companies have obtained and patented biosynthetic human insulin molecules using recombinant DNA technology, such as Eli Lilly and Company in 1982 (38), and Novo Nordisk in 1987 (39). Some studies about animal and recombinant human insulin have shown differences in pharmacodynamics and pharmacokinetics, providing a slow onset and long durability of recombinant human insulin action, which increase the risk of hyperglycemia and hypoglycemia episodes (40).

Recombinant human insulin allowed to solve the problem of insulin availability but did not mimic the pharmacodynamics, pharmacokinetics, and effects from endogenous insulin. By the early 90s, the production of insulin analogous as

molecules derived from insulin structure, allowed to increase the glycemic control and reduce the daily injections thanks to products with high purity. The development of insulin analogous enables a faster onset and shorter duration of effects with a closer insulin release to endogenous insulin (41). Insulin analogous are based in the insulin structure, and they are obtained by substitution of amino acids along A and B chains excluding the N terminus of the A chain and the C terminus of the B chain, which are receptor binding sites (42). Currently, there are two insulin analogous commercially available, insulin glargine and insulin detemir. Insulin glargine mechanism includes the neutralization of the subcutaneous injection solution at pH=4 to produce microprecipitates, allowing a steady insulin release. However, insulin detemir release principles are based on the high affinity of the compound for albumin, which combination delays the absorption of insulin (43). Both insulin analogous have higher durability than 24 hours but, short effects after 5.7 hours for insulin detemir and 10.8 hours for insulin glargine. Thus, patients treated with glargine displayed lower nocturnal hypoglycemic episodes in type 2 diabetes than patients treated with slow-release insulin (44). Besides, several groups are developing investigational insulins, such as insulin degludec, a long-durability insulin with a period of action of 42 hours and a stable pharmacokinetic profile (45) (46), or long-acting insulin LY2605541 (LY), a current version of lispro insulin designed to have a sizeable hydrodynamic size and delaying insulin absorption (47). Moreover, the development of inhaled insulin systems by some pharmaceutical companies can change the administration mode, solving the issues derived of daily injection required by the conventional administration of insulin.

At present, several companies have developed oral treatments to solve the complications derived from insulin administration. Thus, there are some oral non-insulin-based drugs for glucose control during diabetes such as metformin, thiazolidinedione, insulin secretagogues, starch blockers, incretin therapy and amylin analogous. All these treatments are used for glucose control in early stages of diabetes with lower β-cell destruction levels while, for the latest stages, the

conventional treatment is insulin administration by daily injection or computerized pumps. However, exogenous insulin administration is the most frequently used method to treat diabetes because increase life expectancy and helps to normalize blood glucose but, all the insulins and insulin derivatives present hyperglycemia episodes and lipodystrophy at the injection site. Besides, the insulin administration is not a cure to T1DM but is a treatment which allows increasing the life expectancy without replenishing the β-pancreatic cell.

Nowadays, several research groups are focused on healing T1DM with β-pancreatic cell replenishment, either by whole vascularized pancreas transplantation or by islet transplantation. Whole pancreas transplantation is a major intraabdominal surgical procedure used for pancreas replacement which includes the surgical technique and the immunosuppression post-implantation. The first whole pancreas transplantation was made in 1966 (48) with more than 25,000 worldwide whole pancreas transplantations made by 2005 (49). Pancreas transplantation is usually combined with kidney transplantation from the same donor, with extremely restrictive indications for surgery. The whole pancreas transplantation is indicated for T1DM patients who have a negative C-peptide, and terminal renal insufficiency, the absence of tumor lesions, chronic infections and pronounced cardiovascular issues. Besides, pancreas transplantation does not show benefits in patients over 50 years (50). Patients are frequently double kidney and pancreas transplanted, while patients presenting severe hyperglycemia and hypoglycemia episodes and without kidney damages or slight damages derived from immunosuppressants are rarely transplanted (51). However, the ratio of whole pancreas transplantation complications is the highest among all solid organ transplants. In pancreas and kidney transplantation, infections and rejections are frequent, with higher mortality and more extended hospitalization (52) (53). Besides, the intense immunosuppression after pancreas transplantation has been related with the appearance of carcinomas and lymphomas (54). Studies regarding life quality have shown normalization in blood glucose levels with no exogenous insulin administration, but with dietary restrictions and

lifelong immunosuppression (55). Although the whole pancreas transplantation has demonstrated to be a feasible β-cell replenishment option, it involves transplantation of endocrine and exocrine tissue. Since the exocrine tissue contributes to the risk of infection, graft thrombosis and pancreatitis, consequently the isolation of islets of Langerhans from endocrine pancreas for transplantation has provided similar results and minor surgery than whole pancreas transplantation, allowing a decreasing risk of infection for patients.

The transplantation of pancreatic islets became a promising treatment for T1DM since guinea pig islets were isolated and cultured in 1965 (56), establishing the use of collagenase to digest the exocrine pancreas. Some years later, pancreatic islets were transplanted at different implantation sites displaying higher engraftment ratios of pancreatic islets transplanted in the portal vein than in intraperitoneal implantation (57). Transplantation of islets in the portal vein of rats demonstrated a reversion of diabetes (58). At the end of the 70s and early 80s, clinical studies showed portal hypertension, disseminated intravascular coagulation and lack of effect to reverse diabetes after islets transplantation (59). Thus, research groups began to study large animal models again, optimizing the conditions for islet isolation and purification (60) (61), to obtain a higher purified extract that would allow reducing the volume of implantation. The implantation in the portal vein of large animals was optimized too (62), together with the long-time functionality of transplanted pancreatic islets (63). After several failed attempts transplanting human pancreatic islets, in 1990, the first T1DM patient showed insulin independence after pancreatic islet transplantation (64). The insulin independence was observed for less than 1 year after pancreatic islets transplantation, when the graft lost induce patients to T1DM again (65) (66). β-cells in the pancreatic islets were destroyed by the immune system, and a potent immunosuppression treatment was needed to avoid the immune rejection.

In 2000, the Edmonton Protocol, one of the most relevant advances for pancreatic islets transplantation (67), displayed the highest ratio of insulin independence in T1DM patients: 5 years after pancreatic islets transplantation compared with 1

year before Edmonton. The conditions established in this protocol were an adequate islet mass (>10,000 islet equivalents per kg recipient body weight), an immediate infusion of islets following islet isolation and the avoidance of corticosteroids. With transplanted islets immunosuppression by administration of sirolimus, low-dose of tacrolimus and an antiCD25 antibody was achieved (68). T1DM patients showed a lack of insulin independence after 3 to 5 years post-implantation, exhibiting that islet transplantation cannot maintain the insulin independence permanently. Several research groups are still working to improve several parameters that help to success islet transplantation and extended maintenance of insulin independence.

Donor selection is one of the parameters which can improve islet transplantation success by verifying factors from the donors, such as the age, the body mass index, and the cold ischemic time. Donors with more than 50 years old provide more pancreatic islets than younger donors, but with reduced capability to produce insulin (69). However, the digestion of a young pancreas is difficult because of its fibrous nature. It is demonstrated that induced diabetic mice transplanted with human islets older than 50 years show lower diabetes reversion ratios than transplanted with younger human islets (70). The body mass of donors is also essential because high body mass donors have pancreatic islets with lower insulin secretion ratios (71). A short cold ischemic time, the time from tissue extraction from donor to the isolation of pancreatic islets, can also increase the transplantation success (72).

Another parameter of interest for successful transplantation is the islet isolation protocol whose optimization may help to improve the long-term results of islet transplantation. High-quality human pancreatic isolation requires expensive good manufacturing process (GMP) facilities with skilled personnel in islet isolation techniques. The pancreas digestion is a critical step to islets isolation with the use of an adequate collagenase enzyme with non-collagenase impurities is a pivotal part of the process (73) (74). The purification procedure allows dissociating the pancreatic islets from exocrine tissue, reducing the amount of tissue transplanted into the portal vein and avoiding thrombosis, embolism and even death (75). The most common

method to purify pancreatic islets is the centrifugation with discontinuous albumin gradient which separates less dense islets from exocrine tissue (76). But independently of those factors, the major limitation in the actual trials based in β-pancreatic cell replenishment through pancreatic islets transplantation is donor scarcity, requiring to establish new treatments based in β-pancreatic cell replenishment that avoids donor scarcity.

In the last two decades, the application of stem cells for diabetes treatment has been purposed to solve the pancreatic islet donor scarcity and currently is a hot topic of debate (77) (78). Stem cells are defined by self-renew ability, that allows cells to divide for long periods without differentiation, and differentiation capacity, which confer cells the potential to develop into other specialized cells types. The differentiation potential of stem cells can be exploited to obtain β-insulin-producing cells (IPCs) by different stimuli, such as internal chemical stimulation, physical contact or micro-environmental molecules. On this regard, the mesenchymal stem cells (MSCs) are an appropriate cell source since they retain the self-renewal capacity and the ability to differentiate into IPCs of other stem cells, while avoiding the tumorigenicity associated with embryonic or induced pluripotent stem cells. MSCs derived from different tissues, such as bone marrow, umbilical cord or fat, have been used to produce IPCs. For example, bone marrow-derived MSCs were differentiated following a 3-stages protocol during 18 days, expressing at the final stage of differentiation, Pdx1, insulin, and glucagon and secreting insulin in response to different concentrations of glucose (79). However, these IPCs differentiated from bone marrow-derived MSCs only responded to glucose challenge for 2 weeks. Following a differentiation protocol based on stepwise culture conditions described for human ESCs, umbilical cord-derived MSCs have also been differentiated into IPCs, leading to C-peptide expression in transplanted mice for 3 weeks (80). Adipose-derived MSCs have also revealed high proliferation and differentiation capacities. For example, a 21 days protocol to differentiate adipose-derived stromal cells into IPCs revealed typical islet-like cell clusters with positive DTZ staining, expressing

Pdx1 and Glt2 and secreting insulin after glucose challenge (81). However, the differentiation of MSCs into β-pancreatic cells provides low and no sustainable insulin release. On this regard, several studies have shown the differentiation of stem cells within 3D encapsulation systems to enhance insulin secretion. For example, the differentiation of bone marrow MSCs seeded in fibrin glue 3D scaffolds produced 3.5-fold more insulin-positive cells and 3-fold more insulin per cell than those IPCs differentiated in monolayer (82). Another example is the differentiation of adipose-derived MSCs embedded within collagen-HA hydrogels, resulting in a 4-fold increase of insulin release compared to those cultured on monolayer (83).

Collagen (84), hyaluronic acid (85), chondroitin sulfate (86), fibrin (87), fibronectin (88), alginate (89), agarose (90), chitosan (91) and silk (92) are natural polymers currently used in cell encapsulation, being alginate, the biomaterial most commonly used due to its mechanical properties with high tunable possibilities (93). However, alginate cannot provide the extracellular matrix (ECM) that mimic the natural cell environment. On this regard, alginate has been combined with different ECM molecules, such as laminin, collagen I or collagen IV, to create an ECM mimicking environment displaying higher cell viability values (94). Other approaches trying to simulate the cell-matrix interactions provided by ECM are the short synthetic peptides derived from natural proteins that compose the ECM, for example, the arginine-glycine-aspartic acid (RGD) peptide derived from fibronectin. This tripeptide offers advantages over the use of the whole protein like its simplicity, cost-effectiveness, easy manipulation for functionalization and low immune response (95) (96).

