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Pancreatic endocrine differentiation potential and immunomodulatory properties of different postnatal progenitors

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There is no end. There is not beginning. There is only the infinite passion of life.

Federico Fellini

Introduction

1 The Pancreas

1.1 Organogenesis of the pancreas

Foregut endoderm is composed of two regions, the ventral part that gives origin to the thyroid gland, lungs, liver and the ventral part of the pancreas. Meanwhile, the dorsal region gives origin to the intestine and the dorsal part of the pancreas. Thus, during embryogenesis, the pancreas develops from the two buds originating from the endodermal lining of the duodenum, which take the names of pancreatic dorsal and ventral anlagen due to their localization. The ventral pancreas and the common bile duct are carried behind the duodenum and into the dorsal mesentery when the duodenum rotates to the right forming a C-shaped loop. The ventral pancreas soon makes contact and fuses with the dorsal pancreas. The ventral bud forms the uncinate process and the inferior part of the head of the pancreas. The remaining part of the gland is derived from the dorsal bud (**figure 1**). Both the dorsal pancreas and the ventral pancreas possess a large duct. After the fusion, the main duct of the ventral bud makes an anastomotic connection with the duct of the dorsal bud. The portion of the dorsal pancreatic duct between the anastomotic connection and the duodenum normally regresses, leaving the main duct (**duct of Wirsung**) the definitive

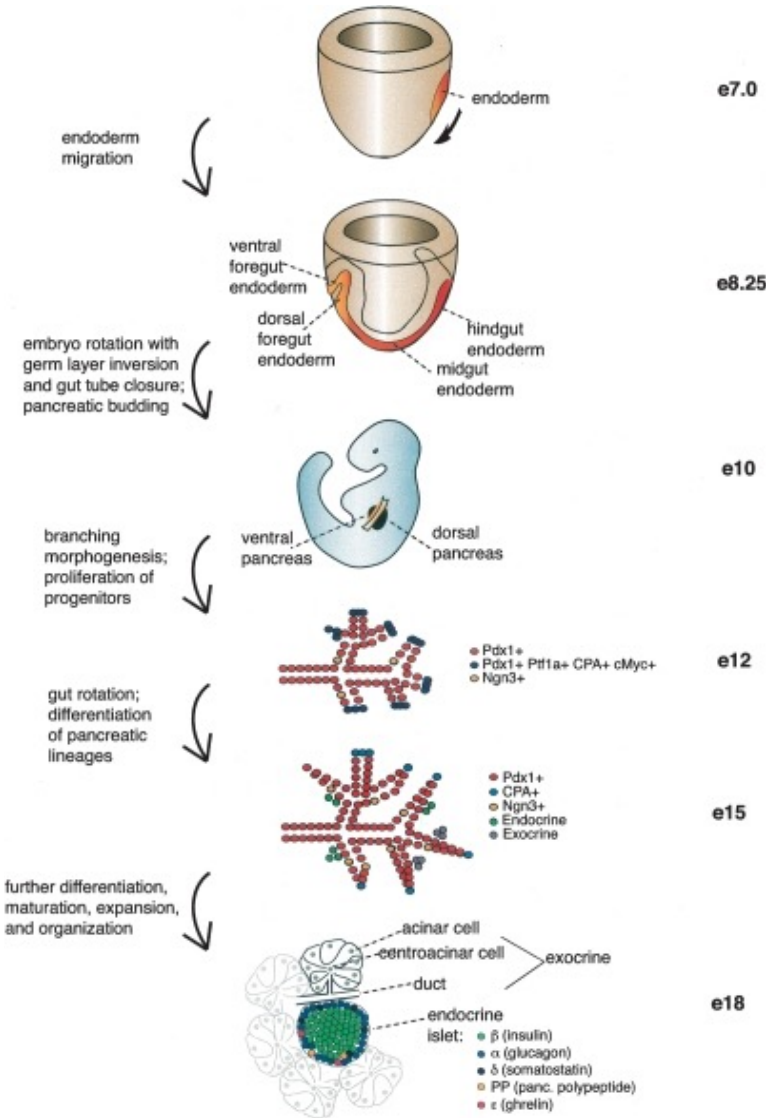


Figure 1. Pancreatic Organogenesis. Oliver-Krasinski *et al.*,2008.

outlet from the pancreas into the duodenum that takes the name of **main pancreatic duct**. When that proximal part of the dorsal pancreatic duct persists. It takes the name of **accessory pancreatic duct** (**of Santorini**). The pancreatic duct, together with the bile duct, enters the duodenum at the site of the **major papilla** (**ampulla of Vater**); the entrance of the accessory duct is named the **minor papilla**. In about 10% of all cases, the duct system fails to fuse and the original double system persists. The system of all these ducts, draining into the main pancreatic duct, enters in contact with the last portion of the biliary tree in the immediate proximity of the duodenum, giving rise to the hepatopancreatic common duct at the site of the major papilla.

The adult pancreas present salmon pink in color and a firm, lobulated smooth surface. The main portion of the pancreas is divided into four parts: head, neck, body and tail. In adult, it measures between 12 and 15 cm in length and it is shaped shaped like a flattened 'tongue' of tissue, thicker at its medial end (head) and thinner towards the lateral end (tail). The adult pancreas is the largest of the digestive glands and performs a range of both endocrine and exocrine function. The main part of the gland is exocrine and secretes a group of enzymes involved in the digestion of lipids, carbohydrates, and proteins. The exocrine portion consists of a large number of acini, which are connected to a secretory duct system. It is a branched ductal and acinar gland, surrounded and incompletely lobulated by delicate loose connective tissue [1-3]. The endocrine portion of the pancreas consists of **islets of Langerhans**, cluster of hormone-producing cells scattered among

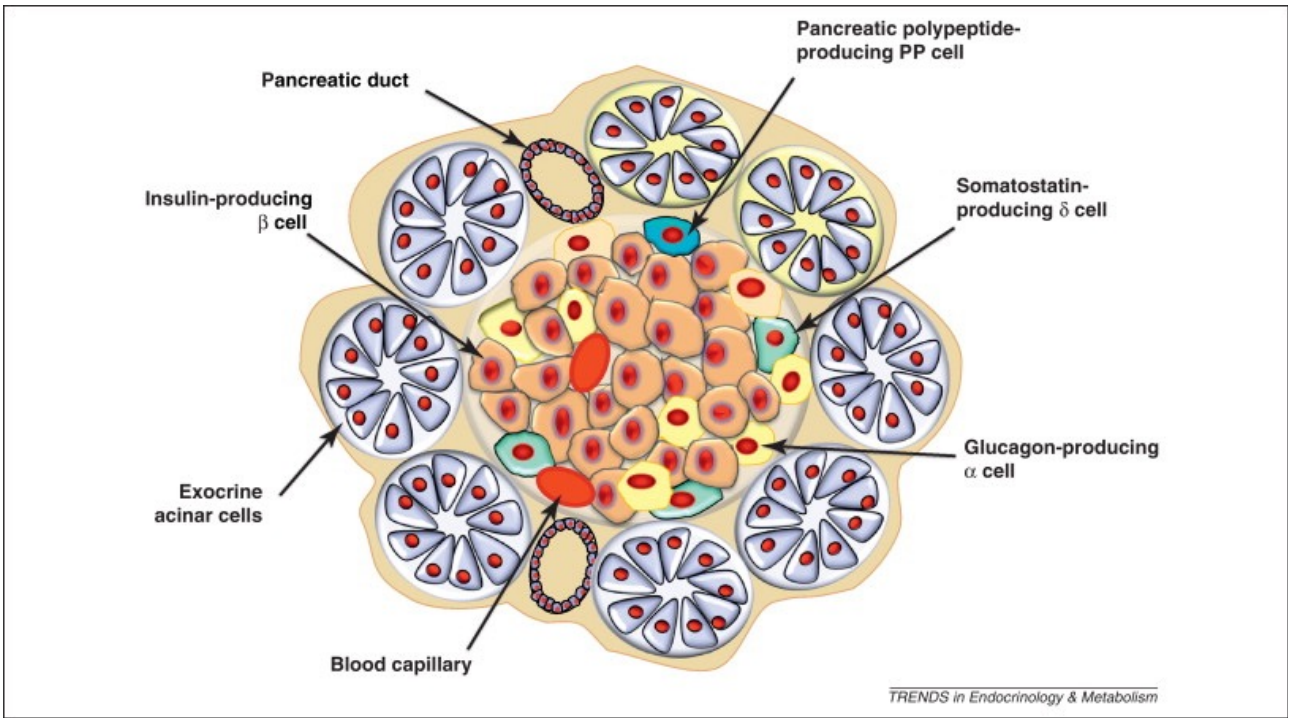


Figure 2. Human pancreatic islet. Efrat *et al*., 2012.

the exocrine tissue (**figure 2**). The human adult pancreas may contain more than a million islets, usually most numerous in the tail. The islets of Langerhans represent only the 1-2% of the total mass of the pancreas and most of them measure 50-200 μm in diameter. Like other endocrine tissues, human islets are are highly vascularized, as endocrine cells secrete their hormones in a network of capillaries to exert systemic effect. Islets typically consist of five types of secretory endocrine cells, namely insulin-producing beta cells, glucagon-producing alpha cells, somatostatin producing delta cells, ghrelin-producing epsilon cells and pancreatic polypeptide-producing (PP) cells [4]. Recently, notable cytoarchitectural differences between mouse and human islets have been reported. Beta cells are the main cellular component of islets in most species, representing 60% to 80% of islet cells in rodents and 50% to 70% in humans. In islets from mice and other rodents, beta cells are located predominantly in the central core while alpha and delta cells in the periphery forming a mantle. In human and monkey islets, alpha cells are not localized in the periphery but are rather dispersed throughout the islet. Beta cells are intermingled with other islet cells in primates (both humans and monkeys), and this has been suggested to increase beta cell function, as primate beta cells respond to low concentrations of glucose (1 mM) to which normal mouse islets are blind [5,6].

1.2 Molecular regulation

Foregut endoderm is composed of two regions, the ventral endoderm and the dorsal endoderm. During embryogenesis, dorsal endoderm is in contact with the notochord and ventral pancreas is close to the cardiac mesoderm. **Sonic Hedgehog** (*Shh*) is highly expressed throughout the gut, where it mediates interactions between the gut endoderm and the surrounding mesoderm. However, during mouse development at stage e8.5, there is a region of the epithelium where Shh is specifically absent: the Pdx1+/Ptf1α+ pre-pancreatic endoderm [7,8]. Knockout experiments showed that the entire pancreas originates from *Pdx1*+ progenitor cells [9]. The concerted action of both the transcription factors ***Pdx1*** and ***Ptf1α*** is necessary for the initiation of the pancreatic program. One of the earliest events is the repression of Sonic Hedgehog (Shh) by the notochord through pro-pancreatic factors like **activin B** and **FGF2**. This in turn promotes Pdx1 expression in adjacent pancreatic endoderm. Thus, *Shh* repression and activation of *Pdx1* and *Ptf1a* are key events of pancreatic specification. Shh could be chemically inhibited in vitro by the steroid alkaloid cyclopamine stimulating the pancreatic differentiation, as the Pdx1 expression is no longer

restricted throughout the posterior foregut [10]. In the ventral portion of the pre-pancreatic region, signals deriving from the cardiogenic mesenchyme (chiefly of the FGF family), repress firstly *Shh* and subsequently induce liver formation, whereas a low concentration of these signals leads to a default differentiation into the ventral pancreas. After the patterning signals from the mesenchyme [11] and the notochord [12], blood vessels impart inductive instructions to the developing endoderm [13]. The early endoderm and the pre-pancreatic region, indeed, express **VEGF** and could thus attract and induce maturation of nearby vessels [14]. The dorsal bud emerges in the proximity of the dorsal aorta, whereas the ventral bud appears close to the vitelline veins. Thus, blood vessels and factors derived from the endothelium represent major morphogenetic agents in pancreatic specification [15]. The pathway controlling cell specification from ***Ngn3*+/Hes1-/*Ptf1α*-** endocrine committed progenitors is still a field of active investigation [16]. Several transcription factors have been identified with a pivotal role in the steps leading to cell differentiation. Animals lacking ***Nkx2.2*** [17] and ***Nkx6.1*** [18], two members of the NK family of homeodomain proteins, have defects in cell formation. *Nkx2.2* is expressed in the early pancreatic epithelium and then becomes restricted to *Ngn3*+ progenitors. Later on, it can be found in endocrine cells with the exception of delta cells [19]. *Nkx2.2* null mutants develop with no detectable beta cells, a major reduction in alpha cells, minor effects on PP-cells and no effects on delta cells [17]. Thus, *Nkx2.2* seems to be a marker of multipotent progenitors early in pancreatic commitment and with a subsequent role in α, β and PP lineages. Similarly, *Nkx6.1* is expressed in the committed pancreatic endoderm and its expression is subsequently restricted to beta cells [20], supporting a role in multipotent and endocrine-committed progenitors [18]. ***Nkx6.2*** seems to have a partial redundancy with *Nkx6.1,* and *Nkx6.1/ Nkx6.2* double mutants cause a 92% reduction in the generation of beta cells [21]. The central role of another transcription factor, the insulin-promoter binding ***BETA2/NeuroD***, is evidenced by the fact that the murine knockout model develops a pancreas with a dramatic reduction in the number of beta cells, impaired islet morphogenesis and additional abnormalities in the exocrine pancreas [22]. ***Pax6*** is another transcription factor with a major role in multipotent endocrine-committed progenitors and alpha cell commitment, since knockout mice exhibit an almost complete absence of alpha cells and a significant reduction and abnormal distribution of all other hormone-producing cells within the islet [23,24]. *Rfx6* is a transcription factor downstream of *Ngn3* involved in the commitment of progenitors for all endocrine cells except polypeptide-producing PP cells [25]. ***Pax4,*** a member of the *Pax* family, appears shortly after endocrine specification, co-localizing with

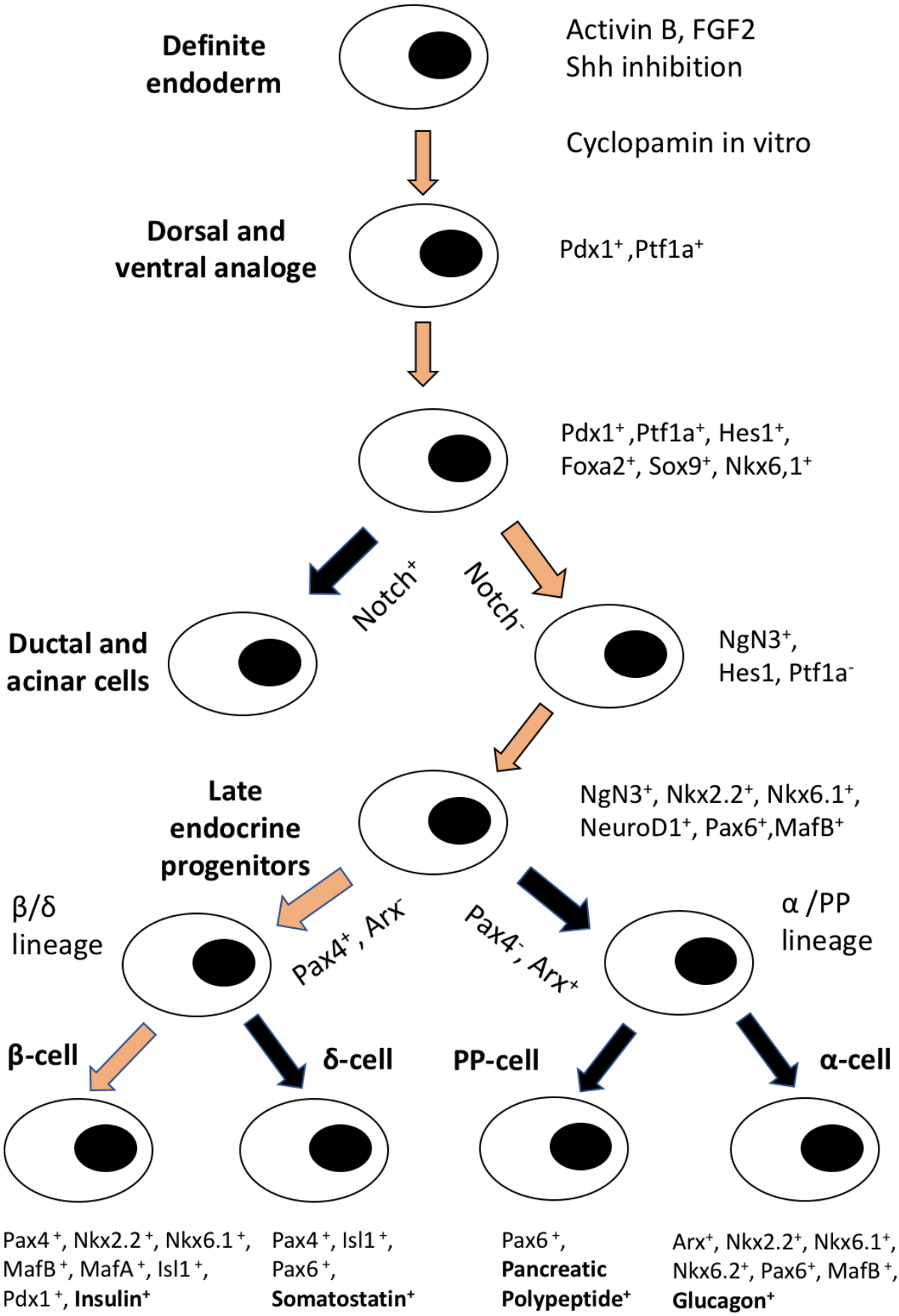


Figure 3. Pancreatic endocrine commitment and differentiation pathway.

Ngn3 [26] and probably representing one of its targets [27,28]. *Pax4* acts downstream of *Ngn3*, *Nkx2.2,* and *Nkx6.1* as a major switch in the definition of β/δ cell progenitors, inhibiting ***Arx*** and the lineage selection towards α/PP cells [29-31]. *Pax4* is subsequently a hallmark of the commitment to the beta lineage, since it is repressed in delta cells. The knockout of this gene results in a total absence of beta cells but not alpha cells [32]; its expression reaches its maximum between e13.5 and e15.5 in the mouse, coinciding with an extraordinary wave of endocrine cell differentiation known as secondary transition [32,33]. Endocrine committed progenitors and immature beta cells express ***MafB***, and as they mature and turn on *Pdx1* at high levels, they switch off *MafB* and start ***MafA*** production [34]. *MafA* has been identified as specific to cell differentiation: it acts in the lineage reactivation of Pdx1 [34] and it is a critical regulator of the insulin gene in the mature cell. MafA is dispensable for the differentiation of insulin expressing cells during development, but its role is crucial for the functional regulation of glucose-mediated insulin secretion in mature beta cell [35].

1.3 Beta-cells function

Under normal conditions, increased plasma glucose levels stimulate the beta cell production of insulin and downregulate the alpha cell production of glucagon. On the other hand, glucose levels stimulate the production of glucagon to avoid hypoglycemia and ensure normoglycaemia [36]. Insulin, a 51-aminoacid peptide, is the key hormone responsible for maintaining glucose homeostasis (**Figure 4**). It keeps blood glucose levels limits by regulating the uptake of glucose by muscle and fat cells as well as regulating the hepatic glucose output. The most important function of beta cells is, therefore, to convert small fluctuations of glucose in blood glucose concentration (typically from 4.5 to 8 mM in man) into large changes in insulin secretion within minutes. Within beta cells, newly synthesized insulin is first produced as the prohormone proinsulin and converted into mature insulin through the action of prohormone convertases (PC1,

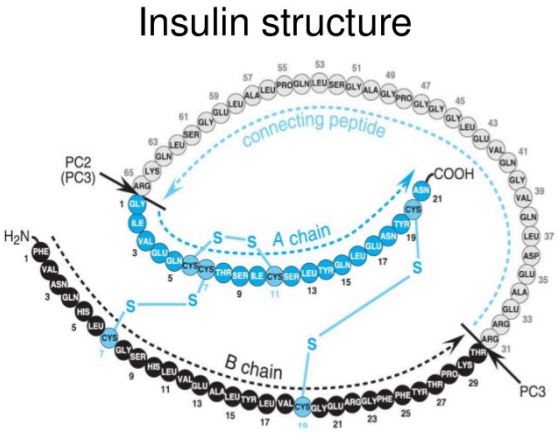


Figure 4. Insulin structure

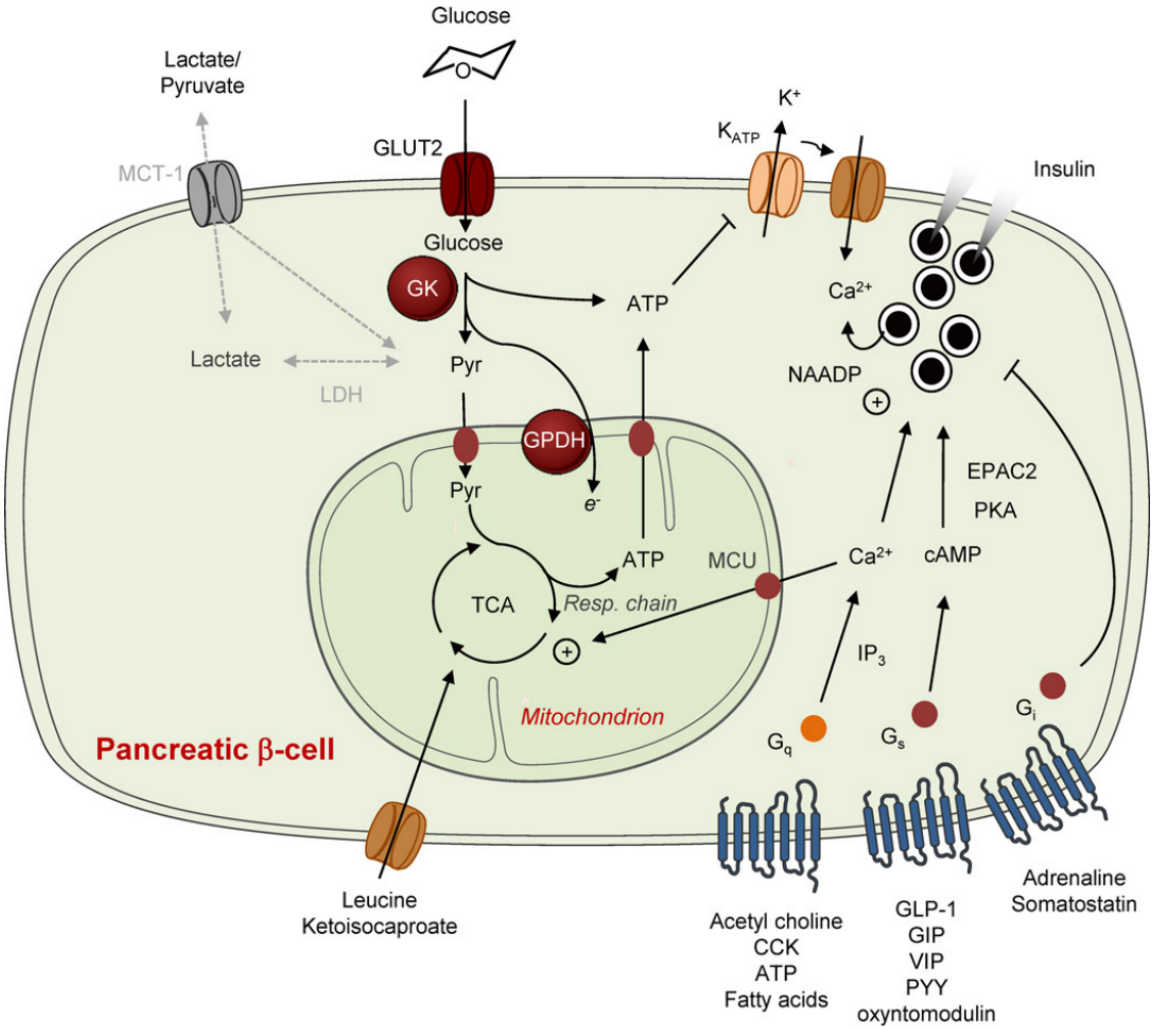


Figure 5. Overview of canonical signalling mechanisms involved in β-cell glucose sensing, and responses to secretory potentiators or inhibitors. Rutter *et al*., 2015

PC2, encoded by Pcsk1 and Pcsk2, respectively) during trafficking through the secretory pathway. Active insulin is then stored in dense core granules (5–10000 per cell), each containing 300000 or more molecules of insulin. The tightly regulated release of only a fraction of the granules through exocytosis (~2% per hour at maximal glucose concentrations) is sufficient to achieve regulation of blood glucose levels within the above narrow physiological range. This tight control is necessary not only to prevent hyperglycemia but equally to suppress the potentially lethal hypoglycemia which would accompany over-secretion of insulin. Glucose uptake from the extracellular environment is mediated by glucose transporters of different types (GLUT2 in the mouse, GLUT1, 2 and 3 in humans) [37]. Central to glucose sensing by beta cells is the stimulation of glycolytic and oxidative metabolism of the sugar mediated from GK. In brief, glucose is taken up by beta cells via glucose transporters, where it is metabolized in glycolysis and Krebs cycle, resulting in an increase in the cytoplasmic ratio of ATP to ADP. This lead to the closure of ATP-sensitive K + (KATP)

channels. The unbalanced influx of positively charged ions, notably Na+, then leads to plasma membrane depolarization, the firing of action potentials and the opening of voltage-gated Ca++ channels [38]. This, in turn, prompts the activation of secretory granule-associated small Nethylmaleimide-sensitive factor receptor (SNARE) proteins and granule fusion with the plasma membrane. Nonetheless, the above description summarizes the essentials of the 'canonical' pathway for glucose-stimulated insulin secretion. In addition, a constellation of further intracellular signaling events, independent of KATP channel closure, is also likely to be essential for normal glucose sensing [39]. A range of physiologically important secretory 'potentiators' also exists. These enhance insulin release only at permissive (i.e. stimulatory; usually above 6 mM) glucose concentrations. The latter group includes the **incretin** hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP), as well as cholecystokinin (CCK), peptide YY (PYY) and oxyntomodulin, released from the gut in response to food transit. GLP-1, for example, reduces blood glucose through inhibition of glucagon secretion from pancreatic alpha cells. Moreover, GLP-1 is also involved in the refreshment of the intracytoplasmatic insulin depots through enhancement of cAMP-mediated proinsulin gene transcription and mRNA stabilization. To accomplish this task, GLP-1 stimulates Pdx-1 gene synthesis and its binding to insulin gene promoter. Pdx-1 gene synthesis is also responsible for the anti-apoptotic properties of GLP-1R agonists on beta cells. These gut-derived factors that enhance glucose-stimulated insulin secretion from islet beta cells are responsible for the augmentation of insulin release in response to food intake versus an identical change in glycemia imposed by intravenous injection of the sugar [40]. An important feature of glucose-stimulated insulin secretions is its 'phasicity'. Multiple waves of action potentials occur in the islets exposed to an increased glucose concentration. In this mechanism, there are two phases of insulin release. The first step is rapid and is characterized by the release of insulin stored in the granules. The second phase is prolonged and sees the synthesis and release of insulin until the blood glucose levels remain high. After lowering blood glucose to normal levels, the insulin granules are regenerated, ready for the next rapid phase. An oral administration of glucose induces a faster and prolonged secretion of insulin compared to intravenous administration because incretins are released into the bloodstream and alert the beta cells the imminent influx of glucose [39].

2 Diabetes

2.1 Type 1 Diabetes

Type 1 diabetes mellitus (T1DM) is thought to be precipitated by an immune-associated, if not a directly immune-mediated destruction of insulin-producing pancreatic beta-cells. Polydipsia, polyphagia, and polyuria along with hyperglycemia remain diagnostic hallmarks. Although T1DM can be diagnosed at any age, it is one of the most common chronic diseases of childhood, and it is slightly more common in boys and men than females. T1DM is believed to be autoimmune in nature and triggered by several factors like seasons of autumn and winter, viral/bacterial infections and environmental pollutants. Peaks in presentation occur between 5-7 years of age and at or near puberty. The prevalence is 0,1-0,5% in the general population, and the incidence is 30-50/100.000 persons. Moreover, it is increasing at an alarming rate of 3% every year.

The diagnosis has historically included fasting blood glucose higher than 126 mg/dL that is the standard value, and any blood glucose of 200 mg/dL or higher. In 2009, the American Diabetes Association had also included glycated hemoglobin. Despite the effort to standardize the diagnosis, particularly among adults diagnosis of type 1 versus type 2 can be challenging. A key distinguishing feature between type 1 and type 2 diabetes is the presence of autoantibodies against beta cells. More than 90% of individuals with a recent diagnosis of T1DM have one or more of the following autoantibodies at disease onset: those reactive to insulin (IAA), glutamic acid decarboxylase (GADA), insulinoma-associated autoantigen 2 (IA2A), zinc transporter 8 (ZnT8A) and also other. Development of type 1 diabetes-associated autoimmunity may occur months or years before the onset of symptoms. Autoantibodies screening can help the identification of people with an increased risk for T1DM.

Type 1 diabetes is clearly a polygenic disorder, with a nearly at least of 40 loci known to affect disease susceptibility. For example, the HLA region of chromosome 6 provides perhaps one-half of the genetic susceptibility, and among HLA types, HLA class II shows the strongest association with T1DM. Most of the loci associated with risk of T1DM are thought to involve immune responses, supporting the idea that the genetic influences include mechanisms that collectively contribute to aberrant immune responsiveness, including the development and maintenance of tolerance [41,42].

2.2 Histopathogenesis

In natural tissue, cells carry out the majority of their life process on a network of proteins and polysaccharides know as **extracellular matrix** (ECM). In addition to its primary functions, the structural function, ECM is responsible for the transmission of chemical and mechanical signals mediating fundamental aspects of the cellular physiology such as adhesion, migration, proliferation, differentiation, and death [4].

While the cellular structure of the pancreas is well described, its ECM has only recently gained attention and has been shown to contribute to pancreas development. Broadly, the ECM is composed of **basement membranes** (**BM**), tight networks of specialized glycoproteins that act to separate tissue compartments but that also direct cellular processes, and the looser **interstitial matrix** (**IM**) typical of the stroma of most organs. In the pancreas, BMs predominate, occurring around each acinar cell of the exocrine pancreas, surrounding blood vessels and ducts, and encasing each pancreatic islet. The IM, which confers tensile strength and elasticity to tissues mainly due to the peri-islet BM and surrounding large ducts and blood vessels. **Laminin and collagen type IV**, networks are the major components of all BMs, both self-assemble into superstructures that are interconnected by heparin sulfate proteoglycan and by the nidogens. Of all BM components, laminin is considered to be the biologically active component, collagen type IV, by contrast, is essential for structural integrity. Peri-islet BM collagen is composed of type IV, again, perlecan, nidgen-1 and -2, and different laminin isoforms. However, endothelial BMs of blood vessels within pancreatic islets are rich in laminin α4 and α5. Moreover, the IM underlying the peri-islet BM is composed of the fibrillar collagen type I and III, collagen type VI, fibronectin, fibrillin2, and matrilin-2 [43,44].

The tissue environmental is known to decisive effects on Treg behavior and immune regulation. In fact, from transplant studies, it is known that the islet ECM plays critical roles also in islet survival and function. However, it is unknown how the immune response and the islet ECM interaction during autoimmune insulitis contribute to the pathogenesis of T1D [45]. Autoreactive T cells in T1D develops in the pancreatic lymphonodes and subsequently migrate in the pancreas where they first must extravasate from the postcapillary venules (PCVs) that surround the islets. Subsequently, they have to migrate through the thin IM and finally penetrate the peri-islet BM before they given

access to the insulin-producing beta cells. Immunofluorescence studies have revealed a global loss of peri-islet IM and BM components only at sites of leukocyte infiltration into the islet [44].

The presence of a chronic inflammatory infiltrate that affects pancreatic islets at symptomatic onset of T1DM is one of the dogmas. Another dogma is that in patients with longstanding disease, the pancreas do not present any more insulin-producing cells and the remaining beta cells are not able of regeneration. Both of these concepts have been debated in the last few years. Recent data suggest that although most patients with longstanding type 1 diabetes have few beta cells if any, there is evidence for beta cell regeneration. Thus there has been considerable interest in understanding the mechanisms that regulate replication of beta cells with the goal of discovering new therapeutic targets to promote their regeneration. Many reports support the idea that the endocrine pancreas is a plastic organ, especially regarding the ability of the beta cell mass to change according to the metabolic demand of insulin in conditions such as pregnancy and obesity. Glucose and insulin are potent stimulators of beta cell growth and function both *in vivo* and *in vitro*. Replication of beta cells is the predominant mechanism that ensures the rapid expansion of beta cell mass early in life; however, the regenerative capacity of beta cells rapidly declines with advancing age. Also during pregnancy, beta cell mass expands to adapt the organism to increasing insulin demand [46-48].

2.3 Therapies

Despite the advances in type 1 diabetes research and therapy, researchers and clinicians are disappointed by a perceived lack of progress. The discovery of insulin in 1921-22 was clearly the most important event in the history of type 1 diabetes therapies; however, exogenous insulin replacement does not always provide the metabolic regulation necessary to avoid complication associated with TD1M. As a result diabetes management in modern countries often includes the use of insulin analogs and mechanical technologies like insulin pump.

The first pancreatic transplantation was tempted by Kelly and co-worker in 1966 [49]; afterward other 25.000 transplantations have been conducted worldwide. Rapid control of hyperglycemia with consequential discontinuation of the exogenous insulin supplementation was noted in successful cases. However, the major drawbacks were significant morbidity related to major surgery and requirement of life-long immunosuppression, which had its side effects including

recurrence of diabetes. These drawbacks led the scientists to work on different paths. Another approach studied and developed by Ricordi and other scientists is the islet extraction and transplantation [50]. However, these protocols could not succeed in rendering insulin-free survival for more than 5 years [51]. Other modalities of encapsulation, immune modulation, and delivery techniques are still being developed. However, success is still far away.

Long-term restoration of immune tolerance is paramount to the survival of residual endogenous or implanted islets. Two types of approaches have been evaluated or implemented to achieve this goal. The first type aims to eliminate parts of the immune system, such as T and B cells. This kind of approach is quite useful for as long as it is applied, but patients became susceptible to infections and cancers. Moreover, autoimmunity usually resurges after the interruption of the treatment. Considering the safety and efficacy of current diabetes care, this is not a viable long-term solution for the majority of T1D subjects. The second type of approach aims to specifically eliminate or tame the immune cells responsible for the beta cell destruction by exploiting their beta cell antigen specificity. Clearly, we need a combined approach, in one hand control and interact with the immune system to avoid a relapse, on the other hand, a source of insulin able to respond to different levels of glucose [52].

2.4 Stem Cells Therapies

The need for an unlimited supply of a substitute for insulin-secreting beta cells led to research on the suitability of stem or progenitor cells to generate insulin-secreting cells *in vitro*. The main objective of these cell-based therapies was the down-regulation of the immune system and the abrogation or at least halt the process of autoimmune destruction of these cells. The other aim was to generate stem cells (SCs) and differentiate them into functional insulin-secreting beta-like cells to treat T1DM.

SCs obtained from several sources have been tested for their beta cell regenerative potential and for the ability to restore immune homeostasis or promote longitudinal islet graft survival. This includes, among other, embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), bone marrow (BM)-derived hematopoietic stem cells (HSCs), umbilical cord blood (UCB)-derived mesenchymal stromal cells (MSCs), Adipose tissue Derived MSCs (ADSCs) and pancreas derived multipotent precursor cells, as well as pancreatic beta cell progenitors that reside in the ductal epithelium and exocrine tissue. The so called “adult stem cells” occur in fetal and post-natal

tissues, although they are restricted to lineages defined by a germ layer (ectoderm, mesoderm, or endoderm). Specific stem cells for liver and pancreas comprise multiple subpopulations of biliary tree stem cells (hBTSCs), found in peribiliary glands (PBGs) throughout the biliary tree. Adult pancreatic SCs can also be another source for pancreatic beta cells.

Impressive progress has been reported in inducing differentiation of human ES [53,54] and iPS cells [55] *in vitro* into insulin-producing cells, using protocols which attempt to mimic pancreas organogenesis, based on knowledge gained in mice. By employing combinations of hormones, cytokines, and small molecules, these protocols direct pluripotent stem cells first into definitive endoderm, then into gut tube cells, pancreatic endoderm [56], endocrine pancreas progenitors, and finally into hormone-expressing cells. However, the differentiation efficiency decreases significantly with each step, and the low numbers of insulin-producing cells generated produce more than one hormone and are not glucose-responsive. These protocols probably lack crucial signals that operate during normal pancreas development *in vivo*, thus hindering the generation of functional beta cells *in vitro* [57].