A major component of the natural ECM is hyaluronic acid (HA), an anionic non-sulfated glycosaminoglycan molecule with variable molecular weights, composed by repetitions of a disaccharide unit of an N-acetyl-glucosamine and a β-glucuronic acid. HA has been extensively tested for hydrogels preparation (97) since this macromolecule has been described for being involved in a wide variety of biological procedures like cell-signaling, regulation of cell adhesion and proliferation, and manipulation of cell differentiation (98). HA is also involved in

the maintenance of islets stability, integrity, anti-inflammatory properties (99), and the proliferation of cells through the formation of stabilized complexes composed of high molecular weight HA and chondroitin sulfate proteoglycans (100). Thus, HA has been extensively used for embedding pancreatic cells in hydrogels (101), providing a native ECM-like microstructure and contributing to structural support and protection of embedded cells (102), while promoting cell viability (103). Based on the outcomes shown by embedded pancreatic cells into HA hydrogels, HA can be proposed as an interesting component in the 3D differentiation of MSCs into pancreatic cells for their future application in T1DM treatment.

2. The mixtures 1% alginate 0.25% HA and 0.5% alginate 0.5% HA resemble 1.5 % alginate rheological properties and are optimal for cell encapsulation.

We began our studies by identifying the best combination of hyaluronic acid and alginate to form hybrid microcapsules with similar physicochemical properties to alginate microcapsules, with the hypothesis that the presence of hyaluronic acid will mimic the natural ECM environment and, therefore, enhance the encapsulated cell viability and functionality. Hence, we selected the formulation of the hybrid microcapsules based on the rheological behavior of a large number of combinations between HA and alginate, next studying more deeply the physicochemical characteristics of those combinations with similar rheological behavior to alginate. We chose 200 kDa alginate since this molecular weight allows the performance of microcapsules where cells can actively proliferate and release therapeutic factors (104). Also, 1.1 MDa HA was chosen since this molecular weight shows the higher cell adhesion and proliferation rates during differentiation of MSCs (105). Thus, we proceeded to mix alginate and HA at different concentrations, evaluating the physical and chemical properties of these mixtures. Rheological studies showed that 1% alginate 0.25% HA, 1% alginate 0.1 % HA, and 0.5% alginate 0.5% HA were the mixtures with more similarities to 1.5% alginate viscosity profile (Fig 2A1-A2). Mixtures containing a higher concentration of HA displayed viscosity curves over

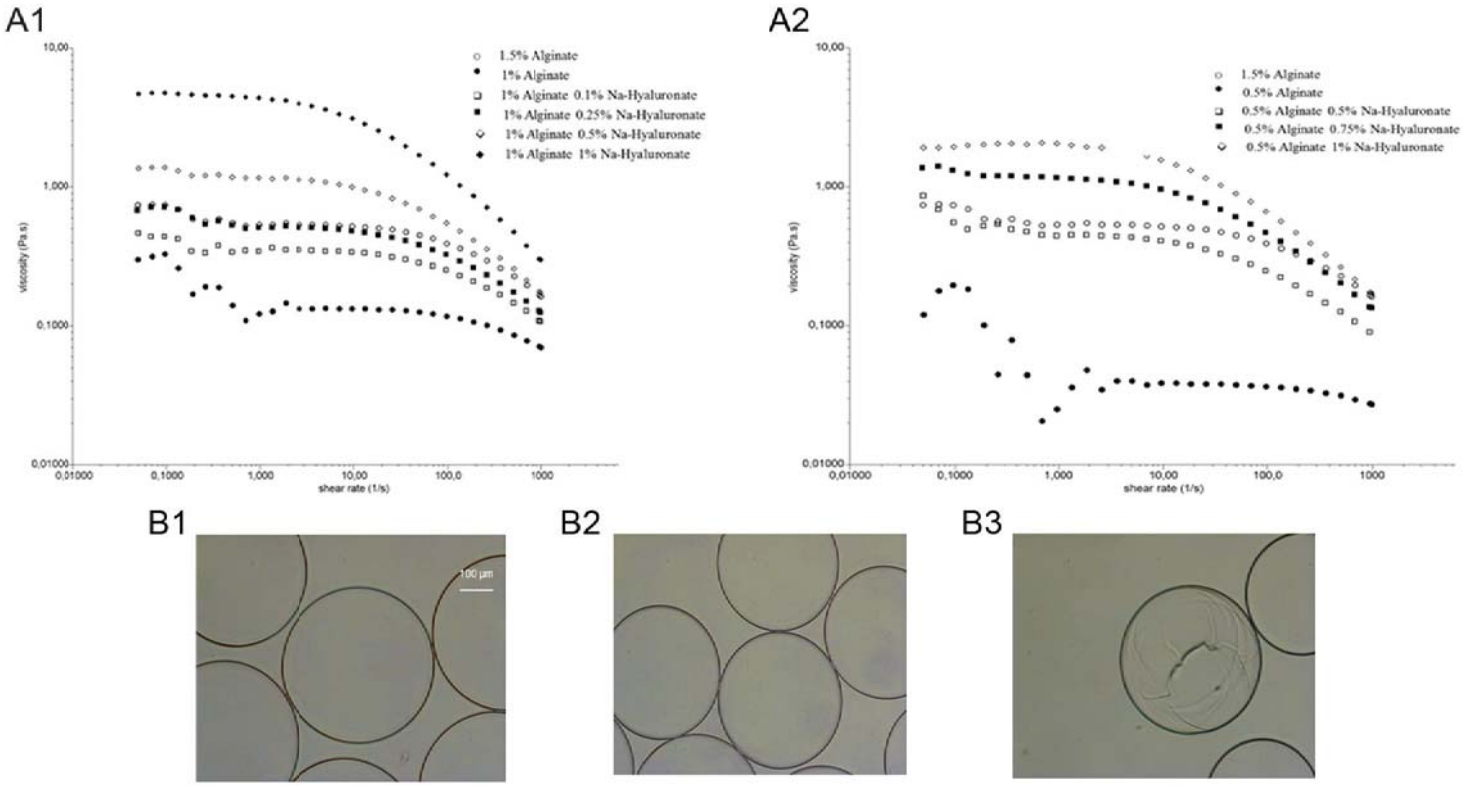

Figure 2.- (A) Rheological behavior comparison to 1.5% alginate of: (1)1% alginate 0.1% HA, 1% alginate 0.25% HA, 1% alginate 0.5% HA, 1% alginate 1% HA and (2) 0.5% alginate 0.1% HA, 0.5% alginate 0.25% HA, 0.5% alginate 0.5% HA and 0.5% alginate 1% HA. **(B)** Micrographs of microcapsules at the following compositions: (1) 1% alginate 0.1% HA, (2) 1% alginate 0.25% HA and (3) 0.5% alginate 0.5% HA. Note: Scale bar represents 100 µm.

1.5% alginate curve, while low HA concentrations were not able to get over this curve, due to the interaction mediated by hydrogen bonding between both alginate and HA macromolecules (106). We selected 1% alginate 0.25% HA, 1% alginate 0.1 % HA, and 0.5% alginate 0.5% HA mixtures to test them as encapsulation biomaterials, using an electrostatic atomization generator and obtaining homogeneous 450 μm spherical microcapsules with smooth surfaces. The microcapsules formation ability of these mixtures correlated with the viscosity profile showed above since the viscosity of the biomaterial is one of the critical parameters for microcapsules formation (107). However, wrinkled surfaces in microcapsules were formed with 0.5% alginate 0.5% HA mixture and were discarded (Fig 2B1-B3). Moreover, we also discarded 1% alginate 0.1% HA mixture for the following assays since it contains a low concentration of HA, and any effect detected with this mixture should be enhanced in the 1% alginate 0.25% HA composition (108).

After the formation of microcapsules, HA should be retained within microcapsules to functionalize them and provide a biological effect on the embedded cells. Therefore, we proceeded to determine if HA was kept inside of the microcapsules. For this purpose, we formed a batch of microcapsules using 1.1 MDa FITC-labelled HA and quantified the HA content by measuring the fluorescence intensity. Alginate and alginate-HA microcapsules containing FITC-labelled HA displayed highly significant fluorescence ($p<0.001$) compared to no FITC-labelling (Fig 3A-C), confirming the presence of HA interacting with alginate in a 3D network within the microcapsules. We hypothesize that in this structure alginate and hyaluronate macromolecules are molded in a 3D structure where alginate acts as a solid crosslinked backbone, and HA is coupled in the network (109) since HA is a macromolecule predisposed to form hydrogen bonds (110).

We next proceeded to study the microcapsules surface using scanning electron microscopy (SEM), since the smooth surface related with the biocompatibility of alginate microcapsules is one of the main properties related for 3D scaffolds (111). The SEM micrographs showed some differences between 1.5% alginate (Fig 3D),

1% alginate 0.25% HA (Fig 3E), and 0.5% alginate 0.5% (Fig 3F) HA microcapsules surfaces, suggesting different biocompatibilities among the mixtures studied. Finally, we analyzed the swelling properties of the microcapsules, not detecting significant differences in core diameter expansion among 1% alginate 0.25% HA, 0.5% alginate 0.5% HA, and 1.5% alginate microcapsules (Fig 3G). These results indicate that the osmotic resistance of the hybrid microcapsules is enough to preserve them from the *in vivo* environment, as was previously tested with 1.5% alginate microcapsules (112).

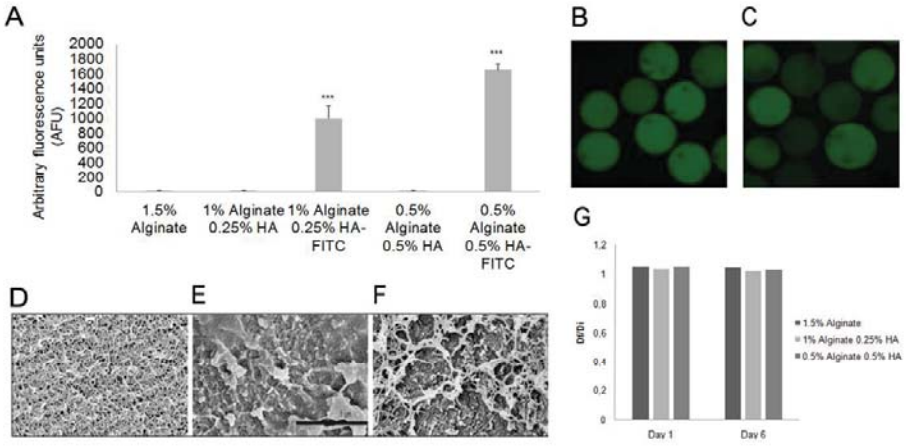

Figure 3.- Physical and chemical characterization of the biomaterial (A) HA-FITC content quantification of 1.5 % alginate, 1% alginate 0.25% HA, 1% alginate 0.25% HA-FITC, 0.5% alginate 0.5% HA and 0.5% alginate 0.5% HA-FITC. Micrographs by means of confocal microscopy of **(B)** 1% alginate 0.25% HA-FITC microcapsules and **(C)** 0.5% alginate 0.5% HA-FITC microcapsules. Microcapsules surface micrographs by SEM **(D)** 1.5% alginate, **(E)** 1% alginate 0.25% HA and **(F)** 0.5% alginate 0.5% HA. **(G)** Results from swelling assay of 1.5% alginate, 1% alginate 0.25% HA and 0.5% alginate 0.5% HA microcapsules expressed as Df/Di: final diameter/initial diameter were Di corresponds to day 0 and Df is indicated in the abscises axe. Note: Values represent mean ± SD. ***: $p<0.001$. Scale bar represents 1 μm.