For successful transplantation of stem cells, they have to be prepared and transplanted in a way that supports long-term function of the graft and ensure the safety of the host. The main goal of tissue engineering is to generate functional tissue. Though we can now generate many cell types from human pluripotent stem cells, these cell types are often immature and do not reach the functionality of tissue *in vitro*. After the tentativeness of initial approaches, which yielded questionable results, current efforts are focused on refining culture conditions designed to more accurately mimic pancreatic development. The lack of appropriate tissue structure is likely part of the under-performance of stem cell-derived products. All stem type and organs in the human body are organized in specific structures composed of multiple cell types. Interactions between different cell types and vascularization, as well as the 3D arrangement of the cells, can play a major role in tissue reconstruction [58].

3 Stem and Progenitors Cells

3.1 Classification

Stem cells are generically defined as undifferentiated cells able of self-renewal through replication as well as differentiation into specific cell lineages. More specifically scientists have identified four criteria to define cells as Stem Cells (SCs). The first is self-renewing, the capability to undergo multiple and sequential division. The second is the ability to generate daughter cells differentiate into multiple cell type. The third criteria is the possibility to functional repopulate a damaged tissue. The last is the contribution to the normal and physiological tissue cell-turnover among life.

Scientists have also classified SCs by their developmental potential. **Totipotent** stem cells are able to generate both embryonic and extra-embryonic tissues, and may, therefore, produce a whole organism, a property retained by the early progeny of the zygote up to the morula at the 8-cell stage. **Pluripotent** cells are capable of forming tissues from all the three embryonic germ layers (endoderm, mesoderm, and ectoderm) but not to give rise to extra-embryonic tissues. They are the cells of the Inner Cell Mass (ICM) and embryonic germ cells. **Multipotent** cells can yield a more restricted subset of cell lineages. The historically most significant example is the hematopoietic stem cells (HSC), from which originate all blood cells. Finally, **unipotent** cells produce a single terminally differentiated cell type in the adult organism and are essential for restoring tissues subject to a constant turn-over. The classical example is given from epidermal stem cells.

Stem cells are also classified as **embryonic** or **adult**, depending on the developmental stage from which they were obtained. After the first studies in the mouse model [59], Thomson in 1998 has isolated the first human ESCs, cells capable of indefinite proliferation *in vitro*, with a highly undifferentiated state and a normal karyotype [60]. The high proliferative activity is due to the action of telomerase, a riboprotein that preserves telomere length during DNA replications, thereby allowing these cells to reach a number of cell divisions considerably higher than that established by Hayflick for somatic cells.

The analysis of ESCs gene expression profile has revealed the expression of three transcription factors, OCT4, SOX2, and NANOG; Subsequent studies have defined them such as master-regulators genes of stem cell phenotype.

OCT4 (octamer-binding transcription factor 4) is a transcription factor of the Pou proteins family, which a highly conserved DNA binding domain (150-160 amino acids). Embryonic expression of

Oct4 appears to be initiated at 8-cell stage, and it is expressed in all cells at 16- and 32-cell stage. Subsequently, it is down-regulate in differentiation and development phases and finally, it is found in germ cells. Oct4 has not been studied extensively after birth, but it has been shown to be expressed in adult in at least some multipotent stem cells and many types of human cancer.

SOX2 (SRY-box related protein) is a transcription factor belonging to the Sox family, which includes approximately 20 proteins sharing a highly conserved DNA binding domain composed of about 80 amino acids. Zygotic Sox2 expression begins at the morula stage where it is preferentially localized to the inner cells of the morula, which will give rise to the ICM. As the ICM develops, Sox2 is express only in the epiblast and interestingly Sox2 expression is initiated after that of Oct4, but Sox2 becomes restricted to the cells that will ultimately give rise to the ICM before Oct4. After birth, Sox2 expression has been reported in numerous tissues and stem cells. Hence, Sox2 appears to be essential for tissue regeneration and survival later in life. It is now generally recognized that transcription factors do not work in isolation, but regulate transcription as part of large protein complexed [61,62].

NANOG (Nanog homeobox protein) is a protein of 305 amino acids and has a conserved homeodomain able to bind localized DNA at cells nuclear level. The role of this factor is confirmed by the fact that its presence is less need of leukemia inhibitory factor (LIF) to maintain the pluripotency of the crop, while its lack of expression involves the spontaneous differentiation of the cells [63].

These factors play a key role during the entire embryonic development of mammals and they are critical for the regulation of auto-maintenance and pluripotency of ESCs. The inactivation of these genes during embryonic development leads to the death of the embryo, while due to the loss of ESCs stem cell phenotype and the subsequent differentiation [61,62].

The ESCs high differentiation potential makes them extremely promising as a therapeutic tool, but at the same time extremely dangerous, so their use is mostly tied for research. In fact, ESCs represent an excellent model for the study of developmental biology and replication processes and cell differentiation. It should also be noted that the isolation of ESCs involves the suppression of embryos from which cells are derived. This has raised a number of ethical questions, which have limited and still restrict their use. In order to overcome both ethics and technical related problem, research has worked to produce pluripotent cells of different origin *in vitro*. In 2006, Takahashi and Yamanaka reprogrammed for the first time murine fibroblasts into pluripotent stem cells, using

retroviral vectors to introduce and express genes coding for four cell specific transcription factors (OCT4, SOX2, Klf4, and C-MYC). These cells have been called induced pluripotent stem cells (iPSCs) and show morphological, proliferative and differentiation properties very similar to those of ESCs [64]. The following year, in the same way, has been reprogrammed human fibroblasts and the first human iPSCs were obtained. The use of these cells does not pose any ethical limitation and appears promising in the field of cell therapy for the possibility of obtain patient-specific stem cell populations. These cells could be recognized as self from the patient immunity system and not rejected. At the moment, the lack of knowledge about the long-term stability of reprogramming severely limits their use *in vivo* and pushes towards the search for new methods of reprogramming or trans-differentiation.

3.2 Adult Stem Cells

True multi or pluripotent stem cells persist in postnatal life and they are called Adult Stem Cells (ASCs). These cells are maintained in relative quiescence within specific niches throughout the body. Although the baseline state of adult stem cells is relative quiescence, they are rapidly mobilized in response to stress or tissue injury. Multipotent stem cells effectively maintain homeostasis of adult tissues throughout life [65]. Over the past years, a series of reports has been published suggesting that the previous dogma of tissue specificity associated with adult stem cells may not be correct. The presumed ability of tissue-specific stem cells to acquire the fate of cell types different from the tissue of origin has been termed adult stem cell plasticity. The prototypical adult stem cell is the HSC, first theorized to exist in 1961 by Till and colleagues [66] and definitively isolated in mouse in 1988 [67]. These cells are multipotent in that they retain the ability to differentiate into all cell types of the hematopoietic system. Another group of adult stem cells is the Mesenchymal Stem Cells (MSCs), a term first coined from Caplan in 1991 [68]. MSCs exhibit fibroblastic morphology and in accord to the International Society for Cellular are defined as plastic adherent multipotent cells that can differentiate *in vitro* into mesodermal lineages such as osteoblasts, adipocytes and chondrocytes (**figure 6**) [69]. They also express the cell surface markers as CD44, CD73, CD90, CD105, whereas they present minimal or no expression of the hematopoietic markers CD11b, CD14, CD19, CD34, CD45, CD79a and of the histocompatibility antigens HLA-DR-DP-DQ. Since the late 1980s when the technology for isolating, culture and

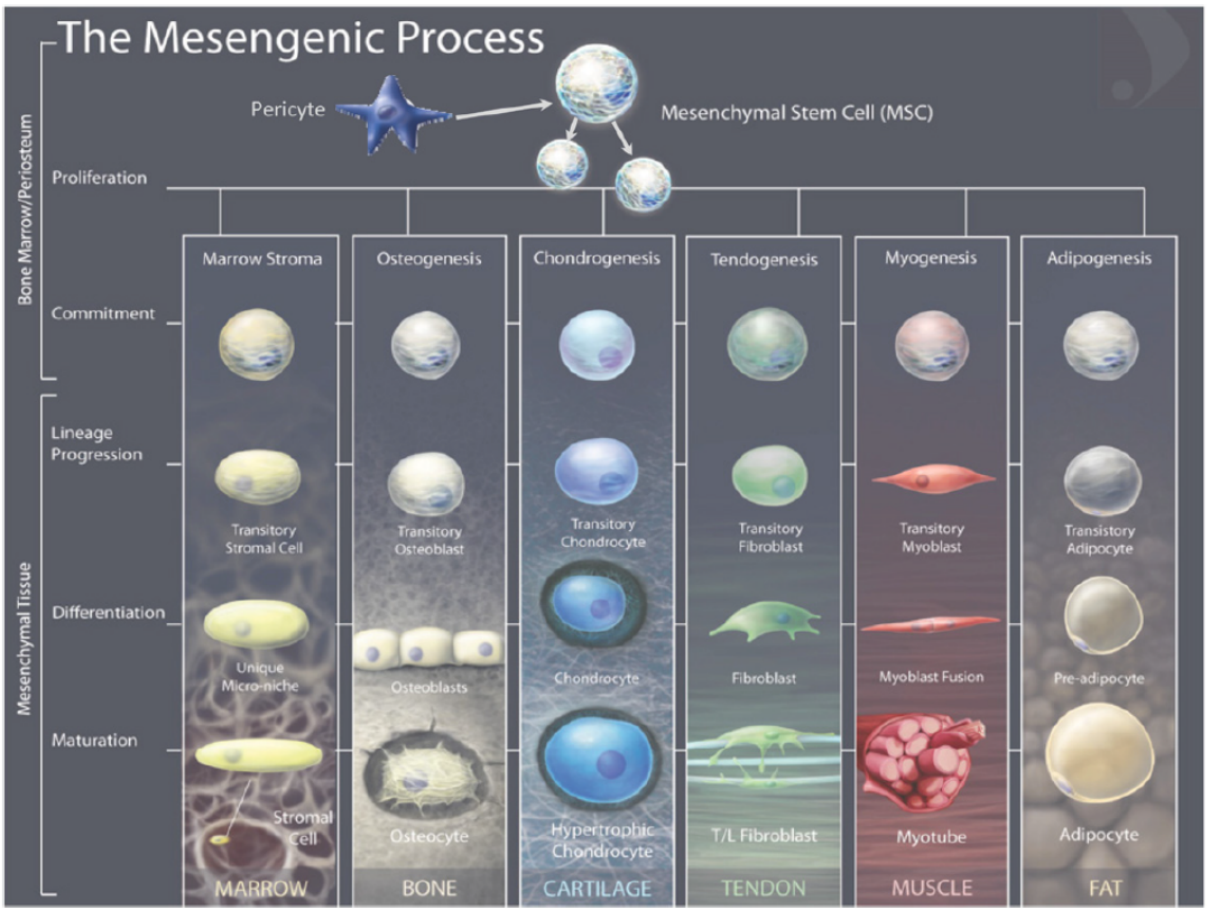


Figure 6. The mesengenic process. Caplan, 2011.

expanding MSCs was perfected and then became practicable in the early 1990s, their use for clinically relevant therapies has evolved. Indeed, two very different theories have been proposed and explored. The original theory was that expanded MSCs, because of their multipotency, could be applied to replace injured, damaged, or diseased mesenchymal tissues. Although this theory was pursued for almost three decades and continues to be explored, no product or treatment is currently available. In defense of this pursuit, newer logics and scaffolds now being experimentally tested hold realistic promise for eventual success and clinical use to replace cadaveric products now used routinely. The documentation that MSCs (perhaps all MSCs) are derived from perivascular cells, pericytes [70], now explains how MSCs can be isolated from almost every tissue in the body [71]. Moreover, the fact that MSCs possess the capacity to secrete immunomodulatory and trophic mediators strongly argues that their natural and normal *in vivo* function is as Medicinal Signaling Cells (MSCs) for sites of injury or inflammation [72].

To date, there are very few examples of proven stem cell therapies: bone marrow transplantation (BMT), regeneration of skin with epidermal stem cells and regeneration of cornea with gimbal stem

cells. The most famous and well studied is bone BMT. The first successful transplant of HSCs between an unrelated donor and recipient was performed in 1969, 14 years later the first trials where all patients led to the deaths. Donnell Thomas spent 14 years learning why donors had to be matched to recipients during transplants [73]. The second was approved in the corneal resurfacing with populations that contain limbal stem cells [74] and skin regeneration with populations that contain epidermal stem cells. There is also strong preclinical evidence and case reports for bone regeneration using bone marrow stromal cells (BM-MSCs). A number of therapies have been envisioned for the treatment of diverse disorders and diseases, such as diseases of the central nervous system, graft versus host diseases, cardiovascular diseases, pulmonary diseases and many other, using primary BM-MSCs. It is now clear that these cells do not transdifferentiate into cells outside of the mesodermal lineage (bone, cartilage, hematopoiesis supportive stroma and marrow adipocytes). However, it is thought, but as yet unproven, that they may exert paracrine, immunomodulatory and immunoregulatory effects on endogenous tissues upon systemic infusion or direct injection. It is not clear that these cells display these properties *in vivo*, and if so, by what mechanisms. Upon intravenous injection, these cells accumulate in the lungs and are then rapidly removed from the body. Thus, they neither transdifferentiate nor engraft, making the putative paracrine/ immunomodulatory feature a property of the population as a whole, and not of the stem cell subset within it [75].

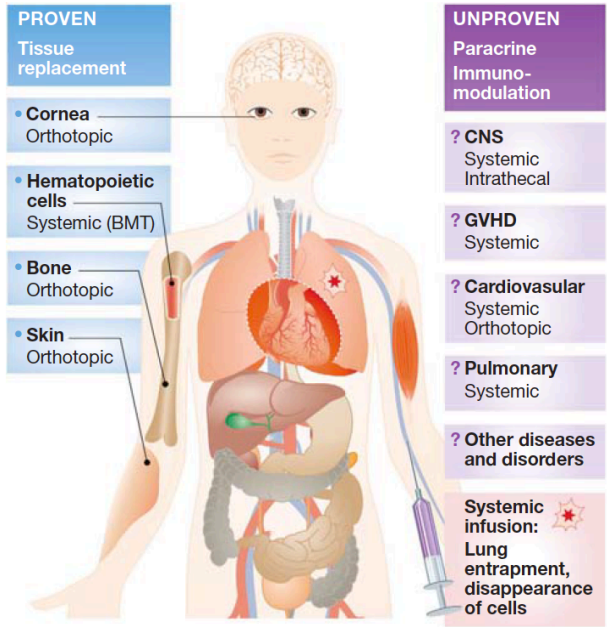


Figure 7. Stem Cell Therapy. Bianco *et al*., 2013.

3.3 Tissue Engineering

Tissue Engineering was defined for the first time in 1993 from Longer and Vacanti as an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function [76]. Since that time, researchers have been able to fabricate increasingly complex tissue/organ constructs,

and some such bioengineered products are used clinically today as the standard treatment for a variety of conditions. The three original pillars of tissue engineering proposed by Langer and Vacanti remain relevant today: first isolated cells, second tissue inducing substances, and third matrices. Cells constitute the fundamental unit of tissues and exhibit a broad spectrum of functional diversity. Two main possible categories of cellular components are stem/progenitor cells and mature differentiated cells. In the next section, we will analyze specific cells for pancreatic differentiation. Previously we have already discussed about factors able to induce and improve pancreatic differentiation, what we miss now is the matrix. To deeply understand we need to start from the in vivo architecture of organs and tissues. In this contest, we will concentrate our attention in epithelial cells organization in vivo. Epithelial cells are connected to each other by intercellular junctions and are located within a specialized ECM, which is known as the basement membrane. Connective tissues, in contrast to the epithelium, contain an abundance of ECM and different population of stromal cells, including fibroblasts, immune cells, and adipocytes. Epithelium and connective tissue are functionally interdependent units within organs. In addition to the cellular inputs, culture formats can be varied independently in order to answer specific biological questions [77]. The addition of basement membrane proteins to the medium in 2D cultures is sufficient to induce tissue-specific differentiation of diverse epithelial cells, including mammary, kidney, and lung. Most experiments rely on a commercial basement membrane protein source, such as Matrigel [78]. In 2D culture system, it is not completely modeled these cell–cell and cell–ECM interactions. These observations led to the development of diverse assays in which single cells are plated on top of Matrigel, with additional Matrigel in the medium [79]. Finally, researchers have also embedded epithelial cells within an ECM gel and observed that cells, in this case, are enables the cells to self-assemble into tissues and to both interpret and remodel the ECM [80].

4 Different Sources of Fetal and Adult Stem Cells

4.1 Pancreatic progenitors

Stem/progenitor for liver, biliary tree, and pancreas exist at early stages of development in the definite ventral endoderm forming the foregut. In humans, they persist postnatally as part of a network and studies had evidenced their contributions to hepatic and pancreatic organogenesis throughout life [81]. Multiple stem cell niches continue in specific anatomical locations within the human biliary tree and pancreatic ducts. In the human extra hepatic biliary tree, the stem/ progenitor niches have been identified as the **peribiliary glands** (**PBGs**). PBGs contain niches of cells, collectively termed **biliary tree stem cells** (**BTSCs**), with phenotypic traits of endodermal stem/progenitors. BTSCs present the expression of transcription factors (SOX17, PDX1, and SOX9), surface markers (epithelial cell adhesion molecule [EpCAM] and LGR5) and cytoplasmic markers, and the capacity of proliferation, self-renewal, and multipotency [81-83]. A subpopulation (nearly 10%) of PBG cells appears to be primitive stem cells. These cells also co-express several pluripotency markers (e.g., OCT4, SOX2, and NANOG) and can self-renew or differentiate into

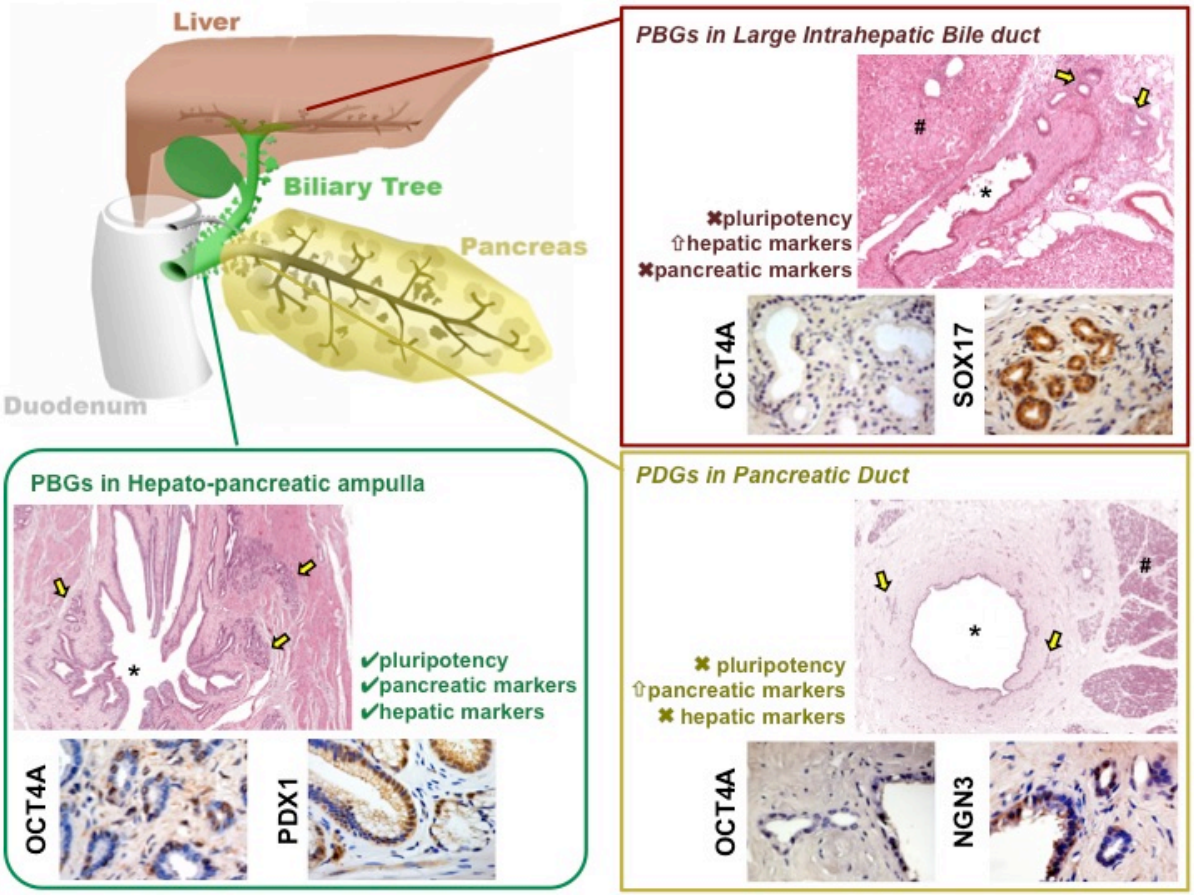


Figure 8. PBG and PDG distribution and proximal to distal axis maturation in humans. Lanzoni *et al*., 2016.

functional hepatocytes, cholangiocytes, and pancreatic islets [82]. The biliary tree, the pancreatic duct and their associated gland PBGs and **pancreatic duct glands** (**PDGs**) demonstrate striking similarities histologically. More detailed analysis of the phenotypic traits implicate two separate but overlapping maturational lineages identified by gradients in gene expression. One is the **proximal** (PBGs) **to distal** (PDGs) **axis** of maturation from the duodenum and extending into the pancreatic ducts (**figure 8**). The other is a **radial axis** of maturation starting at the **fibromuscular** (**FM**) layers within duct walls and extending to cells at the ducts' lumens. The anatomical distribution of PDGs along the pancreatic ducts of larger caliber has similarities with the distribution of PBGs along the biliary tree. PDGs occurs in association with the main pancreatic duct and its immediate branches and are abundant in proximity to the hepato-pancreatic ampulla. Wang and co-worker provided evidence of the proximal to distal maturational lineage axis starting in PBGs in the hepato-pancreatic common duct near to the duodenum, transitioning to pancreatic ducts, thence to PDGs and finally to mature pancreatic islet cells. Cells expressing markers of pluripotency (OCT4, NANOG, and SOX2), self-replicative ability (SALL4) and hepato-pancreatic endodermal commitment (SOX 9, SOX 17, PDX1 and LGR5) are present in the PBGs of the hepato-pancreatic common duct. Moreover, these cells do not express NGN3 and only few cells express Insulin, but on the other hand, they strongly express the proliferative marker Ki67. By contrast cells in the PDGs had no co-expression of pluripotency genes; none expression of NANOG or SALL4; fewer than 5% present a cytoplasmatic expression of either OCT4A or SOX2, and this expression was cytoplasmatic; Moreover, all express PDX1, most expressed NGN3 and insulin (**figure 9**) [82,84].

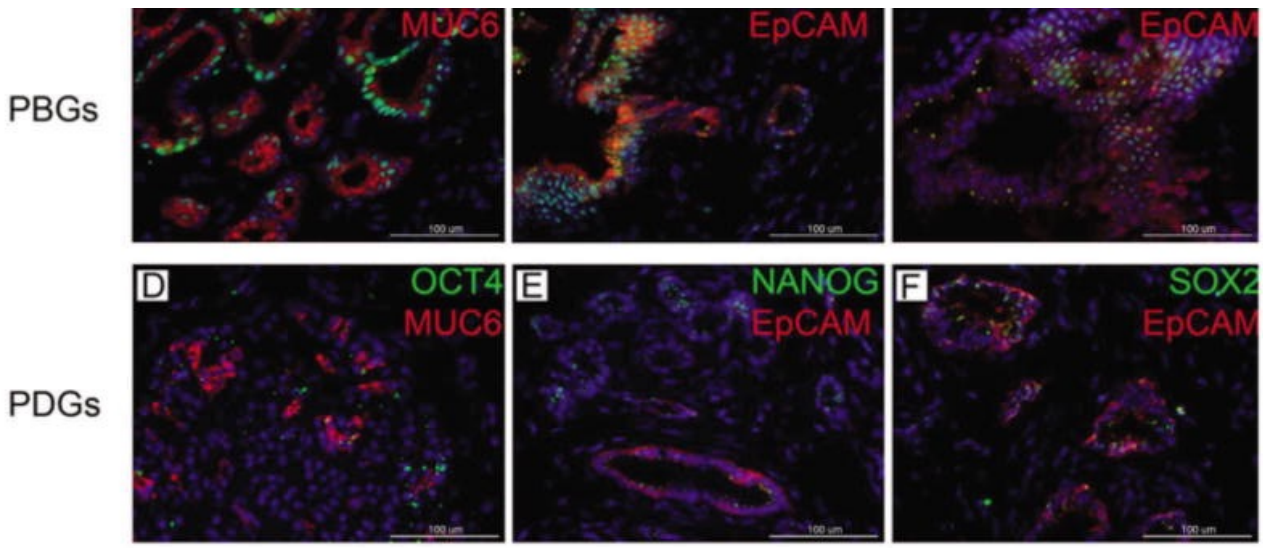


Figure 9. The maturation gradients in gene expression of pluripotent genes. Wang *et al*. 2013.

Very recent study have also highlighted that PDGs represent niches consisting of a heterogeneous population of Sox9+ and Lgr5+ cells. Lgr5 is a marker in multiple organs of adult stem cells and progenitors of extensive proliferative potential. These phenotypic traits of Sox9+ cells within adult PDGs are consistent with those of pancreatic committed progenitor cells. Moreover, insulin+ cells are also located within human PDGs, but there was no overlap in expression of insulin and various progenitor markers [85].

The radial-axis maturational lineage consists of stem cells in the PBGs deep within the walls of the hepato-pancreatic common duct and near the FM layers. The PBGs near these FM layers contained cells that did not express EpCAM, NGN3, insulin or any other islet hormone but co-expressed, within the nuclei, the pluripotent genes, SALL4 and the endodermal commitment genes (SOX 17, PDX1 and LGR5). Proceeding towards the luminal surface of the duct, the expression the pluripotent genes and SOX17 faded and, in parallel, there was maintenance of PDX1 along with appearance of and then increasing expression of EpCAM and insulin [84].

Cells from PBGs could also be isolated and cultured *in vitro* in serum-free Kubota's Medium (KM). This medium has been shown previously to select for early endodermal stem/progenitors, and with minor modifications works well also for mesenchymal stem/progenitors. The human biliary tree stem cells (hBTSCs) formed *in vitro* colonies that expanded readily on plastic generating colonies of growing cells of two main types. *Type 1* colonies were composed of cells with an undulating, swirling morphology. Cells initially did not express EpCAM, but with time in culture, they acquired its expression at the edges of the colonies in parallel with a slight increase in cell size and of marker indicating sight differentiation. *Type 2* colonies were comprised instead of cells that express EpCAM immediately on every cell and formed "carpet" like colonies of tightly packed, uniformly cuboidal shaped cells [82,84]. hBTSCs in culture retained expression of pluripotency markers such as OCT4, SOX2, and NANOG, whereas they did not display markers of endocrine committed progenitor such as NGN3 or of mature pancreatic islet cells like insulin. Wang and co-worker also show that embedding hBTSCs in a 3-dimensional hydrogel it is possible to obtain *in vitro* a conversion of them to neoislet-like spheroids. These spheroids could be then induced towards an islet fate using a hormonally defined medium tailor-made, named HDM-P. In any case, the differentiation was still partial only slight increase of C-peptide was observed. To verify more in deep the functional differentiation in an *in vivo* setting, neoislets were transplanted into

streptozotocin treated mice. This treatment ameliorated the mice condition and corroborated the interpretation of the authors that hBTSCs are precursors to pancreatic committed progenitors [84].

I In perspective, all this evidence makes human BTSCs a candidate source of stem/progenitors for regenerative medicine. Encouraging preliminary data from an ongoing phase 1/2 clinical trial indicated the safety of human BTSCs administration for the treatment of advanced liver cirrhosis [86]. A very recent study has also demonstrated the possibility to expand hBTSCs adding 5% of fetal bovine serum and supplement containing pituitary gland extract to obtain a sufficient cell number for regenerative applications. In the same studies, researchers had analyzed and suggested for the first time the product of Hepatocyte Grow Factor and its role in inducing apoptosis in leukocytes exposed to co-culture with hBTSC. These studies enforce the significance of the HGF-mediated immune modulation possessed by hBTSC, suggesting that the secretion of HGF might enhance the therapeutic potential of hBTSC [87].

There are hints, but no proof, that the network of niches begins with the Brunner's glands, submucosal glands found in the duodenum and located between the major papilla, the entranceway to the hepato-pancreatic duct, and the minor papilla, the port connecting the duodenum to the dorsal pancreatic duct. The Brunner's glands are not found elsewhere within the intestinal tract. Indeed, they are used to define the transition from the duodenum to the beginning of the small intestine. Ongoing studies may determine their possible relevance to the stem cell and progenitor cell niche network in the biliary tree, liver, and pancreas [88].

4.2 Pancreatic Islet derived Mesenchymal Stromal/Stem Cells

Cells with a fibroblasts morphology from pancreatic islets were isolated from different researchers from 2004. Since then they have attracted attention for two principal reasons, first for the possibility to differentiate into islet-like cells [89], second because they could potentially provide a more suitable feeder layer for pancreatic islet co-culture ex-vivo compared with other cells tested before [90].

Human pancreatic islet cell equivalents in culture started to generate spindle-shaped cells. PI-MSCs as BM-MSCs show the multipotent ability to differentiate into the classical mesodermal lineages and the expression of classical mesenchymal markers (CD44, CD90, CD73, CD105) and the absence of hematopoietic markers (CD14, CD34, CD45) [90-92]. This essential

characterization satisfied the minimal criteria fixed by the International Society for Cellular Therapy to define a cell population as mesenchymal stromal cells [69].

In the beginning, it was proposed that these mesenchymal cells originated from beta cells undergoing to an epithelial to mesenchymal transition [93]. This transition would allow the expansion of this cells in the presence of serum whereas, following serum removal, the cells could regain an endocrine phenotype with synthesis and release of insulin [93-95]. In contrast, following reports from several laboratories have published studies based on genetic lineage tracing in mice that highlight the possibility that beta cells could undergo an epithelial to mesenchymal transition, thus suggesting that this cells may originate from preexisting mesenchymal cells *in vivo* [96-98]. The appearance in islet cell cultures of cells positive for both nestin and C-peptide or for both nestin and Ipf-1 would argue for a conversion of b-cells from an epithelial to a mesenchymal phenotype. Similarly, positivity for Ki67 of cells expressing Ipf-1 would suggest that cells of epithelial origin can proliferate at least for a given time. But after the first week in culture, almost all cells were positive for nestin, a-SMA, and vimentin, but resulted negative for C-peptide and Ipf-1. However, taking into account all these results, an alternative explanation could be that even if there was an initial transition of beta cells to a mesenchymal phenotype at early times in culture, these cells may die off and be taken over by a population of mesenchymal cells that continue to proliferate and to persist at the later passages in culture [91].

Moreover, PI-MSCs show the expression of HLA-ABC but are absent for HLA-DR, a major histocompatibility complex class II cell surface receptor. This suggests the ability of this cells to escape the immune system, a very interesting property of MSCs. The ability to suppress lymphocyte proliferation *in vitro* was analyzed, and PI-MSCs have shown the capacity to inhibit both CD4+ and CD8+ T lymphocyte in a dose manner [90].

Very important, different researchers try to differentiate PI-MSCs into islet-like cells with different differentiation protocols. Gallo and co-workers also study the effect of cyclopamine in the inhibition of Hedgehog pathway in PI-MSCs showing it inhibition in a dose manner [91]. They have one common characteristic that is the formation of clusters that improve differentiation efficiency [89,99]. Davani and co-worker have also demonstrated that partially differentiated hIPC clusters, when implanted under the kidney capsules of mice, continued to differentiate *in vivo* into hormone-producing cells. However, they noted that not all hIPC preparations yielded insulin-secreting cells *in vivo* and that in some animals no hormone-expressing cells were found. This suggested that the

implanted cells were not always irreversibly committed to further differentiation and may even de-differentiate to a mesenchymal phenotype [99].

4.3 Placenta

The term placenta is a discoid-shaped organ with a diameter of 15–20 cm and a thickness of 2–3 cm. It is composed of a fetal portion (the chorion) and a maternal portion (the decidua). Fetal component all originate from the blastocyst consists of the chorionic plate, fetal membranes, and umbilical cord. The umbilical cord extends from the amnion toward the fetus. Generally, it contains two umbilical arteries and one umbilical vein embedded within a gelatinous, proteoglycan-rich matrix called Wharton's jelly (**figure 10**), which prevents the compression, torsion, and bending of the umbilical vessels. Meanwhile, the fetal membranes contain the fetus throughout the pregnancy and eventually undergo programmed rupture during the first stage of labour. They consist of the maternal-facing, chorion, and fetal-facing, amnion. The chorion contains the reticular layer, a basement membrane, and the trophoblast cell region, which at term firmly adheres to the maternal decidua. The amnion is a thin, avascular membrane contiguous, over the umbilical cord, with the fetal skin. It comprises five distinct layers. The innermost layer is the amniotic epithelium, which is in direct contact with the amniotic fluid on one side and a basement membrane on the other. The

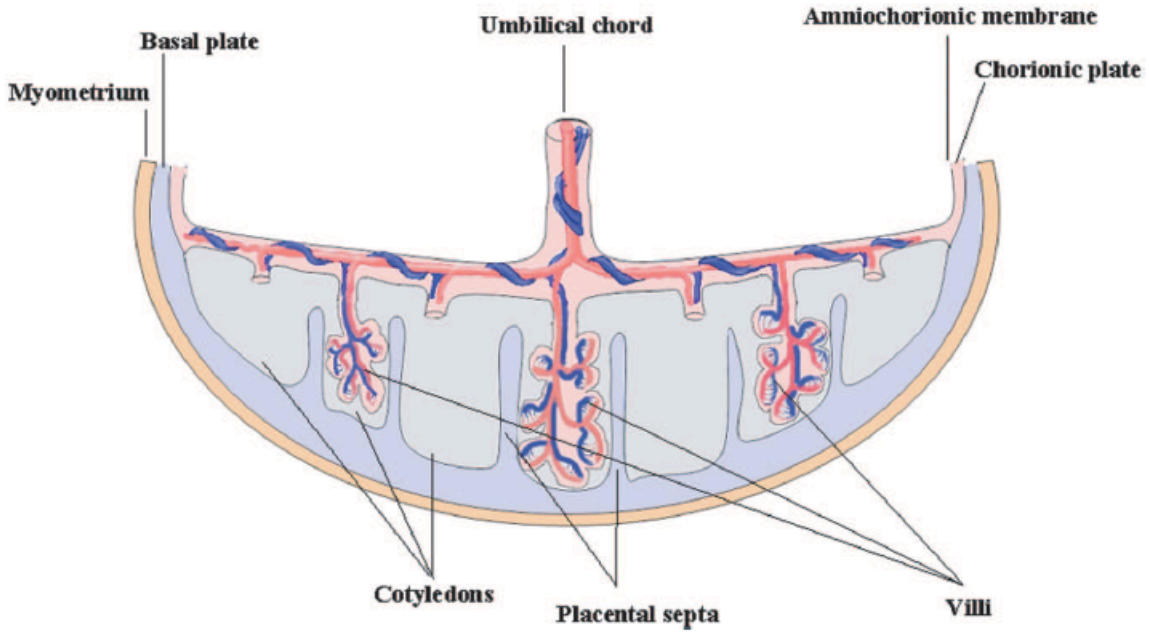


Figure 10. Schematic section of the human term placenta. Parolini et al.,2008.