With all these data in mind, we can conclude that 1% alginate 0.25% HA and 0.5% alginate 0.5% HA mixtures resemble the rheological behavior of 1.5% alginate, and can be extruded to form microcapsules, retaining HA within the microcapsules and therefore could mimic the ECM of a natural cell environment where HA is a major component.

3. HA incorporation within alginate microcapsules promotes cell survival, therapeutic factor release and chondrogenic differentiation of encapsulated MSCs.

Following our goal of using HA as a component in 3D differentiation of MSCs into pancreatic within alginate matrices, and after observing that HA combined with alginate is suitable for cell encapsulation, we studied if the presence of HA favors the viability of encapsulated MSCs before their differentiation, mimicking their natural environment. We selected D1-MSCs genetically modified to secrete erythropoietin (EPO) as sustainable drug delivery technology application, testing the vascular endothelial growth factor (VEGF) and EPO release after encapsulation within 1% alginate 0.25% HA and 0.5% alginate 0.5% HA microcapsules. Thus, we were able to encapsulate MSCs with 1% alginate 0.25% HA, similar to 1.5 % alginate. However, 0.5% alginate 0.5% HA mixture generated microcapsules that agglomerated and released cells outside the capsules, therefore discarding this hybrid biomaterial for our future studies.

We proceeded to assess the early apoptosis percentage by means of annexin V/propidium iodide staining and subsequent quantification by flow cytometry at days 1, 7, and 21 after encapsulation, no detecting significant differences between apoptotic cells percentages of MSCs encapsulated within 1% alginate 0.25% HA and 1.5% alginate at days 1 and 7 (Fig 4A). However, at day 21, we quantified a significant reduction of cell apoptosis ($p<0.05$) when cells were encapsulated within 1% alginate 0.25% HA compared to 1.5% alginate microcapsules. Moreover, cell death percentage quantified by calcein/ethidium staining and subsequent flow cytometry analysis did not show significant differences between 1% alginate 0.25% HA and 1.5% alginate at day 1 after encapsulation (Fig 4B). At day 7 post-encapsulation, we were able to detect a significant increase of cell viability ($p<0.001$), detecting also a highly significant reduction of cell death ($p<0,001$) at day 21 after encapsulation. Apoptosis and cell death results indicated that HA promotes the viability of encapsulated MSCs which is supported by studies showing that the incubation of

CD44+ chondrocytes with HA enhances cell viability, while reduces cell apoptosis by decreasing the mitochondrial DNA damage (113). After cell internalization, HA has been reported as a protective agent against DNA damage (114), leading to justify the cell apoptosis decrease registered in our experiments. Besides, previous results showed that the supplementation of adipose-derived MSCs culture medium with HA increases the cell growth rate, reducing the cellular senescence and promoting the cells differentiation potential (115).

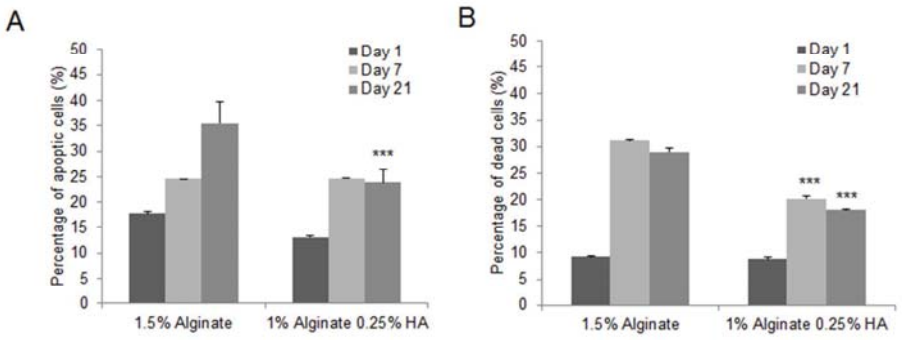

Figure 4.-Viability of D1-MSC-EPO encapsulated in 1% alginate 0.25% HA and 1.5% alginate microcapsules. (A) Early apoptotic cell quantification using flow cytometry after annexin/PI staining. **(B)** Dead cell quantification using flow cytometry after calcein/ethidium staining. Note: *: $p<0.05$ and ***: $p<0.001$.

Cell metabolic activity also allowed us to confirm the suitability of 1% alginate 0.25% encapsulation matrix, with a significant 3-fold increment ($p<0.001$) in encapsulated D1-MSC-EPO within 1% alginate 0.25% HA compared to 1.5% alginate microcapsules (Fig 5A). These data are supported by the previously described enhancement of cell metabolic activity detected on tendon-derived cells exposed to HA, indicating that HA also increases cell metabolic activity when is internalized in CD44+ cells (116). We also identified a significantly lower membrane damage percentage ($p<0.05$) in 1% alginate 0.25% HA compared to 1.5% alginate microcapsules at days 1 and 21 after encapsulation (Fig 5B). Our data demonstrate

that the HA presence in alginate microcapsules, not only improves the viability of the encapsulated D1-MSC-EPO, but also enhances their metabolic activity and their membrane integrity.

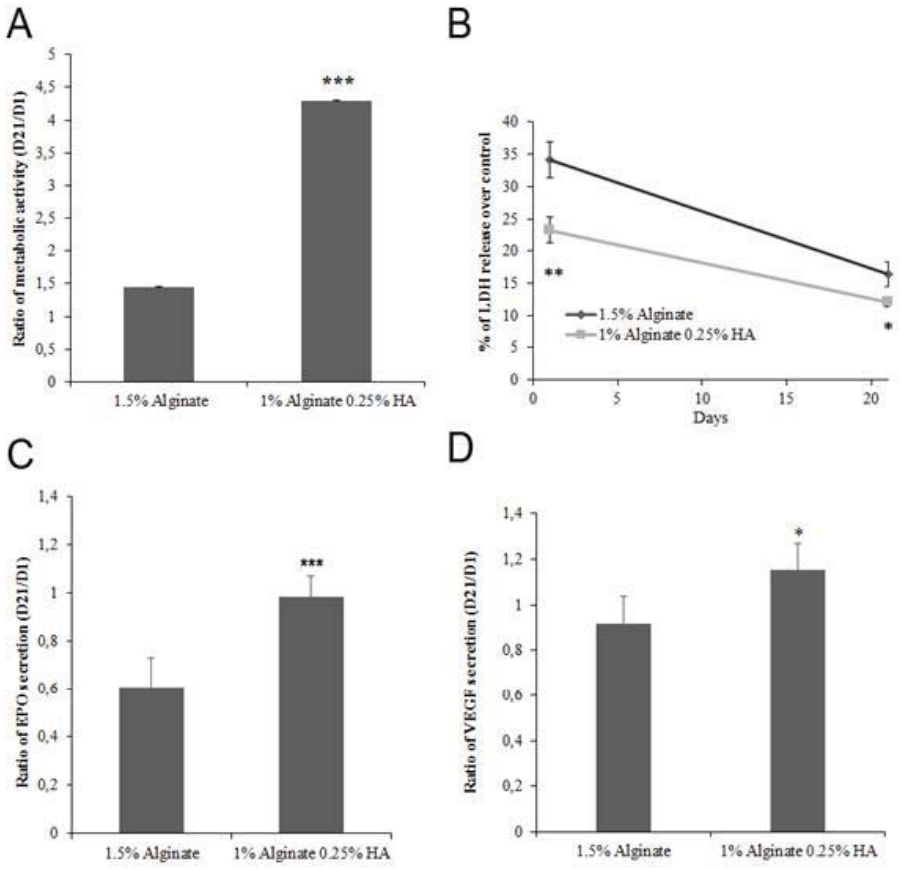

Figure 5.- Biological behavior of D1-MSCs encapsulated within 1.5% alginate and 1% alginate 0.25% HA (A) Ratio of metabolic activity between day 21 (D21) and day 1 (D1) after encapsulation. **(B)** Membrane damage at day 1 and 21 after encapsulation. **(C)** Ratio of EPO release between day 21 (D21) and day 1 (D1) after encapsulation. **(D)** Ratio of VEGF release between day 21 (D21) and day 1 (D1) after encapsulation. Note: Values represent mean ± SD. *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$.

We next quantify the EPO and VEGF release from alginate-HA microcapsules. The ratio of EPO release between days 21 and 1 after encapsulation showed a 2-fold significant increment ($p<0.001$) of encapsulated D1-MSCs-EPO within alginate-HA compared to alginate microcapsules (Fig 5C). Similar to EPO secretion, hybrid 1%

alginate 0.25% HA microcapsules provided higher VEGF release increment ($p<0.05$) than 1.5% alginate microcapsules (Fig 5D). These results indicate that HA presence within alginate microcapsules promotes the release of therapeutic factors, enhancing the capacity of alginate microcapsules as a sustainable drug delivery system, similar to HA-composed hydrogels (117).

Finally, we studied the ability of MSCs to differentiate into the three mesoderm lineages: adipogenic, osteogenic and chondrogenic (38). The differentiation potential of MSCs can be modulated by the environmental conditions, being HA one factor involved in the manipulation of cell differentiation potential. After differentiation into adipocytes, osteocytes, and chondrocytes of encapsulated D1-MSCs within 1.5% alginate and 1% alginate 0.25% HA, stained fat vacuoles were detected in the adipogenic differentiation without qualitative differences between D1-MSCs from 1.5% alginate and 1% alginate 0.25% HA (Fig 6). Moreover, for osteogenic differentiation, we identified a lack of qualitative differences in the calcification of the extracellular matrix (Fig 6). However, in the chondrogenic differentiation procedure, a qualitative increase of sulfated proteoglycan deposits was detected in 1% alginate 0.25% HA when compared to 1.5% alginate microcapsules (Fig 6). These results indicate that HA presence within alginate matrices promotes the chondrogenic differentiation of D1-MSCs through the induction of aggrecan and proteoglycan accumulation, nodule formation, and inhibition of TNF-alpha (118). Therefore, we can conclude that the inclusion of HA promotes the differentiation into chondrocytes, and it can be hypothesized that could also promote the differentiation into other cells types, such as insulin-producing cells (IPCs).

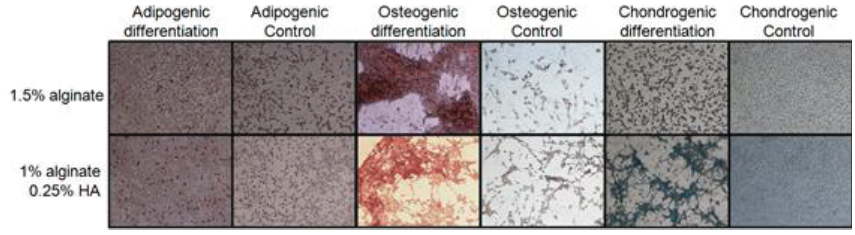

Figure 6. Differentiation potential of encapsulated D1-MSCs EPO in 1% alginate 0.25% HA and 1.5% alginate microcapsules. Microscopic images at 4× amplification 3 weeks after differentiation.