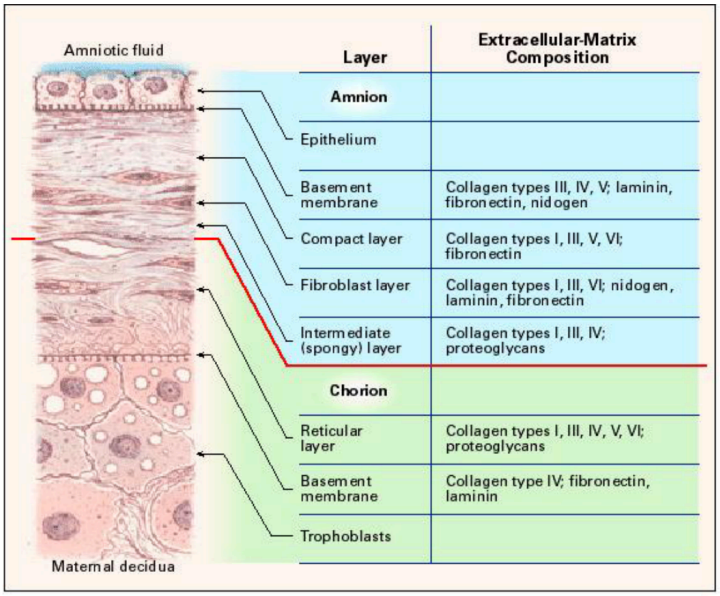


Figure 11. Schematic presentation of the structure of the fetal membrane at term. Niknejad *et al*., 2008

other layers consist of the compact layer, the fibroblast layer, and the spongy or intermediate layer. The human amniotic epithelium (hAE) is an uninterrupted, single layer of flat, cuboidal and columnar epithelial cells in contact with amniotic fluid. It is attached to a distinct basal lamina that is, in turn, connected to the amniotic mesoderm (AM). In the amniotic mesoderm closest to the epithelium, an acellular compact layer is distinguishable, composed of collagens I and III and fibronectin. Deeper in the AM, a network of dispersed fibroblast-like mesenchymal cells and rare macrophages are observed. Very recently, it has been reported that the mesenchymal layer of amnion indeed contains two subfractions, one having a mesenchymal phenotype, which is referred to throughout this review as amniotic mesenchymal stromal cells, and the second containing monocyte-like cells (**figure 11**). The primary functions of the placenta can be categorized under the headings of transport and metabolism, protection and endocrine. The placenta acts to provide oxygen, water, carbohydrates, amino acids, lipids, vitamins, minerals and other nutrients to the fetus, while removing carbon dioxide and other waste products. It metabolizes many substances and can release metabolic products and hormones into maternal and/or fetal circulations to affect pregnancy, metabolism, fetal growth, parturition and other functions. Other essential functions of the placenta are the ability to act as a barrier against many pathogens and viruses and to induce tolerance, avoiding an immunological reaction of the mother against of the fetus. The placenta is, therefore, a physical and functional connection between the mother and the fetus. Through it, the fetal growth and all its functions are finely controlled with the maximum efficiency. The biological and embryological features of the placenta and extra-embryonic tissues also make it a potential stem cell and progenitor source for scientific researchers [100-102].

4.4 hAECs

The amniotic cavity starts already to form as early as 7-8 days post fertilization in the human embryo. The embryonic ectoderm (epiblast) given rise to all fetal tissue, but also to amniotic ectoderm. In human, the amniotic ectoderm is the first structure that is readily distinguishable from the epiblast shortly after implantation, well before the onset of gastrulation [103]. This suggests the possibility that hAECs could have escaped the lineage differentiation that accompanies gastrulation, preserving some or all of the epiblast characteristics such as pluripotency [104]. Due to this reason, in the last decade, hAECs have attracted great interest for clinical application. hAECs are isolated *in vitro* quite easily with enzymatic digestion. In literature, it is possible to find different isolation protocols and recently also in according to the current Good Manufacturing Procedures (GMP) (**figure 12**) [105]. When freshly isolated from human term amnios, hAECs have been shown to express **stem cell surface marker** proteins, such as SSEA-3, SSEA-4, TRA 1-60, TRA 1-81, Thy-1, and c-kit, but not hematopoietic stem cell markers, for example, CD34 [106]. Moreover, both freshly isolated and cultured hAECs express **molecular markers** typical of pluripotent human ES cells, i.e. OCT4, SOX2, and NANOG. Miki and colleagues tried to culture hAECs in the presence of EGF obtaining spheroid bodies. Expression of OCT4 and NANOG mRNA increased during spheroid formation and after five days of culture authors reported higher expression of OCT4 and NANOG in the middle fraction of cells rather than in the adherent fraction (**figure 13**) [106]. Work with whole-mount immunofluorescence analysis of freshly isolated term human amnion revealed that only cells in the amniotic epithelium and not in amniotic mesenchyme presented markers of pluripotent such as SSEA-3, SSEA-4, TRA 1-60 and TRA 1-80 [107].

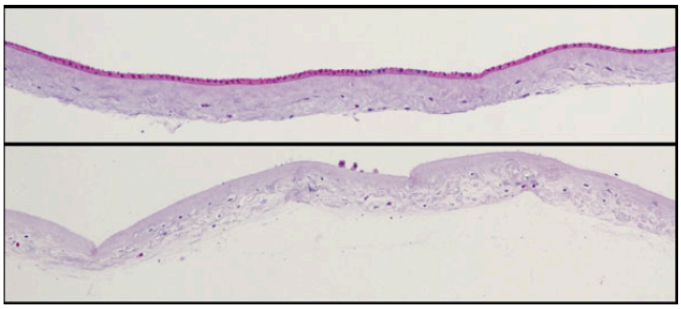


Figure 12. **Human amnios.** Human amnion before (top) and after (bottom) hAEC isolation. Stromal cells are still dispersed in the stroma after digestion. Image modified from Gramignoli *et al*, 2016.

Telomerase is usually limited to immortal cells, such as malignant cells, but also ESCs and germ cells. Unlike ESCs, freshly isolated hAECs do not present it. Furthermore, it has been reported that hAECs are not tumorigenic indeed they do not form teratomas *in vivo* when transplanted into immunodeficient mice. [106,108]. If from one side this finding gives strong advantage to hAECs compared to ES cells regarding their potential use in regenerative medicine, on the other side it could suggest that hAECs lack the capability to differentiate *in vivo*. Evidence for long-term self-renewal is still not available for hAECs, which may be due to the absence of telomerase that limits their ability to divide in culture and, subsequently, their self-renewal. In fact, hAECs have been claimed to be clonogenic by Ilancheran and co-workers [108], which was however not confirmed by the study of Bilic *et al*. [109]. Another very interesting characteristic of hAECs is their **tri-lineages differentiation** ability *in vitro.* Different researchers showed that hAECs could be induced through mesodermal (myocytic, cardiomyocytic, osteocytic and adipocytic), endodermal (pancreatic, hepatic, pulmonary and alveolar epithelium) and ectodermal differentiation (neural) [108,110,111]. Focus on endodermal differentiation Marongiu and co-worker have investigated the differentiation of hAECs *in vitro* and after transplantation into the livers of severe combined immunodeficient (SCID)/beige mice. Following the idea that liver microenvironment may be crucial for hepatic induction, they co-cultured hAECs with Mouse Hepatocytes (mHeps) improving their differentiation. In their work hAECs shown to express mature hepatocytes markers along with metabolically active and inducible CYP3A enzymes. Avoiding a so difficult and inconvenient protocol, they tested if hepatocyte conditioned media might pro-vide a similar inductive influence in a protocol more easily standardizable. Unfortunately, no strong hepatic inductive effect in gene expression was observed with human hepatocyte-conditioned media suggesting that interaction with neighboring cells enhances the hepatic commitment of hAECs. In the same work, they also showed how Activin A, one of the Shh inhibitors during embryogenesis, is not efficient to induce the first stage of the differentiation in common with the pancreatic differentiation, the endodermal commitment [112]. The two main factors were used to induce hAECs through pancreatic differentiation in a 2D system: Activin which, as previously discussed, aims to induce endodermal commitment [113] and Nicotinamide [108,114,115]. Also, other approaches were used as the ectopic expression of mPdx1 [116] and 3-dimensional culture [117]. Final hAECs were also transplanted after pancreatic induction in streptozotocin-induced diabetic SCID Mice [118]. All this data support the idea that

hAECs could be induced and differentiated through different commitments from all the three germ lineages. Therapies, aimed at reducing tissue inflammation and scarring to promote host tissue repair, are another important potential application of stem cells. Studies in murine models of lung and liver fibrosis have shown that primary hAECs reduce inflammation and fibrosis and induce tissue remodeling and repair. Another key feature is that primary hAECs appear to be amenable to allogenic transplantation. Successful transplantation across histocompatibility barriers is probably facilitated by low HLA Class IA antigen expression and absence of HLA Class II antigens in hAECs. Moreover, they do not express the co-stimulatory molecules CD80 (B7-1), CD 86 (B7-2), CD40, with and without of one of the most potent pro-inflammatory cytokine, INF-γ [119]. MHC molecules are responsible for the presentation of non-self antigens to receptors of T lymphocytes, and every individual expresses a particular set of MHC molecules. The primary cause of rejection in transplant is that different MHC molecules are recognized as foreign antigens by the immune system. The reduced presence of leukocyte antigens thus prevents recognition of the AECS on the part of the immune system. Some studies show that following the differentiation in the sense liver or pancreas *in vitro* increases the expression of HLA class IA and II molecules appear on a small percentage of cells [108]. The differentiation may, therefore, result in an alteration of the cells immunomodulatory features, which must be carefully assessed pest differentiation. Primary hAECs have also been shown to exert potent immunosuppressive properties inhibiting T cell proliferation, although the mechanisms remain unclear [111]. More recent investigations into their immunomodulatory properties have shown that hAECs inhibit cells of the innate and adaptive immune system, as shown by the inhibition of neutrophil and macrophage migration by secrete factors [107,120] and reduction of both T and B cell proliferation *in vitro* [121,122]. A very recent interesting study *in vivo* has demonstrated this effect also in a model of mouse autoimmune encephalomyelitis [123].

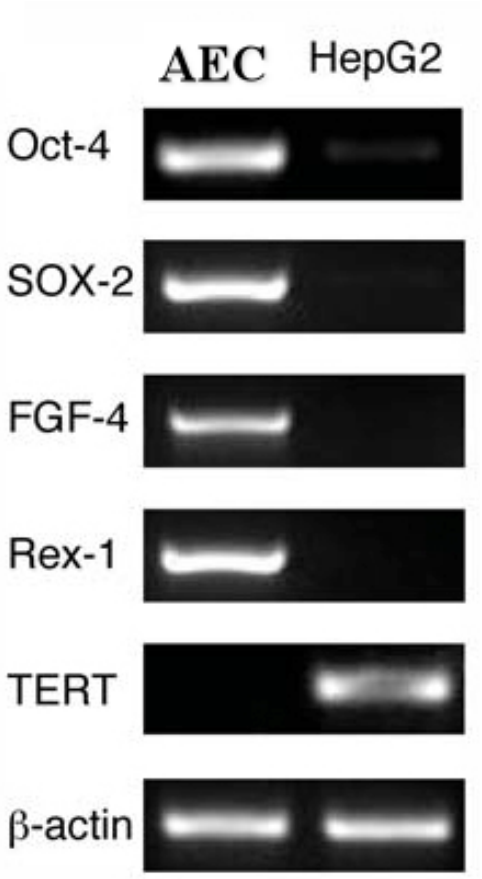


Figure 13. Stem Cell Characteristics of amniotic epithelial cells. Miki *et al*., 2005.

The great aim of education is not knowledge but action.

Herbert Spencer

Aim of the study

Pancreatic islet beta cells are endocrine cells that produce the hormone insulin and that control the metabolism of glucose. An autoimmune attack can cause a loss of beta cells, leading to a disease termed Type 1 Diabetes (T1D). There is no definitive cure for T1D. Treatment with exogenous insulin is a life-saving intervention, for patients with T1D, but it can cause serious adverse effects-including life-theatening hypoglycemic events. Over time, severe complications can arise in T1D patients. Frequently, these complications cannot be managed effectively. Since the discovery of insulin, many steps have been taken towards a safer and more effective treatment for T1D. Transplantation of cadaveric pancreas and isolated islets showed that beta cell replacement could treat T1D and prevent complications very effectively, but both strategies have important limitations. Stem cell-based strategies represent a promising way of research for the replenishment and preservation of beta cells. Stem cell strategies recently entered the initial stages of clinical testing. Certain types of stem and progenitor cells can be harnessed to generate insulin-producing cells for beta cell replenishment. Other types of stem/progenitor cells can be utilized to inhibit autoimmune mechanisms and rejection of transplanted beta cells. Moreover, certain cell types can exert trophic effects on beta cells. In this study, we investigated multiple stem and progenitor cells to better define their potential as cell therapies for T1D. In the first part of the study, we analyzed the potential of human amniotic epithelial cells (hAECs). hAECs can be recovered in large numbers from otherwise discarded placental tissues. These cells are endowed with stemness characteristics and they can inhibit immune attacks. Moreover, their use does not pose ethical problems. We observed that hAECs can be stimulated to partially commit toward pancreatic islet endocrine phenotypes. We developed an *in vitro* model to generate 3D islet-like structures. We aimed at testing whether organization into 3D organoids was able to boost beta cell maturation. Subsequently, we tested the ability of differentiated hAECs to secrete c-peptide (a peptide cleaved from proinsulin) in response to different glucose concentrations. We then evaluated the immunomodulatory properties of hAECs in 2D and 3D culture systems, before and after pancreatic endocrine differentiation. In the second part of the study we investigated the pancreatic differentiation potential of adult human endodermal progenitor cells from different anatomical sites. We studied cells isolated from human Brunner's Glands (BGs), Biliary Tree (BT) and Main

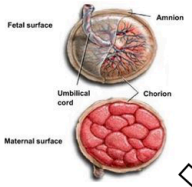
Pancreatic Duct (MPD). Endodermal progenitor cells were differentiated in a traditional 2D culture system and a co-culture system with Pancreas derived Mesenchymal Stromal/Stem Cells (PI-MSCs). This model proved useful to stimulate differentiation. Therefore, in the last part of the study, we combined hAECs and PI-MSCs in an attempt to boost endocrine maturation and obtain islet-like organoids.

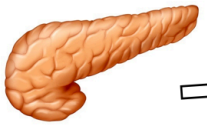
Beta-cell replacement

Immunomodulatory strategies

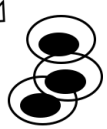
Epithelial cells:

- hAECs
- MPD
- BT
- BG

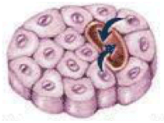


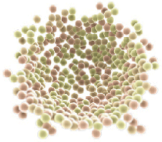


Differentiation into islet beta-cells



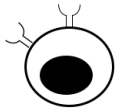
Insulin production

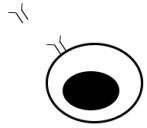




Beta-cell loss

T-lymphocytes



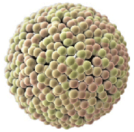


B-lymphocytes

- MSCs
- hAECs

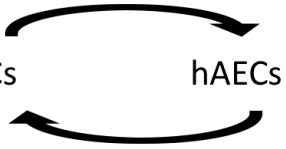
Mesenchymal cells:

- PI-MSCs



Spheroid formation

Epithelial + Mesenchymal cells:



Above all, don’t fear difficult moments. The best comes from them.

Rita Levi Montalcini

Materials and Methods

Isolation and culture of hAECs

Term placenta was obtained by caesarean section from healthy donor mothers after written informed consent and according to the policy of the Local Ethical Committee (Policlinico S.Orsola-Malpighi, prot n.1645/2014, rif. 35/2014/U/Tess). After sample collection, fetal membranes were separated from the chorionic plate and washed in Phosphate Buffered Saline (PBS, Lonza, Walkersville, MD, USA) added with 1% Penicillin-Streptomycin solution (Lonza, Walkersville, MD, USA). The human amniotic membrane was mechanically peeled off the chorion, the other fetal membrane. Amniotic membrane was then washed with abundant PBS (Lonza, Walkersville, MD, USA) in order to remove blood from the membrane. After that membrane was minced into small pieces (2 cm^2 approximately) and incubated for two cycles of 30 minutes at 37°C with 10/15 mL of 1X trypsin-EDTA (Lonza, Walkersville, MD, USA). After digestion, the trypsin activity was inhibited with 2,5 mL of heat-inactivated Fetal Bovine Serum (FBS, Lonza, Walkersville, MD, USA). The cell solution was then centrifuged for 10 minutes at 1500 rpm and the cell pellet was resuspended in the culture medium. Single cell suspension was tested for viability with erythrosin b (Sigma-Aldrich Co., St. Louis, MO, USA). Only samples with > 90% viability were used for further assays. Finally, cells were seeded at 60.000 - 100.000 cells/cm^2 and incubated at 37°C, 5% CO_2. Cells were cultivated in a Serum Rich Medium (SRM), Dulbecco's Modified Eagle's Medium - high glucose (DMEM, Lonza, Walkersville, MD, USA) added with the 10% FBS and 10ng/mL Epithelial Grow Factor (EGF, Sigma-Aldrich Co., St. Louis, MO, USA) or in Q286, a Serum Free Medium (SFM, PAA Laboratories GmbH, Pasching, Austria) added with 10 ng/mL Epithelial Grow Factor (EGF, Sigma-Aldrich Co., St. Louis, MO, USA).

In order to evaluate the cell proliferation potential in the two media, at first passage hAECs were plated at 60,000 cells/cm^2 in 24-well plates and cell growth was assessed using Alamar Blue assay (Invitrogen, Carlsbad, CA, USA). Alamar Blue 10% was added to the basal medium and incubated for 4 hours. Finally, fluorescence was measured using Victor Multilabel Counter (Perkin Elmer, Boston, MA, USA). The same procedure was repeated on days 3, 5, 8, 10, 12, and 15. Data are plotted as the percentage of Alamar reduction analyzed at specific time points.

Immunophenotypic characterization

The immunophenotypic characterization of hAECs was performed through flow cytometric analysis on primary cultures. Cells were detached with Trypsin-EDTA, fixed for 10 minutes with IntraPep Kit (Beckman-Coulter, Brea CA, USA) and incubated with antibodies for 30 minutes at 4°C (1 µg/mL). The antibodies used were: anti-CD105-FITC (Beckman-Coulter, Brea CA, USA), anti-panCytokeratin-PE (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-SSEA-4-APC (R&D Systems, Minneapolis, MN, USA), anti-OCT4 primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and secondary anti-mouse IgG-FITC (Beckman-Coulter, Brea CA, USA). To reveal Oct-4 and SSEA-4 cells were also permeabilized with the IntraPrep Kit (Beckman-Coulter, Brea CA, USA). Cells were finally washed and analyzed on a flow cytometer (Navios FC, Beckman Coulter). Acquired data were elaborated with Kaluza FC Analysis software.

Three-dimensional (3D) cultures

hAECs 3D culture was performed at first passage seeding the cells at 60.000 cells/cm^2 in a complete medium added with 2.5% reduced growth factor basement membrane extract (BME) from Engelbreth-Holm-Swarm (EHS) mouse sarcoma (Trevigen, Gaithersburg, MD, USA). Resuspended cells were seeded on a thick layer of 100% reduced growth factor BME measuring approximately 1 mm in thickness. Multicellular spheroids formed after 5–6 days, partially embedded in the gelled layer of basement membrane. Cell culture medium was changed every 3 days. In order to evaluate cell proliferation potential in the two media cell spheroids growth was assessed using Alamar Blue assay (Invitrogen, Carlsbad, CA, USA). Alamar Blue 10% was added to the basal medium and incubated for 8 hours. Finally, fluorescence was measured using Victor Multilabel Counter (Perkin Elmer, Boston, MA, USA). The same procedure was repeated on days 5, 8, 13, and 15. Data are plotted as the percentage of Alamar reduction analyzed at specific time points.

RNA isolation and expression

Total RNA extraction from samples in 2D culture was performed using the PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA, USA) and from samples in 3D culture system using TRIzol (Invitrogen,

Carlsbad, CA, USA) according to the manufacturer's instruction. RNA concentrations were measured by absorbance at 260 nm with a NanoDrop instrument and 2 µg of each sample were used as a template for a Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). For the 2D culture system, Real Time PCR assays were performed in a StepOne System (Applied Biosystems, Foster City, CA, USA) with KAPA SYBR FAST Master Mix (KAPA BIOSYSTEMS, Woburn, MA, USA). For the 3D system, the cDNA was subjected to 40 cycles of PCR with EconoTaq® PLUS GREEN 2X Master Mix (Lucigen Corporation, USA). The mRNA expression of the following genes was investigated: KI67, OCT-4, SOX2, NANOG and β-ACTIN (control). The PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide. Primers are shown in **Table 1**.

Table 1. PCR Primers

GENES	PRIMERS	SEQUENCES Real-Time PCR	SEQUENCES RT-PCR
KI67	F	TCC TAA TAC GCC TCT CAA AAG	
	R	GAT TTC TGA ACC TGA CTC TTG	
OCT 4	F	AGA GAA AGC GAA CCA GTA TC	GGTGAAGCTGGAGAAGGAGAAGCTG
	R	TTA CAG AAC CAC ACT CGG	CAAGGGCCGCAGCTTACACATGTTC
SOX2	F	CAG ACT TCA CAT GTC CCA G	ACCAGCTCGCAGACCTACAT
	R	CTC CCA TTT CCC TCG TTT	TGGAGTGGGAGGAAGAGGTA
NANOG	F	AGA AAT ACC TCA GCC TCC AG	AAGGCCTCAGCACCTACCTA
	R	CGT CAC ACC ATT GCT ATT CTT	ACATTAAGGCCTTCCCCAGC
β-ACTIN	F	CCT TCT ACA ATG AGC TGC G	GGACTTCGAGCAAGAGATGG
	R	CCT GGA TAG CAA CGT ACA TG	AGCACTGTGTTGGCGTACAG

In vitro pancreatic stepwise differentiation

Prior to differentiation, hAECs were allowed to grow for 5 days in SRM and SFM until the sufficient spheroid size was reached. Pancreatic induction of 3D cultured cells was performed using a stepwise protocol divided into three stages. Stage 1: hAEC spheroids were pre-differentiated for 4 days with 100 ng/mL Activin A (Sigma-Aldrich Co., St. Louis, MO, USA); Stage 2: SRM and SFM were supplemented with 100 ng/mL Activin A and Nicotinamide (NAM, 10mM, Sigma-Aldrich Co., St. Louis, MO, USA) for 4 additional days; and Stage 3: Terminal differentiation was performed

switching the medium to SRM and SFM with NAM (10 mM) and Retinoic Acid (RA, 2 µM, Sigma-Aldrich Co., St. Louis, MO, USA) for 10 days. The glucose concentration of SFM was ≥15 mM.

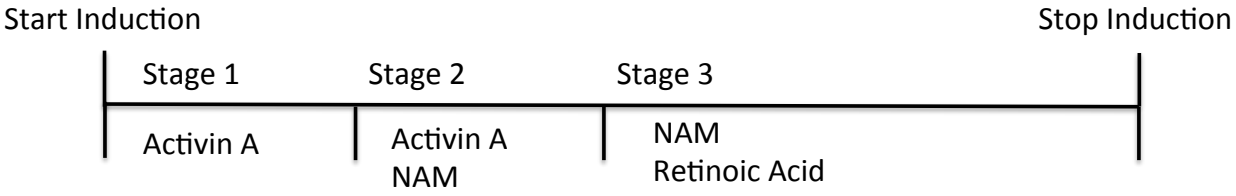


Figure 1. Step-wise differentiation protocol

Immunofluorescence analysis and confocal microscopy

3D hAEC spheroids were fixed with 2% Paraformaldehyde (PFA, Sigma-Aldrich Co., St. Louis, MO, USA) for 20 minutes at room temperature. After washing 2 times for 10 minutes in PBS, spheroids were permeabilized in 0.5% Triton X-100 (Sigma-Aldrich Co., St. Louis, MO, USA) in PBS for 10 minutes and then rinsed again 2 times for 10 minutes in PBS. Antigen blocking was performed for 1 hour at room temperature in blocking solution: 5% normal goat serum (Vector Laboratories, Burlingame, CA, USA), 0.2% Triton x-100, 0.1% Bovine Serum Albumin (BSA, Sigma-Aldrich Co., St. Louis, MO, USA). Primary and secondary antibodies were diluted in freshly prepared blocking solution. For anti-phalloidin, the antibody was incubated for 1 hour at room temperature. The other primary antibodies were incubated at 8°C overnight and secondary antibodies for 40–50 minutes at room temperature. The following primary antibodies and dilutions were used: FITC-Phalloidin 1:500 (#P5282, Sigma-Aldrich Co., St. Louis, MO, USA); mouse anti-insulin 1:100 (ab46707, Abcam, Cambridge, UK); rabbit anti-glucagon (ab8055, Abcam, Cambridge, UK); rabbit anti-C-peptide 1:100 (#4593, Cell Signaling Technology, Beverly, MA, USA); rabbit anti-CK19 1:100 (PA5-16726, Thermo Scientific, Waltham, MA, USA); mouse anti-laminin 1:100 (MA1-21194, Thermo Scientific, Waltham, MA, USA). Secondary antibodies were: DyLight 594 goat anti-mouse 1:200 (Jackson Immunoresearch Labs, West Grove, PA, USA); Alexa Fluor 488 goat anti-rabbit 1:400 (Molecular Probes); CF 488 goat anti-mouse 1:400 (Biotium, Fremont, CA, USA); DyLight 594 goat anti-rabbit 1:200 (Thermo Scientific, Waltham, MA, USA). Nuclei counterstaining was performed with Fluoroshield+DAPI (Sigma-Aldrich Co., St. Louis, MO, USA). Imaging was performed by confocal microscopy (Leica TCS SP2, Leica Microsystems, Mannheim, Germany) and images were processed using Leica Confocal Software.

Transmission electron microscopy (TEM)

Pancreatic induced hAECs cultured in 3D matrices and undifferentiated control cells were gently washed with Tyrode's buffer, pH 7.3, and centrifuged at 10,000 x g for 5 minutes at 10°C. The resulting pellets were fixed overnight with 2.5% Glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in Tyrode's buffer, postfixed for 2 hours in 1% Osmium Tetroxide (Electron Microscopy Sciences, Hatfield, PA, USA), dehydrated, and embedded in Spurr resin (Electron Microscopy Sciences, Hatfield, PA, USA). Since cells were not homogeneously distributed within matrices, semi-thin sections were routinely stained with Toluidine Blue and observed with a Zeiss Axiophot light microscope (Carl Zeiss, Jena, Germany) to locate cells in each cut layer. Ultrathin sections were performed on areas selected for the presence of spheroids and observed with a Jeol 2010 electron microscope (Jeol, Tokyo, Japan) at 200 kV after staining with Uranyl Acetate and Lead Citrate (Electron Microscopy Sciences, Hatfield, PA, USA).

Glucose-stimulated release and expression of C-peptide

To confirm the synthesis of insulin, C-peptide release from differentiated and undifferentiated hAEC spheroids was measured in hypoglycemic and hyperglycemic conditions. First, cells were incubated in Krebs-Ringer solution with bicarbonate and HEPES (KRBH; 129 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5 mM $NaHCO_3$, 10 mM HEPES, 0.1% (wt/vol) BSA (Sigma-Aldrich Co., St. Louis, MO, USA) for 70 minutes as a preliminary washing step. Next, spheroids were incubated in KRBH with 2mM D-Glucose (Sigma-Aldrich Co., St. Louis, MO, USA) for 1 hour and then switched to hyperglycemic stimulation condition consisting of KRBH with 20 mM D-Glucose and 30 mM KCl for 1 hour. C-peptide levels in supernatant from basal (2 mM D-Glucose) and stimulation condition (20 mMDGlucose + 30 mM KCl) were measured using the Human Ultrasensitive C-peptide enzyme-linked immunosorbent assay (ELISA) Kit (Mercodia, Uppsala, Sweden) and normalized to total amount of cellular DNA using CyQUANT Cell Proliferation Assays Kit (Invitrogen, Carlsbad, CA, USA). The fold stimulation was calculated as the ratio of the C-peptide concentration in supernatant from hyperglycemic condition to the C-peptide concentration from hypoglycemic condition. Glucose-stimulated spheroids were immediately assayed for the presence of C-peptide with immunofluorescence analysis.

Pancreatic Islet Mesenchymal Stromal/Stem Cells (PI-MSCs)

PI-MSC isolation and culture

Cadaveric pancreas and duodenums were obtained through Organ Procurement Organization. After samples collection, pancreatic islets were isolated in according to the Ricordi's method at the facility of the Diabetes Research Institute of Miami. The viability and the purity of pancreatic islets were analyzed through Trypan blue exclusion and Dithizone staining. Pancreatic islets were then transferred in the lab, 2000 equ were seeded in a T25 culture flask in DMEM Low Glucose (Lonza, Walkersville, MD, USA) and incubated at 37°C 5% CO2. Medium was changed every 3-4 days, after 10 days the confluence were reached and cells were expanded. For all the experiments cells were expanded at least five passages and also cryopreserved to be sure that no endocrine cells are present in culture.

In order to evaluate cell proliferation potential in the two media, at passage five PI-MSCs were plated at 5.000 cells/cm^2 in 24-well plates and cell growth was assessed using Alamar Blue assay (Invitrogen, Carlsbad, CA, USA). Alamar Blue 10% was added to the basal medium and incubated for 4 hours. Finally, fluorescence was measured using Victor Multilabel Counter (Perkin Elmer, Boston, MA, USA). The same procedure was repeated on days 2, 3, 5, and 7. Data were plotted as the percentage of Alamar reduction analyzed at specific time points.

Immunophenotypic characterization

PI-MSCs were analyzed to investigate their immunophenotypic profile by flow cytometry (FACS Navio FC, Beckman-Coulter, Fullerton, CA, USA) and data obtained analyzed by Kaluza FC Analysis software. The antibodies used were for stromal markers anti-CD44-FITC, anti-CD73-PE, anti-CD90-phycoerythrin-cyanine 5 (PC5) and anti-CD105-PE (Beckman-Coulter, Brea CA, USA), for hematopoietic markers anti-anti-CD34-FITC and anti-CD45-allophycocyanin (APC); for epithelial marker anti-panCytokeratin-PE (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and finally HLA-DR.

In vitro differentiation

At passage 5 PI-MSCs were tested for their ability to differentiate into the three classical mesenchymal lineages: osteogenic, adipogenic and chondrogenic lineages. Control cells were cultured in standard medium, DMEM 10% FBS.

Adipogenic differentiation

For adipogenic differentiation, PI-MSCs were cultured on 24-well plates (40,000 cells/well) with adipogenic medium (hMSC, Mesenchymal Stem Cell Adipogenic Differentiation Medium, Lonza, Walkersville, MD, USA*).* The medium was changed twice a week for three weeks. At the end of the induction, differentiation was assessed using Oil Red O staining. Cells fixed in 10% formalin at room temperature for 15 minutes were washed in distilled water and incubated with Oil Red O solution. Subsequently, the cell monolayer was washed three times with demineralized H_2O.

Osteogenic differentiation

For osteogenic differentiation, PI-MSCss were cultured on 24-well plates (15,000 cells/well) with osteogenic medium (StemPro Osteogenesis Differentiation Kit Gibco, Invitrogen, Carlsbad, CA, USA*).* The medium was changed twice a week for two weeks. At the end, differentiation was assessed using Alizarin Red stain (AR-S, Sigma-Aldrich Co., St. Louis, MO, USA). Cells fixed in 10% formalin at room temperature for 15 minutes were washed in distilled water and incubated with AR-S (40mM, pH 4.1). The monolayer was then washed three times with dH_2O.

Chondrogenic differentiation

To induce chondrogenic differentiation, aliquots of 250,000 cells were pelleted in polypropylene conical tubes in chondrogenic medium (StemPro Chondrogenesis Differentiation Kit, Gibco, Invitrogen, Carlsbad, CA, USA). This medium was replaced twice a week for 3 weeks. Pellets were formalin-fixed, embedded in paraffin, examined morphologically with hematoxylin eosin staining and assessed using Alcian Blue stain.

Immunomodulation assay

Immunomodulation assay on hAECs

In order to investigate the immunomodulatory properties of hAECs on activated Peripheral Blood Mononuclear Cells (PBMCs), hAECs were plated in 6-well plates at a density of 50,000 cells/cm^2 and allowed to stabilize in culture for 1 day. PBMCs were isolated by density gradient centrifugation (Ficoll-Paque, Sigma-Aldrich Co., St. Louis, MO, USA) from healthy donors and co-cultured on hAECs monolayers at a ratio of 2:1 in RPMI with 10% FBS (Lonza, Walkersville, MD, USA). PBMCs were activated by the addition of Phytohemagglutinin (PHA, 5 µg/mL, Sigma-Aldrich Co., St. Louis, MO, USA) and incubated for 72 hours at 37°C, 5% CO_2. PBMCs without PHA stimulation were used as negative control and PBMCs stimulated by PHA in the absence of hAECs as positive control. The immunomodulatory ability of hAECs was quantified by different assay. In order to study the ability of hAECs to inhibit the PBMCs proliferation was analyzed the BrdU incorporation by activated PBMCs. After 72 hours of co-cultures between hAECs and PBMCs, the latter were recovered, 100 µl were seeded in triplicate in a 96-well plate and then BrdU incorporation levels were quantified using a colorimetric immunoassay, according to the manufacturer's instructions (Cell Proliferation ELISA, BrdU colorimetric kit, Roche, Basel, Switzerland). After the 72 hours, PBMCs recovered were also fixed (with 70% ethanol at 4°C) and stained with Propidium Iodide (Beckman-Coulter, Brea CA, USA) at room temperature for 10 minutes to analyze the cell cycle phase distribution of PBMCs after co-culture with hAECs. In order to study one of the possible mediators of this effect, the HLA-G expression by hAECs was analyzed. hAECs cultured with and without activated PBMCs were fixed with intraPep kit (Beckman-Coulter, Brea CA, USA) and the HLA-G expression (ab7904, Abcam, Cambridge, UK) was measured through flow cytometric analysis. Finally, also T-Reg sub-population was analyzed. PBMCs were fixed as above and analyzed with the *T-REG Analysis kit* (Beckman-Coulter, Brea CA, USA) for the three markers CD4, CD25, FOXP3.

Three dimensional immunomodulation assay

The immunomodulatory activity of hAECs was also analyzed after stepwise pancreatic-endocrine differentiation in 3D culture. PBMCs were isolated as previously described and plated after one day

of pre-activation on hAECs spheroids at the end of pancreatic differentiation protocol (hAECs/ PBMCs ratio 1:2). PBMCs were activated by the addition of PHA (5 µg/mL) and hAECs spheroids/ PBMCs co-cultures were incubated for 72 h at 37°C, 5% CO_2. The negative control consisted of PBMCs without PHA stimulation and the positive control was PBMCs with PHA in the absence of hAECs spheroids. Finally, PBMCs were resuspended, recovered, and plated in 96-wells plate for BrdU incorporation analysis. BrdU incorporation was evaluated using a colorimetric immunoassay, according to the manufacturer's instructions (Cell Proliferation ELISA, BrdU).

Immunomodulation assay on PI-MSCs

In order to investigate also the immunomodulatory properties of PI-MSCs, they were plated in 6-well plates at a density of 10,000 cells/cm^2 and allowed to stabilize in culture for 1 day. PBMCs were isolated and co-cultured on PI-MSCs monolayers at a ratio of 10:1 in RPMI with 10% FBS (Lonza, Walkersville, MD, USA). PBMCs were activated by addition of PHA (1 µg/mL) and incubated for 5 days at 37°C, 5% CO_2. The negative control was PBMCs without PHA stimulation and the positive control consisted of PBMCs stimulated by PHA in the absence of PI-MSCs. The immunomodulatory ability of PI-MSCs was quantified by different assays. In order to study the capability of PI-MSCs to inhibit the PBMCs proliferation was analyzed the BrdU incorporation by activated PBMCs and their cell cycle. Furthermore, to investigate one of the possible mediators of this effect, the HLA-G expression by PI-MSCs was analyzed as previously described.

Endodermal cells isolation and culture

Main Pancreatic Duct and Biliary tree cells isolation and culture

Pancreas was obtained from cadaveric donors through Organ Procurement Organization. Main pancreatic duct (MPD) was removed from the pancreas during islet isolation in the facility of the Diabetes Research Institute, Miami. The tissue was conserved in RMPI (Lonza, Walkersville, MD, USA) 10% FBS (Lonza, Walkersville, MD, USA) added with Trypsin inhibitor (Sigma-Aldrich Co., St. Louis, MO, USA) in order to avoid tissue auto-digestion from the exocrine part of the pancreas until the isolation procedure was performed. Then tissue was transferred to the lab and all the

exocrine parts were removed. Duodenum was maintained in ice-cold UW or Kubota Medium with 1% Antibiotics during shipment and processed within 24 hours. The organ usually appears surgically closed in the cranial and caudal side. The biliary tree was separated from the duodenum going to cut to ampulla of Vater without perforate the duodenum inner side to avoid contamination. Both the tissue were then minced in small piece and digested with collagenase II 2 mg/mL (Invitrogen, Carlsbad, CA, USA) in a 37°C water bath for 10 minutes, shaking the tubes every 5 minutes. The suspension was flowed through a 100 µm strainer, both the strained flow-through cell suspensions and the undigested tissue over of the strainer was saved. The undigested portion over of the strainer was collected and the collagenase digestion and strainer steps were repeated until most tissue was dissolved. Normally five cycles are necessary. Then cells were centrifuged, washed in PBS and the final pellet was resuspended in complete medium. Complete medium was Kubota Medium (KM, table 1) 10% FBS, for MPD cells were also added 20 µg/mL of Trypsin inhibitor (Sigma-Aldrich Co., St. Louis, MO, USA) were added. Cells were cultured on 6-well plates coated with collagen IV (Sigma-Aldrich Co., St. Louis, MO, USA) for 4-6 days before further analyses.