4. Encapsulated IPCs viability within hybrid alginate-HA microcapsules is enhanced, without affecting insulin release.

The combination of alginate and HA to form hybrid alginate-HA microcapsules for IPCs encapsulation has not been studied yet. Since our primary goal is the differentiation of MSC into IPCs using hybrid HA-alginate encapsulation, before performing the 3D differentiation, we studied if this type of microcapsules were suitable for IPCs encapsulation, determining how HA affects their viability. Hence, we studied for the first time the beneficial *in vitro* outcomes of IPCs encapsulation within microcapsules composed by alginate and high molecular weight HA, which is commonly synthesized by different islet endocrine cell types under regular conditions, becoming an abundant component of the mouse peri-islet ECM (119). For this purpose, we selected Ins1E cells as IPCs model, encapsulating 5x106 cells/mL within 1.5% alginate and 1% alginate 0.25% HA matrices to evaluate the behavior of encapsulated cell at days 1, 7, and 14 after encapsulation.

The quantification of early apoptosis percentage displayed a significant reduction ($p<0.05$) at days 1 and 7 after encapsulation within alginate-HA, compared to alginate, while, at day 14, the significance was even more pronounced ($p<0.01$) (Fig 7A). In the same way, live/dead cell percentage showed a lack of differences between alginate and alginate HA matrices at days 1 and 7 after encapsulation (Fig 7B). However, cell death percentage was significantly reduced ($p<0.001$) with HA presence in the encapsulation matrix compared to alginate matrix without HA at day 14 post-encapsulation, confirming the results observed in the apoptosis at this time point. These results are supported by several reports demonstrating that β-cell survival can be improved by the exposure of IPCs to whole ECM, or individual ECM components (120). For example, β-cell apoptosis can be reduced by culturing cells on laminin-5 enriched ECM scaffolds (121), and a cell viability enhancement has been detected when pancreatic islets are encapsulated within collagen-HA compared to collagen embedded or unembedded islets (122). Hence, the ECM plays an essential role in the β-cell culture, and the extensive presence of HA in the pancreatic islets'

natural environment suggests that the exogenous addition of HA can potentially enhance cell survival. Micrographs obtained after the staining of microencapsulated Ins1E cells at days 1, 7 and 14 after encapsulation confirmed an enhancement of cell viability after 14 days of encapsulation in the presence of HA (Fig 7C).

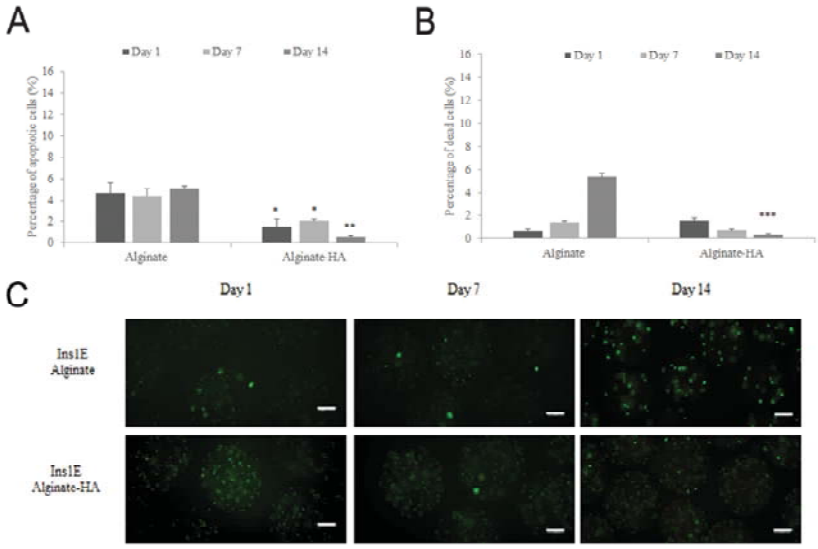

Figure 7.- Viability of Ins1E encapsulated cells. (A) Early apoptotic cell percentage quantification of Ins1E cells by means of flow cytometry after annexin/PI staining. **(B)** Cell death percentage quantification of Ins1E cells by means of flow cytometry after calcein/ethidium staining. **(C)** Fluorescence microscopy micrographs of encapsulated cells after calcein/ethidium staining. Note: Values represent mean ± SD. *: $p<0.05$; **: $p<0.01$ and ***: $p<0.001$. Scale bar: 100 µm.

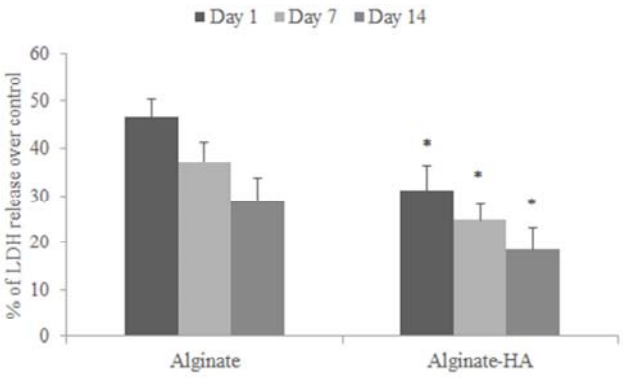

Figure 8.- Membrane integrity of Ins1E encapsulated cells. Cell damage percentage quantification of Ins1E cells by means of the Lactic Dehydrogenase in vitro toxicology kit. Note: Values represent mean ± SD. *: $p<0.05$.

In accordance with the previous results, membrane damage was progressively reduced from days 1 to 14 in both matrices, displaying a significant reduction of

membrane damages ($p<0.05$) in alginate-HA compared to alginate microcapsules at each time point (Fig 8). On this regard, some authors reported an enhancement of cell viability and membrane integrity when rat islets were cultured in HA-containing solutions (123), maybe due to the similarities of dynamic viscosity of HA hydrogels and natural soft tissues (124).

Since β-cell functionality, determined by the insulin secretion ability and the glucose responsiveness of β-cells can be modified by the cell environment provided by encapsulation biomaterial, we tested the insulin secretion ability of IPCs encapsulated within 1.5% alginate and 1% alginate 0.25% HA matrices. Data showed a progressive insulin production enhancement from day 1 to 14 without significant differences between alginate and alginate-HA microcapsules at each time point studied (Fig 9). The function of HA in the pancreatic islets ECM remains still uncleared. Some authors point HA as a molecule with the ability to promote the insulin secretion of IPCs, increasing the insulin secretion of HIT-T15 cells through the enhancement of connexin 43-mediated gap-junctional intercellular communications (121). However, HA represents also a negative stimulus for IPCs under determined conditions, such as the reduction of insulin production quantified in the pancreatic islet's low density after HA accumulation in autoimmune diabetes (125). The blocking of the laminin-5 binding site of β-cells cultured on complete pancreatic ECM resulted in a significative reduction of insulin, indicating that ECM components are essential for β-cell function development (126).

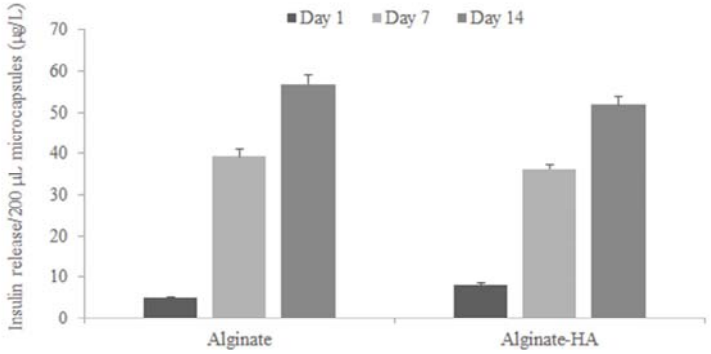

Figure 9- Insulin release of Ins1E encapsulated cells. Insulin release of Ins1E cells determined by ELISA after 24 hours of complete medium incubation. Note: Values represent mean ± SD.

We also studied the IPCs insulin response to glucose concentration, not detecting statistically significant differences between both microencapsulation matrices at days 1, 7, and 14 after encapsulation (Fig 10). These data indicate that HA is not affecting the IPCs functionality, and cells maintain their glucose responsiveness along the time.

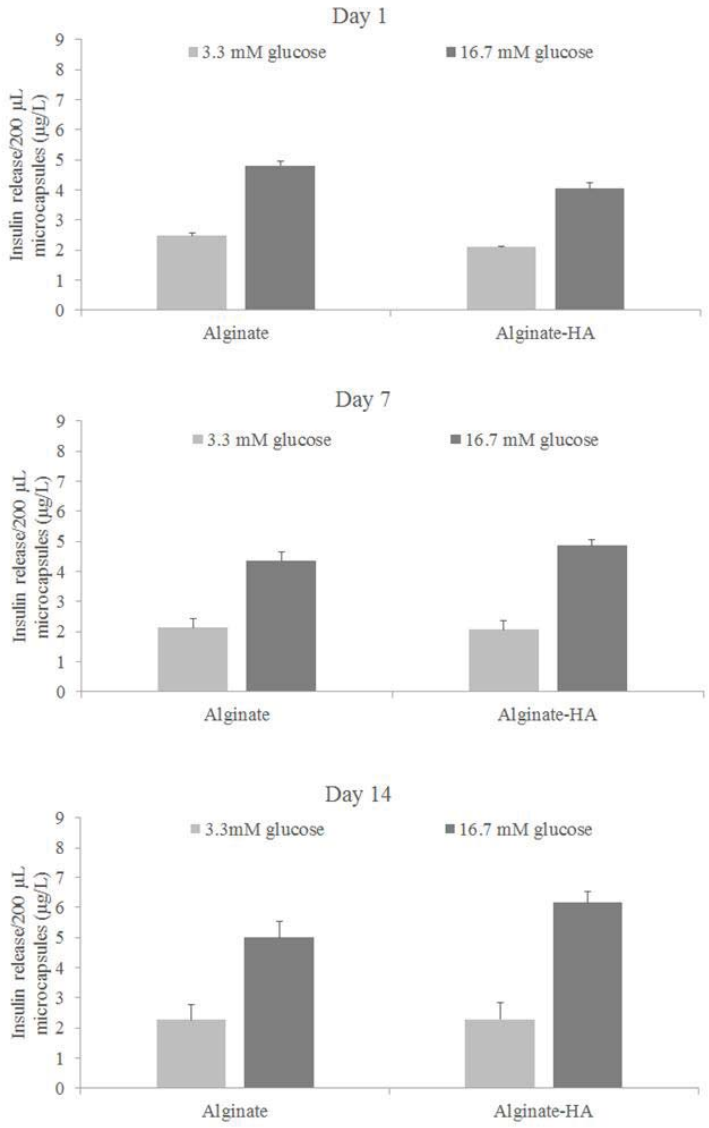

Figure 10.- Insulin release after glucose stimulation of Ins1E encapsulated cells.Insulin release of Ins1E cells determined by ELISA after 2 hours of incubation with 3.3 mM glucose Krebs-Ringer Bicarbonate Buffer and 2 hours of incubation with 16.7 mM glucose Krebs-Ringer Bicarbonate Buffer after 1, 7 and 14 days of encapsulation. Values represent mean ± SD.

Other biomaterials mixtures, such as HA-collagen hydrogels, have shown similar results holding the glucose sensitivity of encapsulated rat islets up to 28 days. After transplantation, these hydrogels were able to reverse diabetes for 80 weeks without the immune rejection or fibrotic capsule formation (122). With these data, we can conclude that HA does not affect negatively to insulin release of encapsulated cells within hybrid alginate-HA microcapsules, while improving their viability and membrane integrity compared to alginate microcapsules.

To study a closer real environment approach, we generated pseudoislets composed of Ins1E cells by liquefying the microcapsules core to aggregate IPCs and forming cell clusters (Fig 11A). We quantified the cell clusters survival without detecting significant differences in cell death percentage between alginate and alginate-HA encapsulated pseudoislets at days 1 and 7 after encapsulation (Fig 11B). Nevertheless, a statistically significant reduction ($p<0.01$) was quantified in alginate-HA encapsulated pseudoislets compared to alginate microcapsules at day 14. Similar to IPCs, the incorporation of ECM components to β-cell cultures or encapsulation biomaterials have shown to improve the β-cell survival and functionality. For example, hybrid hydrogels composed of alginate-collagen IV, alginate-fibronectin or alginate-laminin promoted cell viability of encapsulated pancreatic islets compared to alginate hydrogels by cell-matrix interactions restoring (127). Other studies pointed ECM molecules, such as collagen IV or laminin, as protecting agents of pancreatic islets against stress oxidation associated with ROS molecules and nitric oxide. This reduction in the stress oxidation damages resulted in a survival enhancement of pancreatic islets embedded within biomaterials containing ECM molecules compared to those enclosed without the presence of ECM molecules (128). Besides, the scaffolds formed by the combination of poly-lactide-co-glycolide and collagen IV can also improve mouse islet survival, restoring normoglycemia of diabetic transplanted mice after 3 days. However, diabetic mice transplanted with mouse pancreatic islets encapsulated with alginate displayed a restoring time to euglycemia of 17 days (129). Altogether, we can conclude that the presence of HA within hybrid

alginate-HA microcapsules enhance cell survival of pseudoislets similar to IPCs. However, we consider that more exhaustive studies should be performed to clarify the HA role on β-cells functionality, considering factors such as HA derivations, concentration or molecular weight.

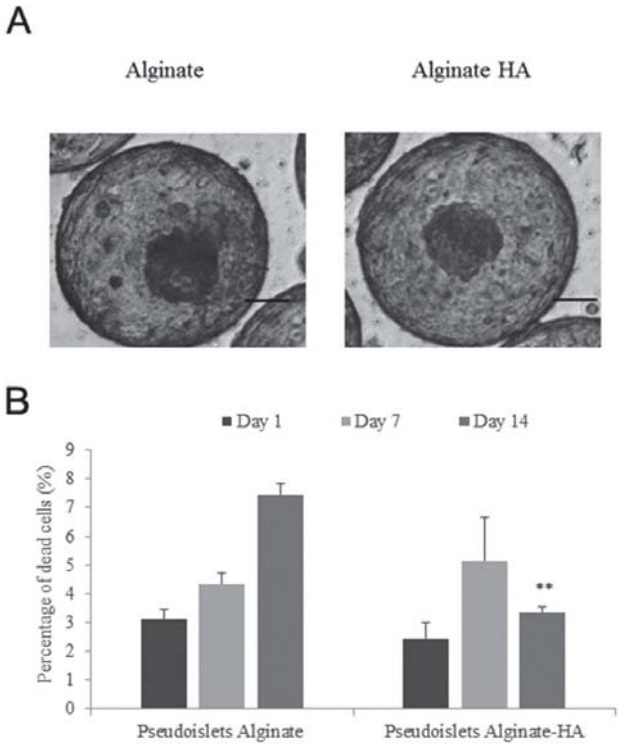

Figure 11- Viability of Ins1E pseudo-islets formed within liquefied microcapsules. (A) Brightfield micrographs of pseudo-islets formed with Ins1E cells after 14 days. **(B)** Cell death percentage quantification of liquefied microencapsulated Ins1E cells using flow cytometry after calcein/ethidium staining. Note: Values represent mean ± SD. **: $p<0.01$. Scale bar: 100 µm.

We hypothesized that Akt pathway could be involved in the mechanisms that HA activities to promote cell survival. Thus, it seems that HA through CD44 could activate the pathway PI3K/Akt, therefore phosphorylating Akt (130), which has important function within the nucleus (131), regulating transcription by phosphorylating FKHRL1, a member of the Forkhead transcription factor family. This leads to FKHRL1's interaction with 14-3-3 proteins and FKHRL1's sequestration in the cytoplasm, away from its transcriptional targets (Fig 12). In

fact, under conditions of growth factor deprivation, the PI3K/Akt pathway is inactivated, FKHRL1 is unphosphorylated at its Akt sites, and FKHRL1 accumulates in the nucleus where it may activate death genes, including the Fas ligand gene, and thereby participate actively in the process of apoptosis (132). Moreover, the phosphorylation of Akt induces Nrf2 accumulation. Under normal conditions, Nrf2 is constantly degraded via the ubiquitin–proteasome pathway in a Kelch-like ECH-associated protein (KEAP1)-dependent manner (133). But after the induction of the Akt phosphorylation by HA through the interaction with receptors CD44 and RHAMM (Hyaluronan-mediated motility receptor), the Keap1–Nrf2 connection and subsequent Nrf2 degradation process ceases, Nrf2 is stabilized, and can function as a transcriptional regulator through binding to antioxidant response element (ARE) on the promoter region of the target genes (134) (Fig 12).

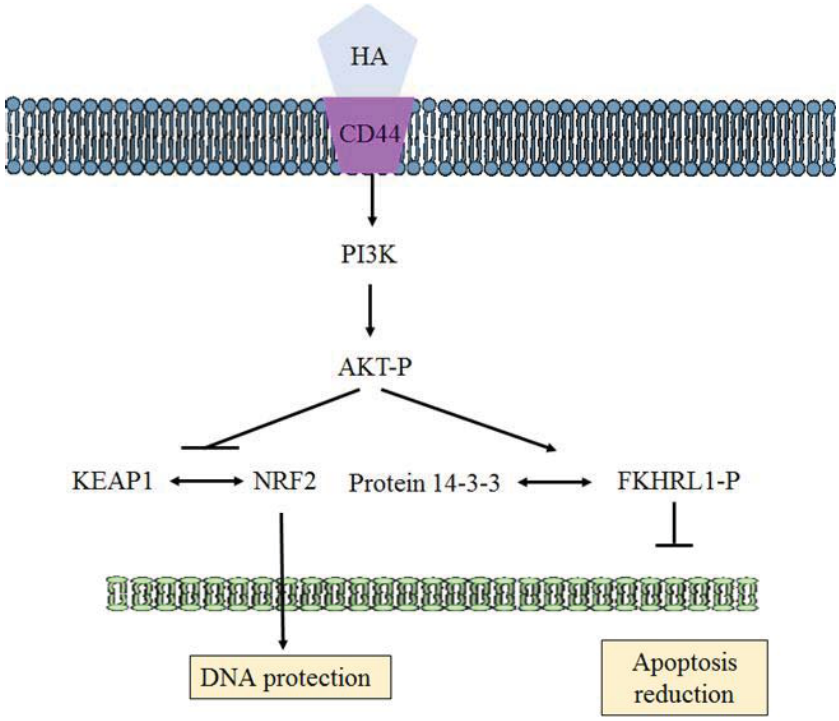

Figure 12.- Apoptosis pathway activated by HA and mediated by CD44. The interaction of HA and CD44 activates Akt phosphorylating (Akt-P) downstream FKHRL1 that interacts with 14-3-3 proteins sequestrating in the cytoplasm away from its transcriptional apoptotic targets. Moreover, Akt-P induces Nrf2 accumulation by inhibiting KEAP1 interaction and ubiquitin-proteasome pathway and allowing its function as transcriptional regulator.

5. HA within 3D alginate matrices promotes differentiation of MSCs into IPCs.

After determining that the presence of HA within alginate microcapsules is not only suitable for encapsulated MSCs and IPCs but also improves their viability, we aimed to direct the differentiation of hybrid alginate-HA encapsulated MSCs towards IPCs, with the goal of increasing their insulin release by getting more mature IPCs. We first isolated mouse bone- and pancreas-derived MSCs derived and used D1-MSCs as a positive control. All MSCs sources were characterized following the minimal criteria established by ISCT (135): plastic adherence, expression of cell surface defined markers and differentiation potential. Corroborating the first criteria proposed by ISCT to define MSCs (135), the adherence to plastic was observed in all the isolated cells (data not shown). Next, flow cytometry characterization showed positive staining for CD73 and SCA1 markers and a lack of expression of CD34, CD45, CD11b, CD19, or HLA-DR surface molecules as it has been previously defined by the ISCT (Fig 13) (135). However, we found some differences in the expression patterns among MSCs sources regarding CD105, CD44, and CD146. Thus, D1-MSCs and bone-derived cells displayed a lack of CD105 expression while pancreas-derived cells resulted in CD105+ (Fig 13). The ISCT described MSCs as CD105+, but some studies indicate that CD105- cells represent a subpopulation of MSCs and not any differentiated cell type. The differentiation of CD105- cells into the three mesodermal lineages results in adipogenic and osteogenic capacities enhancement compared to CD105+ cells, while chondrogenic differentiation is not affected (136). As expected for CD44, D1-MSCs and pancreas-derived cells expressed the surface marker, but bone-derived cells did not (Fig 13). However, this lack of CD44 expression in MSCs that exhibits multilineage differentiation abilities has been previously reported (137). Relating to CD146, only bone-derived cells expressed CD146 as defined by ISCT, with a lack of expression in D1-MSCs and pancreas-derived cells (Fig 13). Nevertheless, some studies have reported an absence of differences in cell expansion, proliferative capacity, differentiation potential or CFU formation between CD146+ or CD146- MSCs (138).

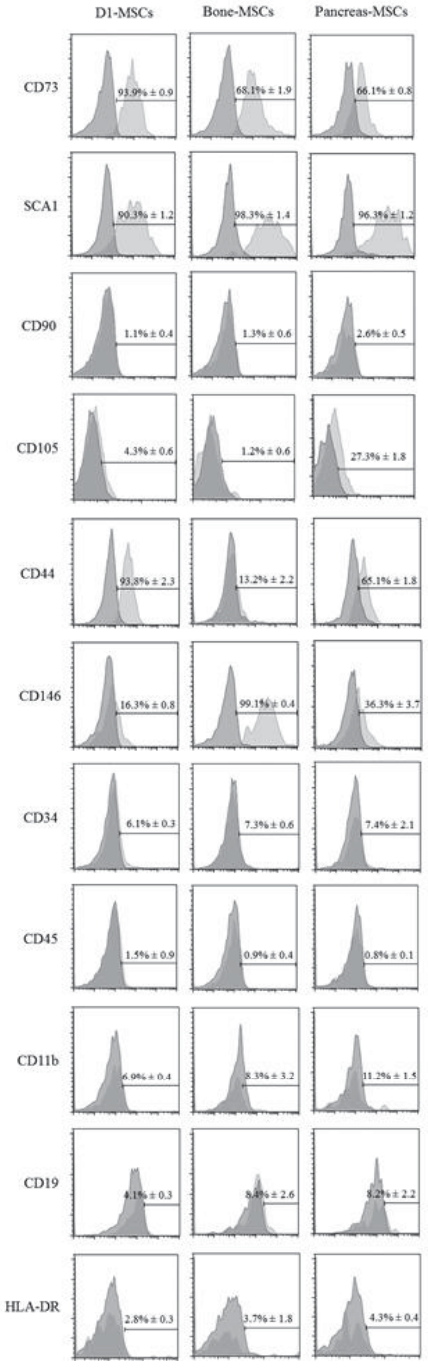

Figure 13.- Phenotype characterization of D1-MSCs, bone-derived cells, and pancreas-derived cells by flow cytometry. Dark and light histograms show isotype control and specific antibody stained sample respectively. Values represent mean ± SD for the positive stained cell population.