Table 2. Kubota Medium (KM)

Component	Concentration or quantity	Seller	Catalog number
RPMI 1640	500 mL	Gibco	11875-093
BSA	0,5 g	Sigma-Aldrich	A8806-5G fatty acid free
Niancinamide	270 mg	Sigma-Aldrich	N0636
Insulin	2,5 mg	Sigma-Aldrich	I5500
Transferrin	10 µg/mL	Ado, Bovine, Sigma	T1283
Selenium	0,5 mL (3.1-10M)	Sigma-Aldrich	211176
Free Fatty Acids	38 µL (7,6 Equ/L)		
L-Glutamine	5 mL (2 mM)	Gibco	25030-081
Antibiotics	5 mL	Gibco	15240-062 AAS
Hydrocortizone	500 µL (10E-7 M)	Sigma-Aldrich	H0888
beta-mercaptoethanol	1,75 µL (5E-5 M)	Sigma-Aldrich	M6250
Zinc Sulfate heptahydrate	0,5 mL (10E-10 M)	Specpure	JMC156
High Density Lipoprotein	10 µg/mL	Sigma-Aldrich	L8039

Cells from Brunner's glands isolation and culture

Duodenum was obtained from cadaveric donors through Organ Procurement Organization. Tissue was maintained in ice-cold UW or KM with 1% Antibiotics during shipment and processed within 24 hours. The organ usually appears surgically closed in the cranial and caudal side. 5 mm slit was cut at the cranial side of the duodenum and the organ was squeezed pushing with the clamp in order to remove the mucus and the content of the lumen. The organ was positioned vertically, cranial side up, and filled with PBS 5% Antibiotics (100-150 mL) by pipetting through the slit. If there is a leak, the opening was clamped at the ampulla of Vater. Then the washing solution was removed by flipping the organ, squeezing and pouring the content on a discard becker. This wash step was repeated 2 more times. The organ was positioned vertically, cranial side up, filled with Sodium Deoxycholate 1% solution in PBS (SDC, Sigma-Aldrich Co., St. Louis, MO, USA)) and incubate at room temperature for 3 minutes. Then the detergent solution was removed by flipping the organ and pouring the content on a discard becker. The organ was filled with PBS 2% Antibiotics by pipetting through the slit. The washing solution was removed by flipping the organ and pouring the content on a discard becker. This wash step was repeated 2 more times. The duodenum was cut open and flattens out using sterile tweezers, the mucosa was exposed and the ampulla of Vater identified. A rectangle of tissue that goes from approximately 2,5 cm cranially to the minor ampulla, to 2,5 cm caudally to the ampulla, and from side to side of the duodenum was collected. The mucosa was than scraped off and removed using a flat sterile scalpel. The tissue was finally washed vigorously in a becker with abundant PBS 2% Antibiotics for three times. The tissue was minced into small pieces and digested in a pre-warmed Collagenase II 2 mg/mL (Invitrogen, Carlsbad, CA, USA) solution per every 1 mL of tissues. Three cycles of 15 minutes of digestion in the 37°C water bath were performed, shaking the tubes every 5 minutes. At the end of every cycle, the suspension was flowed through the strainers. The undigested portion over of the strainer was collected and Collagenase digestion cycle was repeated. The strained flow-through cell suspension was centrifuge for 5 minutes at 1500 rpm. All the cell pellets were then resuspended and collect together in KM added with 2% antibiotic. Cells were finally counted and plated in 6-well plates coated with collagen IV (Sigma-Aldrich Co., St. Louis, MO, USA), in KM supplemented with 10% FBS. Cells were culture for 3-4 days in KM 10% FBS and subsequently the medium was replaced with fresh serum-free Kubota medium or induction medium.

Transwell culture

Freshly isolated endodermal cells were seeded on the bottom side of a transwell with a porosity of 0,4 um (Corning, NY, USA) coated with collagen IV (Sigma-Aldrich Co., St. Louis, MO, USA). Cells were allowed to stabilize for one night, the day after transwells were flipped and cells cultured and induced for 5 days. At day 5 of induction PI-MSCs were seeded on the top side of the transwell at a density of 10.000 cells/cm2. A schematic representation is show in **figure 1**.

Pancreatic differentiation

After three/four days of culture cells were induced through pancreatic differentiation. Control cells were cultured in serum-free KM and induced cells in HDMP+ medium (**Table 2**). Cells were induced for five days with one medium changed. In the second part of the induction after five days PI-MSCs were seeded at 10.000 cells/cm^2 on endodermal cells and maintained in culture for other two days.

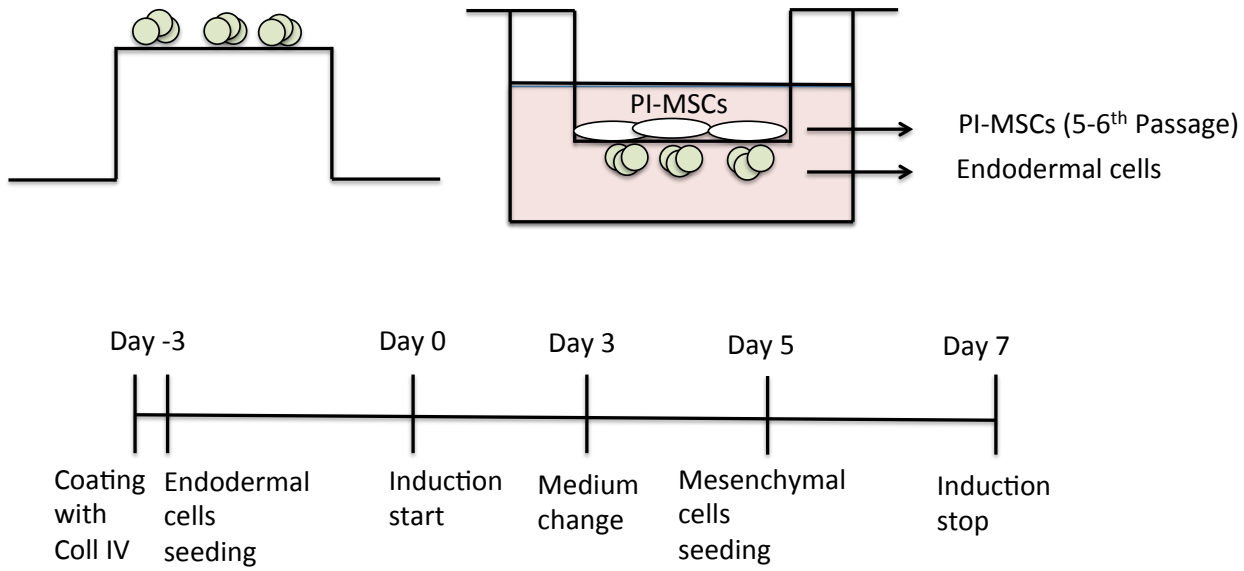


Figure 2. Pancreatic differentiation and transwell protocol

Table 2. HDM-P+

Component	Concentration
KM without hydrocortison (KM-h)	
Glucose	22mM
B27	2%
Retinoic Acid	10 µM
Cyclopamine	2,5 µM
Exendin 4	50 ng/mL
Ascorbic Acid	10ng/mL

Real Time PCR

At the end of the differentiation protocols the RNA was extracted with Total RNA Purification Kit (Norgen, Thorold, ON, Canada). The RNA was then quantified with Nanodrop and the same amount was used for the retrotranscription. The cDNAs were then amplified with TaqMan Fast Universal PCR Master Mix (Life Technologies) and Real-Time PCR assays were performed in a StepOne System (Applied Biosystems, Foster City, CA, USA). RNA isolated from pancreatic islets was used as positive control and all the gene expression was normalized to not induced cells. The gene analyzed and the corresponding primers are shown in **Table 3**.

Table 3. Primer

Primer	# number	Amplicon	Assay location	Exon	Ref seq
INS	Hs00355773_m1	126	252	2-3	NM_000207.2
GLG	Hs00174967_m1	98	791	5-6	NM_002054.4
PDX 1	Hs00236830_m1	73	517	1-2	NM_000209.3
NKX 6.1	Hs00232355_m1	93	677	1-2	NM_006168.2
Pax 6	Hs01088114_m1	86	1078	7-8	NM_000280.4

C-peptide quantification

Supernatants were collected and stored at -20°C at different times during the pancreatic differentiation. At the end of the induction was also performed a static incubation assay. Cells were firstly washed three times with PBS and after incubated with DMEM Low glucose (2.5 mM) for 1 h. The supernatant was collected and stored at -20°C. Cells were washed again three times with PBS. A second incubation with DMEM high glucose (20 mM) for 1 hour was performed and the supernatant was collected. Cells washed three times PBS. Finally a last incubation with DMEM Low glucose (2.5 mM) for 1 hours was performed and the supernatant was collected. Samples collected were analyzed for c-peptide quantification with cobas c-peptide kit.

Co-culture system

2D co-culture system

hAECs were isolated as previously described and at passage 1 were cultured in ultra low attachment (ULA) T25 flask at a density of 750.000 cells/mL for 72 hours. In the meantime PI-MSCs were seeded at a density of 10.000 cells/cm^2 in DMEM L. 10% FBS and left to stabilize in culture for one night. The day after PI-MSCs were treated for 1 hour with mitomycin C 20 µg/mL (Sigma-Aldrich Co., St. Louis, MO, USA) to arrest their proliferation. Finally, hAECs were seeded diluted 1:10 on top of PI-MSCs and allowed to adhere for one night before further analysis.

Immunofluorescence

Cells were fixed for 10 minutes in 2% Paraformaldehyde at room temperature. After three washes with PBS, cells were permeabilized for 10 minutes with PBS 1% Triton (Triton X-100, Sigma-Aldrich, Co., St. Louis, MO, USA), then incubated in blocking solution 1X PBS 1% BSA (Sigma-Aldrich, Co., St. Louis, MO, USA) for 30 minutes. Cortical actin was stained using FITC-Phalloidin (1:250, Sigma-Aldrich, Co., St. Louis, MO, USA) diluted in blocking solution incubating for 1 hours at room temperature. After three washes in 1X PBS coverslips were mounted using ProLong Gold Antifade Mountant with Dapi (Thermo Fisher Scientific, Monza, MB, Italy)

Spheroids creation and characterization

hAECs were cultured in ultra low attachment T25 flasks at a density of 750.000 cells/mL for 3 days in DMEM H. Glucose with 10% FBS and EGF 10ng/mL. After 72 hours PI-MSCs were added in a ratio of 1:4 and maintained in culture for further analysis.

Cell spheroids were also sorted with a tag less proprietary cell sorter Celector® (Stem Sel ltd, Bologna, Italy). Celector® is based on a field-flow fractionation (FFF) derived technique. FFF is a chromatographic-like, soft-impact separation method that performs partition according to chemical-physical properties, such as mass, size, charge, density, shape, and rigidity. Separation is achieved within a capillary channel by the combined action of the laminar flow (mobile phase) and a field applied perpendicularly to the flow. Cells with different physical properties are driven by the applied field in a specific position across the channel thickness thus they are eluted at a well defined velocity. Different field types can be used in FFF. In this study was used a proprietary method based on a novel modification of the Gravitational-FFF process exploiting the Earth's gravity field for separation. The fractionation process is based on the differences in cell features that are dynamically acquired during flow-assisted fractionation under the combined action of the flow stream, the gravitational field, and the hydrodynamic lift forces (Non Equilibrium Earth Gravity Assisted- Dynamic Fractionation, NEEGA-DF) [124]. After fractionation is completed, cells can

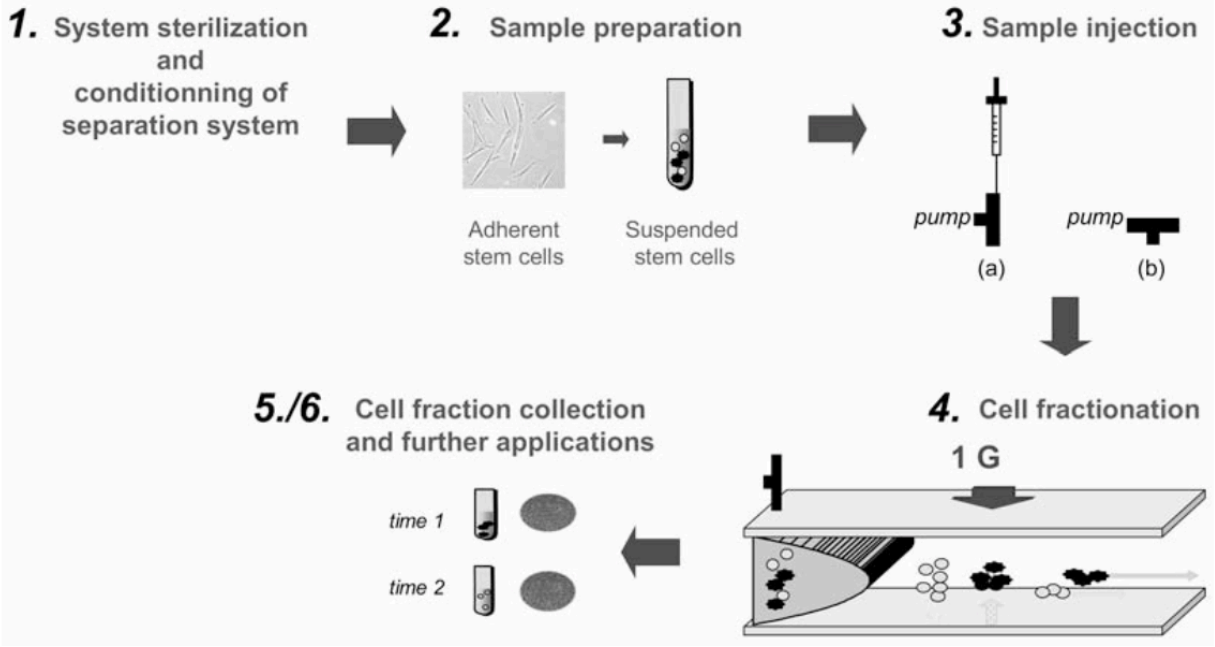


Figure 3. **Scheme of the fractionation process.** Roda B *et al*., 2009.

return to the adherent state, and the native physical features are fully restored. This allows high cell recovery and full maintenance of cell viability and differentiation features [125-127]. The NEEGA-DF method is implemented in a prototype named Celector®. The instrumental set-up is mainly composed of a mobile phase reservoir, an injection valve, pumps for fluids delivery, the separative channel, an optical system for real-time monitoring of the eluted cells and a fraction collector. In this study, 100 µl of spheroid suspension were injected into Celector® with a continuous mobile phase flow at 1 mL/minute flow rate. Sterile filtered PBS added with 0.1% BSA, 100 U/mL penicillin, and 100 µg/mL streptomycin was employed as mobile phase. The fractionation process can be real-time monitored by the optical system since channel wall are made of transparent plastic materials and the cell distribution over elution time can be obtained (fractogram) Three fractions of eluted cells were collected at different time points. F1 cell fraction was collected from 0 to 2 minutes, F2 from 2 to 4 minutes, and F3 from 4 to 10 minutes. After the fractionation process, cells were plated in a 24-well plate and monitored for the following days. The same process was performed with hAECs spheroids alone and with mixed spheroids (hAECs/PI-MSCs).

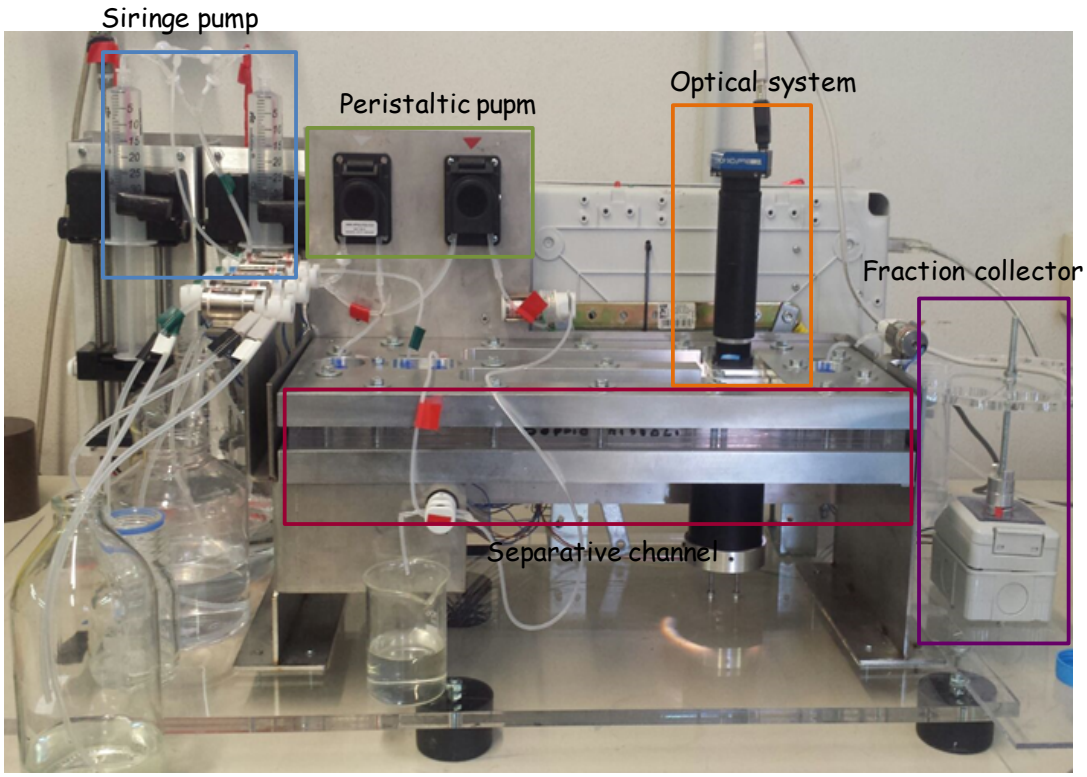


Figure 4. Celector

Statistical analysis

Data are presented as means standard deviation and were analyzed by one and two-way ANOVA or t-test using Graph Pad Prism software. The significance threshold was $p<0.05$.