Finally, we differentiated the isolated cells into the three mesodermal lineages, adipogenic, osteogenic and chondrogenic to confirm the third minimal criteria of MSCs defined by ISCT. The results displayed fat vacuoles after the staining of adipogenic differentiation (Fig 14A), and the classical calcification of the osteogenic ECM (Fig 14A). The presence of sulfated proteoglycan deposits also indicated the differentiation of cells into functional chondrocytes (Fig 14A). These differentiation capacities pointed out all the isolated cells as MSCs (138). Also, some authors have suggested the colony forming unit ability as another critical capacity for MSCs characterization (139). On this regard, the isolated primary cells and D1MSCs displayed colony forming units' percentages upper to 40%, threshold proposed to maintain the clonogenic ability and be considered MSCs (140) (Fig 14B). With all these results, we confirmed that all the cell sources characterized fit the minimal standards established by ISCT to be considered MSCs.

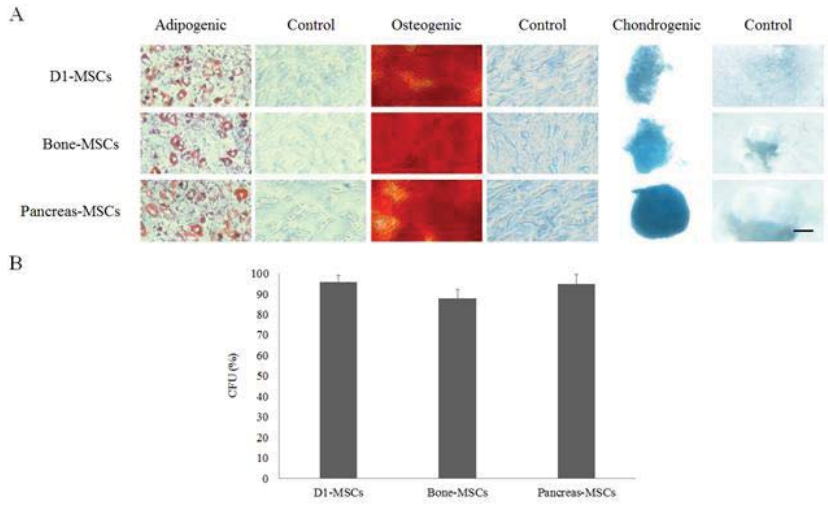

Figure 14- Differentiation and colony forming units potential of D1-MSCs, bone-derived cells, and pancreas-derived cells. (A) Micrographs after 3 weeks of adipogenic, osteogenic and chondrogenic differentiation. **(B)** Colony forming units percentage quantification. Scale bar: 10 µm. Values represent mean ± SD..

After ensuring the MSCs nature of the isolated primary cells, we proceeded to encapsulate the three MSCs sources within 1.5% alginate and 1% alginate 0.25%

HA microcapsules and test the differentiation ability of these cell sources into IPCs. We used a previously published 3-step protocol to induce the differentiation of the MSCs into IPCs (38), consisting of a first step inducing to differentiation into definitive endoderm by the combination of fetal bovine serum and retinoic acid. Next, the addition of N2, B27, epidermal growth factor, nicotinamide, and activin A to culture medium led to pancreatic endoderm formation. In the third step of the protocol, the supplementation of culture medium with exendin-4 increased the differentiated IPCs maturation state. During the differentiation protocol, some samples were collected for gene expression monitoring along the stages, while cell viability, metabolic activity, and insulin release were assessed at the final stage of differentiation (Fig 15).

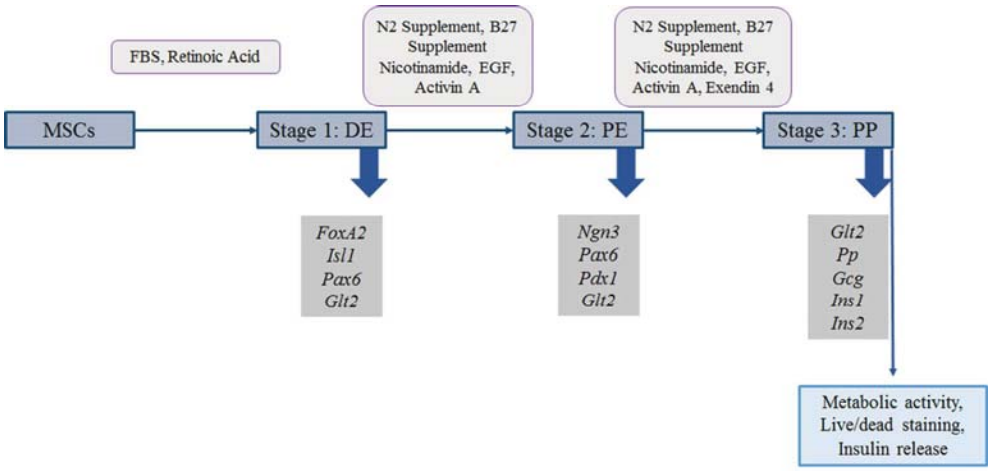

Figure 15.- Graphical abstract of differentiation protocol. MSCs: mesenchymal stem cells; DE: definitive endoderm; PE: pancreatic endoderm; PP: pancreatic progenitor. Arrows indicate that a sample aliquot was analyzed for genes expression. At the final stage a live/dead staining, metabolic activity, and insulin quantification were performed

Comparing cell metabolic activity of differentiated and undifferentiated cells, the reduction of metabolic activity was significantly lower ($p<0.01$) for each MSCs encapsulated within alginate-HA matrix compared to alginate microcapsules, suggesting a protective effect of HA of encapsulated differentiated cells (Fig 16A), or reflecting a higher rate of MSCs differentiation characterized with the notorious higher metabolic activity from β-cells (141). Micrographs after calcein/ethidium

staining confirmed the reduction of viable cells in both matrixes, compared to their respective undifferentiated cultures, and independently of the MSCs source studied (Fig 16B).

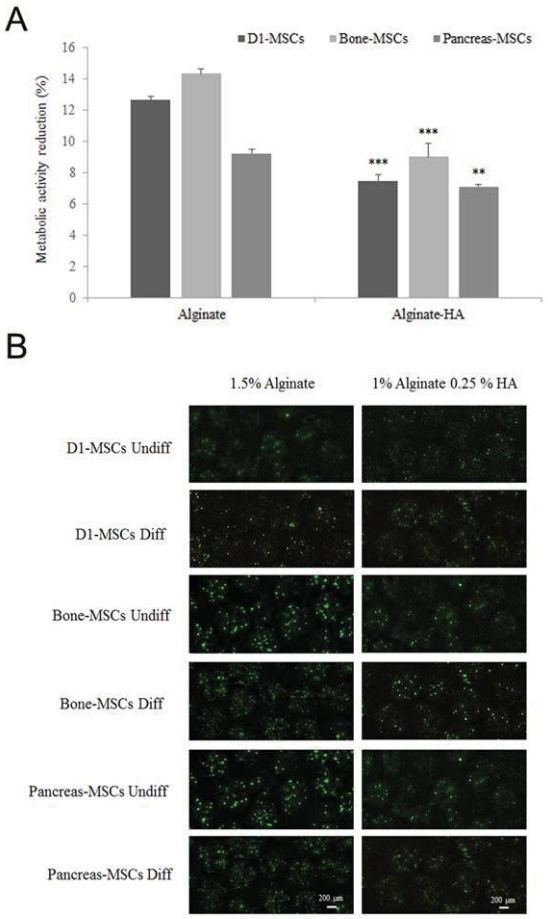

Figure 16.- Metabolic activity and viability of differentiated MSCs. (A) Metabolic activity ratio between final stage-differentiated and undifferentiated 1.5% alginate and 1% alginate 0.25% HA microcapsules cultures. Note: ***: $p<0.001$ and **: $p<0.01$ **(B)** Micrographs of differentiated cells after calcein/ethidium staining. Scale bar: 200 µm.

Finally, we monitored the gene expression of the main markers involved in the differentiation towards IPCs to check the differentiation progression along the 3 stages of differentiation (Fig 15). The gene markers that we selected were forkhead box protein A2 (*Foxa2*), islet 1 factor (*Isl1*), neurogenin (*Ngn3*), paired box protein (*Pax6*), pancreatic duodenum homeodomain (*Pdx1*), glucose transporter 2 (*Glt2*),

pancreatic polypeptide (*Pp*), glucagon (*Gcg*), insulin 1 (*Ins1*), and insulin 2 (*Ins2*). *FoxA2* gene expression displayed a progressive expression reduction along the 3 differentiation stages, independently of the MSCs source studied (Fig 17A). These results are consistent with the embryoid development since this marker has been described in the early endoderm (142), downregulating until a minimal expression in adult foregut and hindgut endoderm-derived tissues, such as the pancreas (143). Also, a soft *FoxA2* expression at the final stage of the differentiation plays a key role in the maintenance of adult β-cell function by the regulation of other β-cell transcription factors expression (144). Concerning to *Isl1* expression, during embryogenesis, its expression appears at the beginning of the endoderm formation, while its late expression indicates a lack of maturity of the resulting β-cells (145). We detected a lack *Isl1* expression in all the MSCs sources, followed by an upregulation at definitive endoderm stage, and a progressive downregulation to almost disappear at the final stage (Fig 17B).

Ngn3 expression resulted in an upregulation ($p<0.01$) at stage 2, the pancreatic endoderm formation, and downregulated again to minimal detection at the end of stage 3 (Fig 18A). However, D1-MSCs showed significantly higher upregulation ($p<0.05$) of *Ngn3* at stage 1 of differentiation, instead of at stage 2, may be related with the *Ngn3* expression variations in the embryo development. Thus, some consecutive activations of *Ngn3* expression during the murine pancreatic tissue development directing the formation to glucagon-producing α-cells when *Ngn3* is activated between E8.5 and E 12.5, to pancreatic polypeptide-cells and insulin-producing β-cells when *Ngn3* is activated between E12.5 and E16.5, or to somatostatin-producing δ-cells when *Ngn3* is activated between E14.5 and E16.5 (146). In fact, encapsulated D1-MSCs within alginate-HA microcapsules, that expressed earlier *Ngn3*, were able to express glucagon at the final stage of differentiation (Fig 19B). Pax6 showed a consistent gene expression mainly detected at the first stages (147), progressively downregulated to almost no detection at the final stage, independently of the MSCs source studied (Fig 18B).

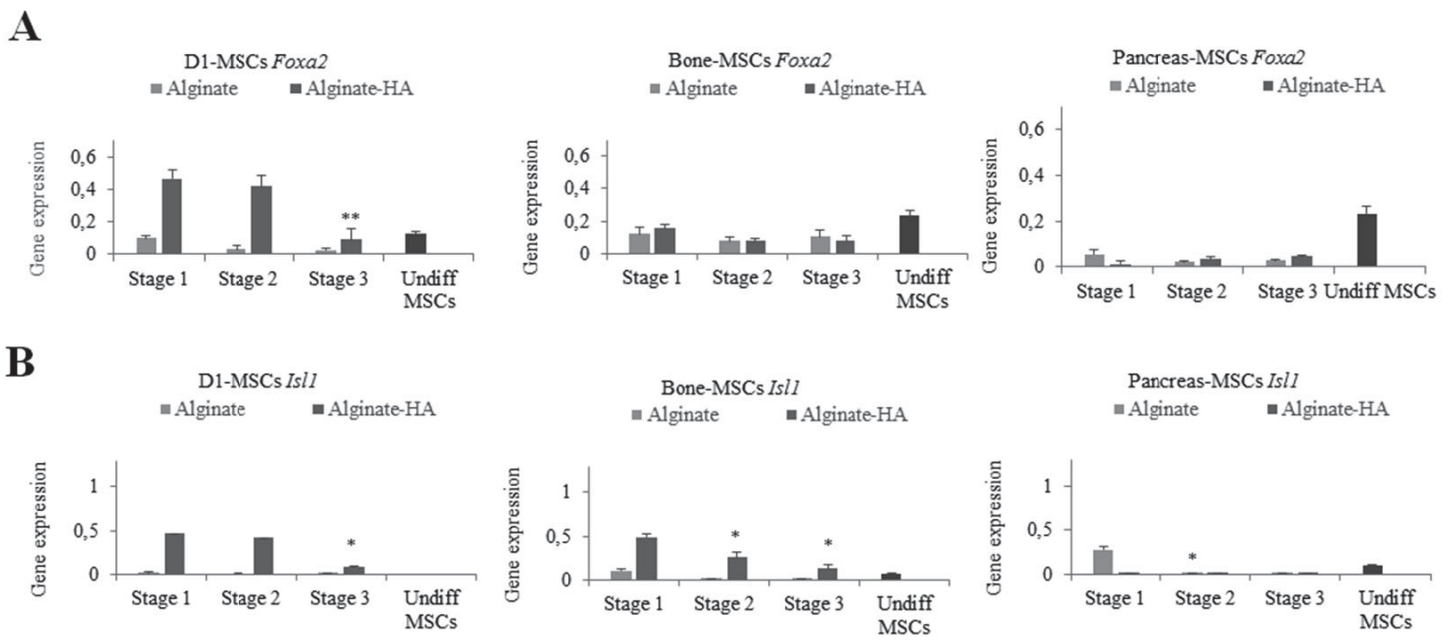

Figure 17.- Gene expression of main IPCs differentiation interest genes. Relative semi-quantification of **(A)** *FoxA2* and **(B)** *Isl1*, at the 3 stages of differentiation from 1.5% alginate and 1% alginate 0.25% HA microcapsules. Data represent the fold regulation between the gene of interest and the housekeeping. Note: **: $p<0.01$ and *: $p<0.05$

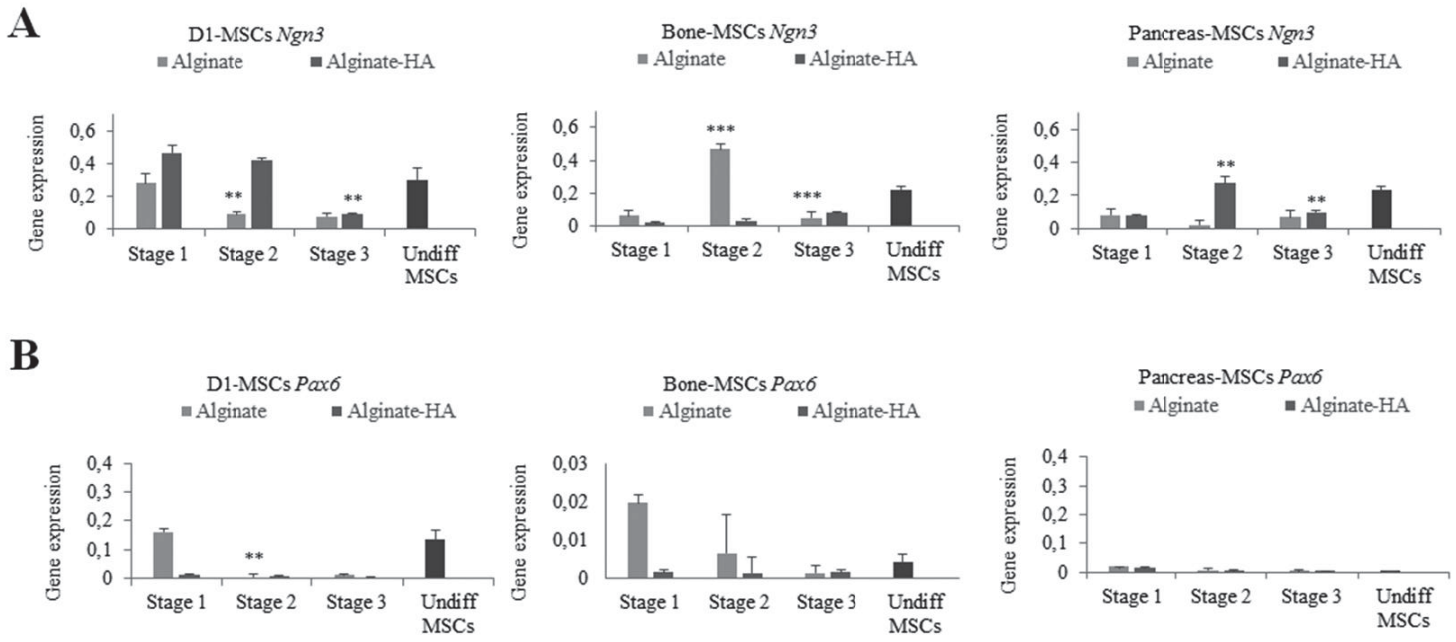

Figure 18.- Gene expression of main IPCs differentiation interest genes. Relative semi-quantification of **(A)** *Ngn3* and **(B)** *Pax6* at the 3 stages of differentiation from 1.5% alginate and 1% alginate 0.25% HA microcapsules. Data represent the fold regulation between the gene of interest and the housekeeping. Note: ***: $p<0.001$ and **: $p<0.01$

Pdx1 is a critical gene during β-cell development (148), and its expression in IPCs (149) or pancreatic islets (150) influences the cell differentiation directly, resulting its presence indicative of cell functionality. This marker also impacts the differentiation of IPCs through the modulation of the temporal expression of *Ngn3* expression (151). *Pdx1* expression in D1-MSCs was upregulated at stage 2, the definitive endoderm stage, followed by a significant downregulation ($p<0.01$), no detecting any upregulation in bone marrow and pancreas-derived MSCs (Fig 19A). The lack of *Pdx1* expression in mice embryos results in pancreas agenesis but, an absence of *Pdx1* expression during a differentiation procedure is not indicative of IPCs deficiency (152). Some authors obtained *Pdx1* negative IPCs which maturated after mice transplantation, displaying similar *in vivo* functionality than β-cells (153). Concerning *Glt2*, the glucose transporter isoform required for glucose sensing in β-cells (154), we did not quantify differences along the differentiation stages among the MSCs or encapsulation matrices studied (Fig 19B). These results were expected because during embryogenesis the expression of *Glt2* by epithelial cells is required for the aggregation of β-cells in the islets of Langerhans (155).

Regarding the endocrine hormones, *Pp* was almost not detected along the differentiation stages into β-cells from the different MSCs sources (Fig 20A), being upregulated in bone marrow-derived MSCs and D1-MSCs at the final stage of differentiation, but not in pancreas-derived MSCs, indicating that the differentiation of bone-derived MSCs and D1-MSCs led to a heterogeneous population with a *Pp* cells presence, while the differentiation of pancreas-derived MSCs did not. Likewise, D1-MSCs significantly upregulated *Gcg* ($p<0.01$) at the last stage of differentiation while pancreatic-derived MSCs and bone-derived MSCs displayed a lack of *Gcg* expression (Fig 20B). The quantification of both pancreatic polypeptide and glucagon-secreting cells is not indicative of a lack of maturity since some authors described poly-hormonal populations during embryogenesis (156), after stem cell differentiation procedures (157) or even in established insulin-producing cell lines (158).

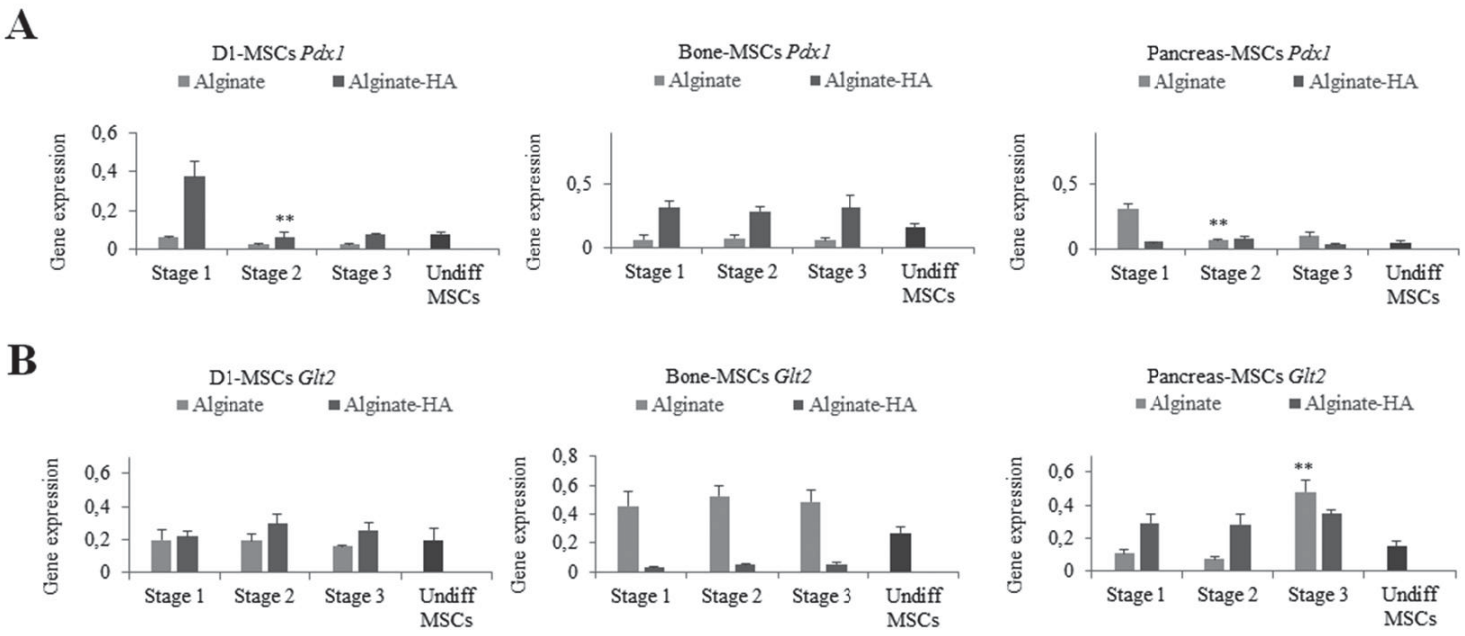

Figure 19.- Gene expression of main IPCs differentiation interest genes. Relative semi-quantification of **(A)** *Pdx1* and **(B)** *Glt2* at the 3 stages of differentiation from 1.5% alginate and 1% alginate 0.25% HA microcapsules. Data represent the fold regulation between the gene of interest and the housekeeping. Note: **: $p<0.01$

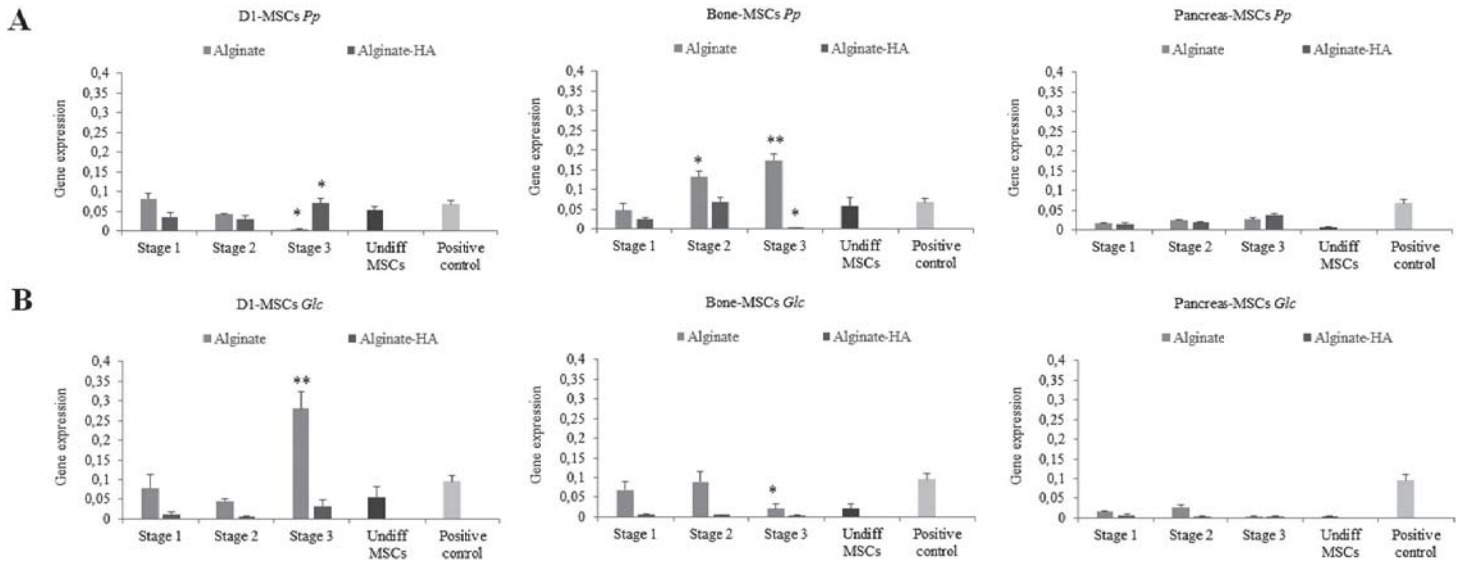

Figure 20.- Gene expression of main IPCs differentiation interest genes. Relative semi-quantification of **(A)** *Pp* and **(B)** *Gcg* at the 3 stages of differentiation from 1.5% alginate and 1% alginate 0.25% HA microcapsules. Data represent the fold regulation between the gene of interest and the housekeeping. Note: **: $p<0.01$ and *: $p<0.05$

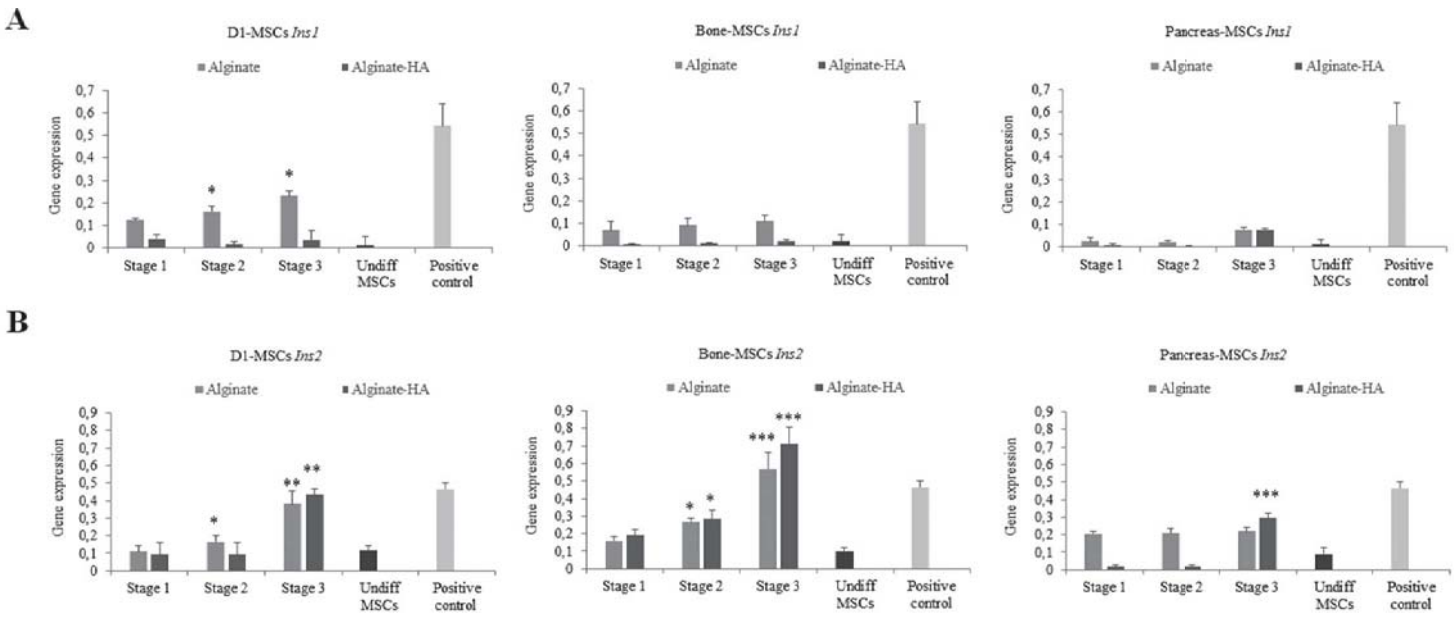

Figure 21.- Gene expression of main IPCs differentiation interest genes. Relative semi-quantification of **(A)** *Ins1* and **(B)** *Ins2* at the 3 stages of differentiation from 1.5% alginate and 1% alginate 0.25% HA microcapsules. Data represent the fold regulation between the gene of interest and the housekeeping. Note: ***: $p<0.001$; **: $p<0.01$ and *: $p<0.05$

Finally, we quantified the expression of *Ins1* and *Ins2* since murine and rat insulin protein is encoded in a two-gene system. Data showed a statistically significant upregulation ($p<0.01$) of *Ins2*, leading to cells with the ability to produce insulin after the differentiation procedure, independently of the MSCs source or the encapsulation matrix (Fig 21A-B). The insulin protein quantification confirmed the insulin release of alginate and alginate-HA microencapsulated cells in the supernatants. All differentiated MSCs displayed an insulin release enhancement compared to undifferentiated MSCs (Fig 22). The HA presence significantly promoted ($p<0.05$) the insulin release to each MSCs compared to alginate microcapsules. Moreover, encapsulated pancreas-derived MSCs within alginate-HA biomimetic matrix displayed a significantly higher insulin release increment ($p<0.001$) after differentiation compared to alginate encapsulated cells, maybe explained by the pancreas-derived MSCs epigenetic features closer to pancreatic progenitors, leading to a positive influence on an effective differentiation of MSCs into IPCs (159).

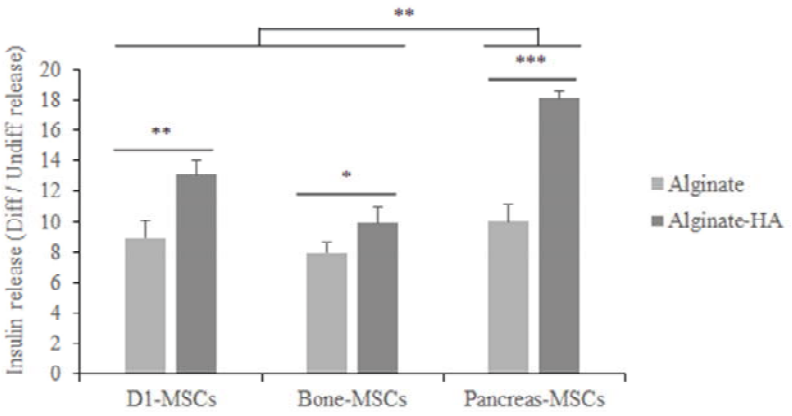

Figure 22.-Insulin production of differentiated IPCs. Insulin release increment after differentiation of MSCs within 1.5% alginate and 1% alginate 0.25% HA microcapsules. Insulin ratios were calculated as the quotient between insulin release at differentiated and undifferentiated states. Note: Values represent mean ± SD. ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$.

Altogether, we can conclude that the 3D differentiation within alginate microcapsules of MSCs towards IPCs, using differentiation protocols previously described for monolayer cultures, is promoted by the presence of HA through the

recreation of an *in vivo* environment. Moreover, the lack of expression of *Pp* and *Gcg*, together with the higher insulin release quantified, afford us to conclude that pancreas-derived MSCs are more optimal source for differentiation towards IPCs than bone marrow-derived MSCs. Finally, it is remarkable that the combination of pancreas-derived MSCs and hybrid alginate-HA matrices provides the closest approach to β-cells embryogenic development, highlighting the importance in pharmaceutical technology of the appropriate selection of cell source and biomimetic biomaterial reproducing the biological stimulus from natural ECM.

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Chapter 7

Conclusions

According to the results obtained in the previously described experiments, we can conclude that:

1) 1% alginate 0.25% HA and 0.5% alginate 0.5% HA mixtures are suitable biomaterials for cell microencapsulation, resembling the rheological behavior of 1.5% alginate, while retaining HA within the microcapsules, therefore, mimicking the ECM of a natural cell environment where HA is a major component.

2) HA presence within alginate microcapsules at 0.25% (w/v) concentration improves encapsulated D1-MSC EPO viability, metabolic activity, and membrane integrity, enhancing the therapeutic factors release, and therefore, improving the capacity of alginate microcapsules as a sustainable drug delivery system. Moreover, its inclusion promotes the differentiation into chondrocytes, among the three mesodermal lineages.

3) IPCs viability and membrane integrity are improved by alginate encapsulation in the presence of 0.25% (w/v) HA concentration, not affecting negatively to their insulin release. Similarly, encapsulated pseudoislets viability within hybrid alginate-HA microcapsules is enhanced

4) 3D differentiation within alginate microcapsules of MSCs towards IPCs, using differentiation protocols previously described for monolayer cultures, is promoted by the presence of HA through the recreation of *in vivo* environments, being pancreas-derived MSCs the most appropriated cell source for differentiation towards IPCs, among the MSCs sources studied.

5) More exhaustive studies that clarify the role of HA on β-cells functionality or β-cells development, considering factors such as HA derivations, concentration or molecular weight, will shed light on the future 3D differentiation protocol from MSCs into more mature IPCs, by implementing biomimetic biomaterials and 3D technologies.