When the telescope ends, the microscope begins.
Which of the two have the grander view?

Victor Hugo
Les Miserables

Results and Discussion

human Amniotic Epithelial Cells (hAECs)

Isolation and culture

Human placenta is obtained in accordance to the local ethical committee. Immediately after delivery, the placenta is transferred and processed in the laboratory. As shown **in figure 1-a,** it is possible to separate the two fetal membranes (amnion and chorion) by a simple mechanical traction. Before the enzymatic digestion, two cell populations are present in the amniotic membrane, the epithelial component and the stromal component disperse in the extracellular matrix (**figure 1-b**). The enzymatic digestion of the tissue with trypsin enables the detachment and collection of amniotic epithelial cells, and the removal of the stromal component, as confirmed by microscope visualization (**figure 1-c**). Therefore, the isolated cells are termed human Amniotic

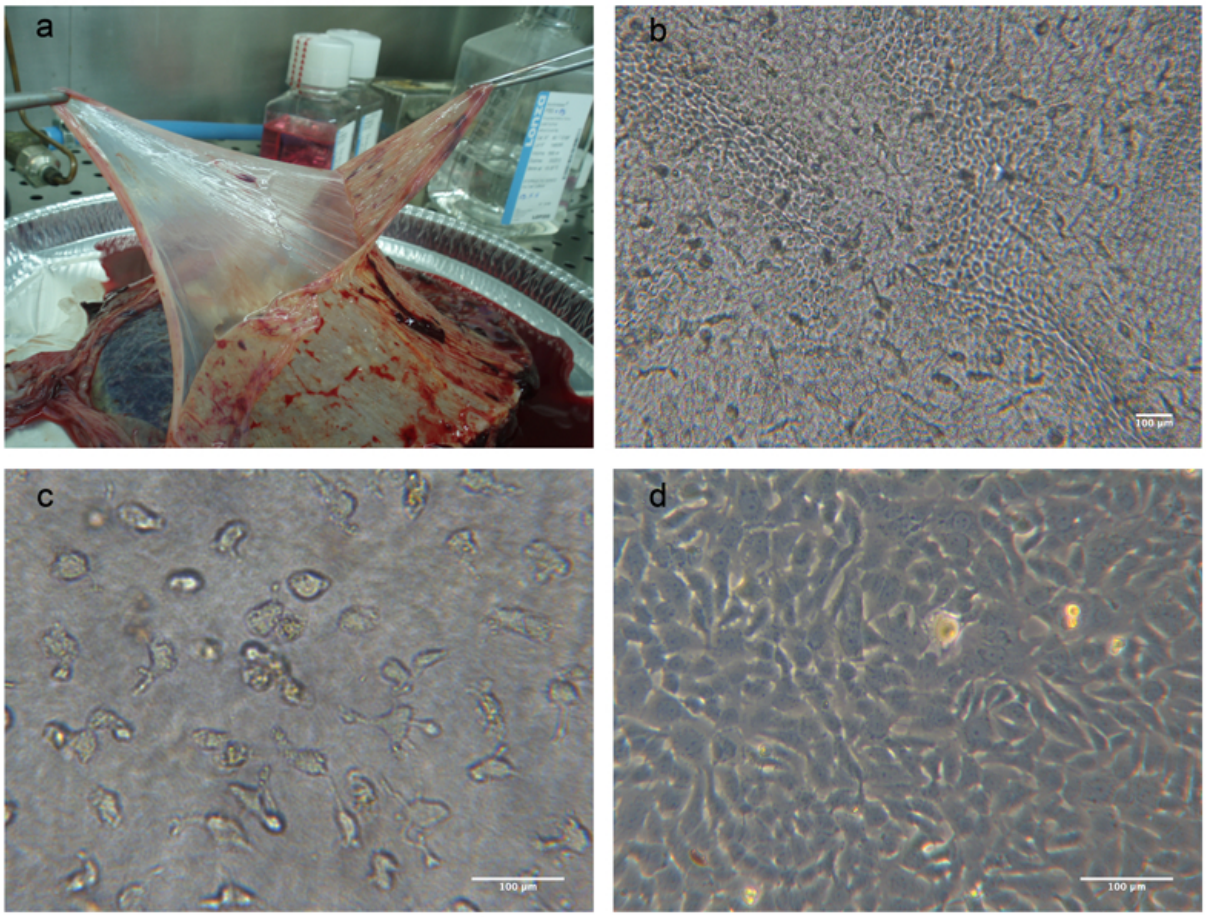


Figure 1. Cells isolation. (a) Separation of the two membranes from the placenta. (b) Amniotic membrane before the enzymatic digestion, 4X. (c) The amniotic membrane after the enzymatic digestion, 10X. (d) a monolayers of hAECs, 10X.

Epithelial Cells (hAECs). They were seeded in a range of 60.000-100.000 cells/cm^2, normally in DMEM H. Glucose added with 10% FBS and EGF 10 ng/mL, and incubated at 37°C with 5% of CO_2. hAECs were expanded in culture, and in 4-6 days a confluent monolayer is obtained with the classical epithelial cobblestone-shaped morphology (**figure 1-d**).

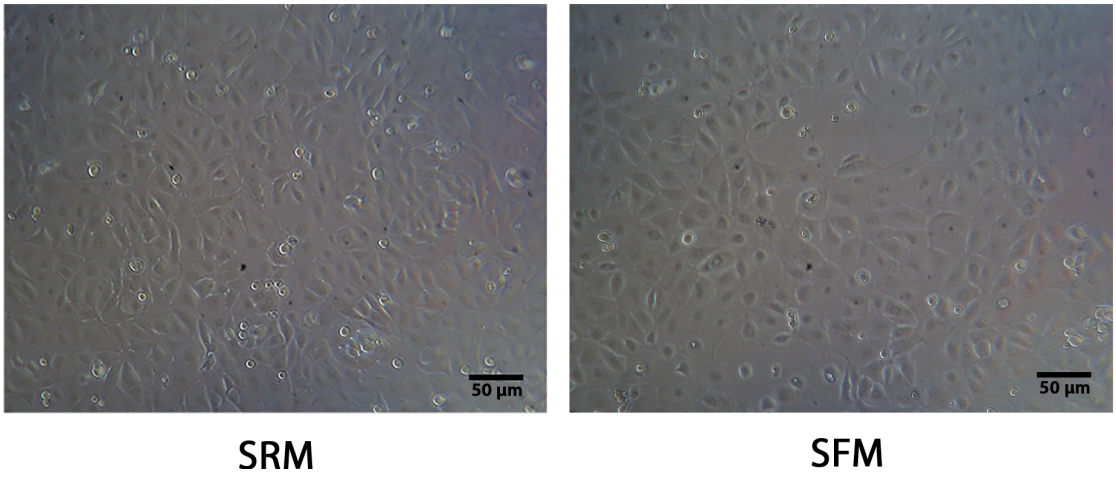
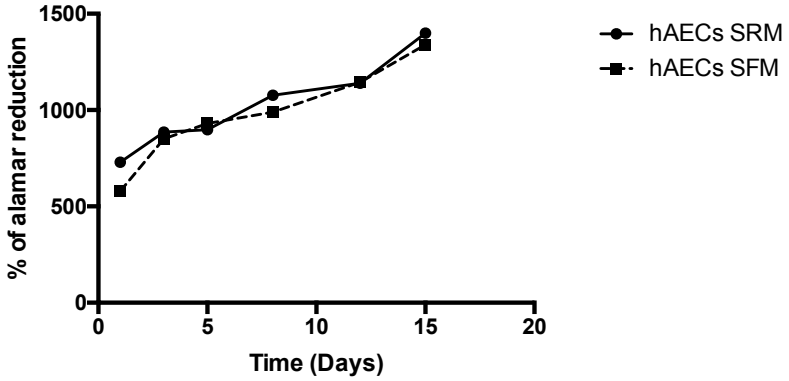


Figure 2. Light microscopy images of hAECs. On the left hAECs cultured in SRM and on the right hAECs cultured in SFM, 4X Magnification.

Cells were cultured in Serum Rich Medium (SRM), DMEM H. Glucose 10% FBS + EGF 10 ng/mL but also in a Serum Free Medium (SFM), Q286 + EGF 10 ng/mL. Q286 is a further developed DMEM enriched with selected serum components and growth enhancers, tailor made for epithelial cells. We have decided to test a serum-free medium for its importance in clinical applications in terms of reproducibility, standardization, and absence of ethical problems related to the use of animal origin component. hAECs can be isolated and cultured in SRM and also in SFM as shown in **figure 2**. Moreover, cells obtained do not show morphologic differences. To evaluate the culture medium influence on hAEC growth kinetic and proliferation ability, we performed a cell growth assay for 15 days. The resulting representative growth curves show no significant differences between SRM and SFM, only a slight decrease in SFM (**figure 3**).

Figure 3. hAECs proliferation. *In vitro* expansion of hAECs in SRM and SFM culture media. Cells were isolated and seeded at passage 1 at 60,000 cells/cm^2, and cell proliferation was analyzed for 15 days. Data are plotted as the percentage of alamar reduction analyzed at specific time points.



Immunophenotypic and molecular characterization

In order to analyze the immunophenotypic profile and the stemness characteristic of hAECs in the two different media, a cytofluorimetric analysis was performed. F**igure 4** shows the expression of two stemness markers Oct4A (SRM 99.1 ± 0,2% and SFM 99,3 ± 0,3%) and SSEA-4 (SRM 97.3 ± 0,4% and SFM 98,9 ± 0,2%) that are expressed in both culture conditions. It is also possible to appreciate the expression of a classical epithelial marker, Pan-Cytokeratin (SRM 99.9 ± 0,1% and SFM 99,9 ± 0,1), and the low expression of a classical stromal marker, CD105 (SRM 94.5 ± 0,5% and SFM 93,7 ± 0,6). These data proved the preservation of the hAEC immunophenotype and stemness characteristics in SFM.

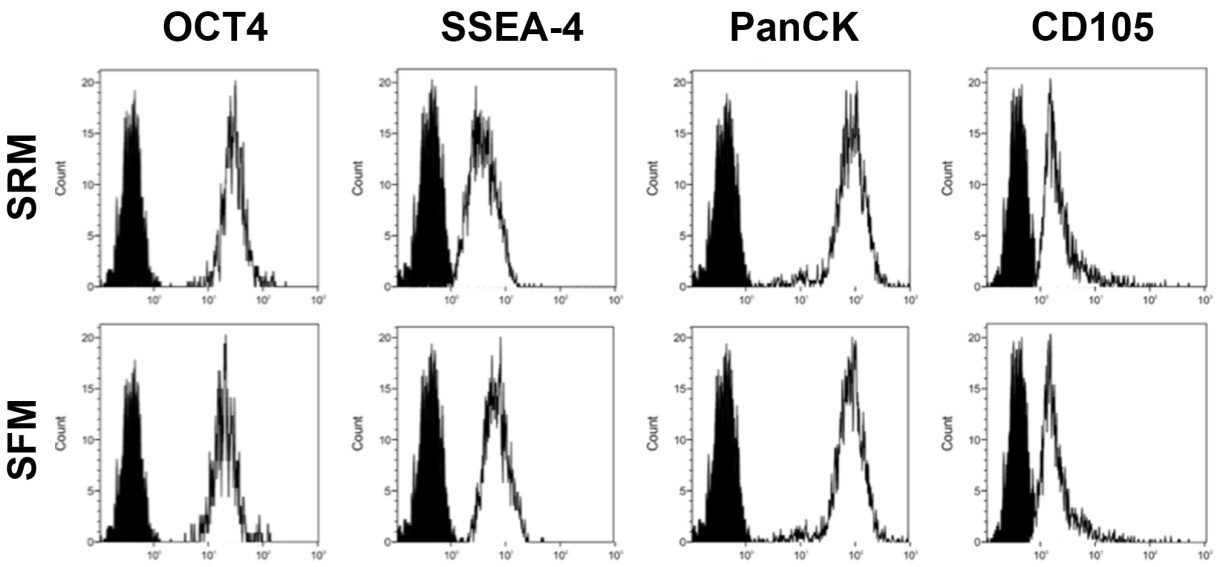
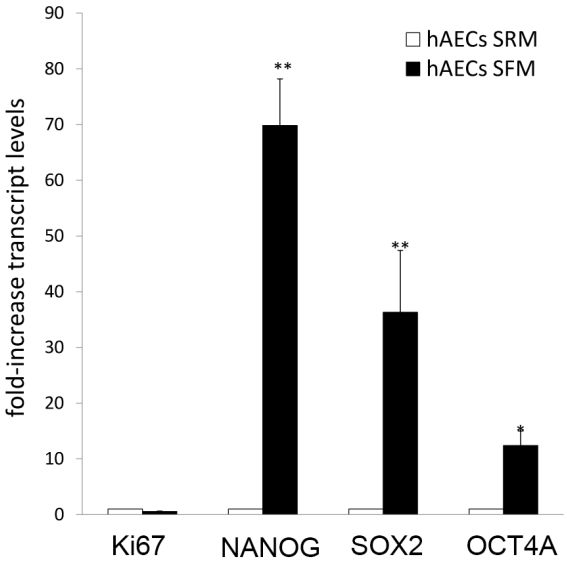


Figure 4 Cytofluorimetric characterization. Expression of the two stemness marker OCT4 and SSEA-4. High expression of the epithelial marker PanCK and low expression of the stromal marker CD105. Unstained controls are presented as filled black histograms, the specific cell markers as white histograms. Modified from Okere *et al.,* 2015.

To further evaluate the SFM impact on hAECs characteristics, the mRNA expression of a proliferative marker, Ki67, and of three classical stemness markers NANOG, SOX2, and OCT4A, was analyzed. Ki67 expression shows a not significant decrease in SFM, which is confirmed by the observation of a mild reduction proliferative capacity *in vitro* (f**igure 5).** However, the gene expression levels of stemness markers were clearly and significantly higher in SFM than in SRM, namely 70-fold for NANOG, 36-fold for SOX2, and 12-fold increase for OCT4a mRNA. This suggests that SFM could preserve hAECs in a more undifferentiated status.

All these data confirm firstly the possibility to isolate and expand hAECs in a standard culture medium without the addition of the fetal bovine serum. Second SFM seems not to affect the classical characteristics of the hAECs. This allows their application for future cell therapies.



F**igure 5. Gene expression.** Gene expression levels for Ki67, NANOG, SOX2, and OCT4a. mRNAs contents of hAECs cultured in SFM (black bars) are expressed as the fold increase against control cells cultured in SRM (white bars). Means ± SD; n = 5. *P <0.05; **P <0.01. Modified from Okere *et al.,* 2015.

3D Culture

In order to test the possibility to induce hAECs through the pancreatic endocrine lineage, we have decided to create a 3D culture system. This allows us to recreate *in vitro* a more similar environment to the *in vivo* one. After the first passage in culture, cells were seeded on a thick bed consisting of 100% gelled basement membrane extract (BME) and cultured for different days. After seven days it is possible to observe the formation of multicellular structures that increase over time and reach a definitive architecture after two weeks. **In figure 6** it is shown how SFM was more efficient in maintaining a stable spheroid morphology after two weeks in culture, while the serum rich condition caused the development of irregular and larger cell clusters.

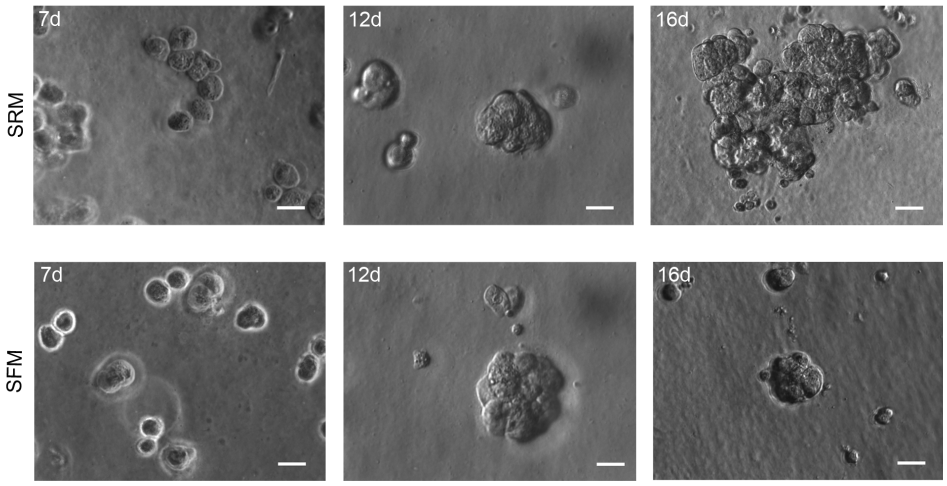
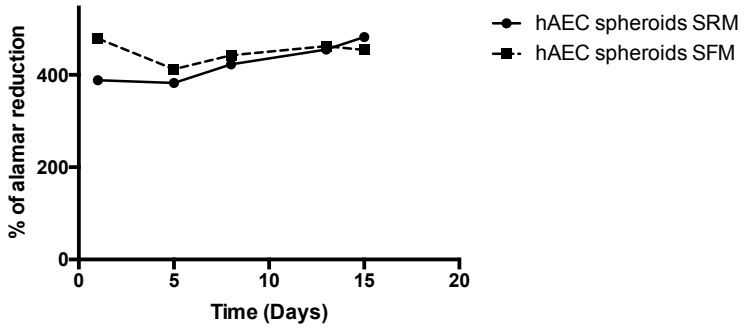


Figure 6. Three dimensional culture. On the top of the image cluster formation in serum rich medium at different time point. On the bottom it is possible to observe the cluster formation in serum free medium. Scale bars = 20 μm. Modified from Okere *et al.,* 2015.

To evaluate the viability and the growth of hAECs in a 3D culture model, we have performed a cell growth assay for 15 days. The resulting curve does not show a growth but only a stabilization of the hAEC spheroids in culture. Moreover, they do not show significant differences between SRM and SFM (**figure 7**).

Figure 7. hAEC spheroids growth kinetics. *In vitro* culture of hAEC spheroids in SRM and SFM culture media. Cells were isolated and seeded at passage 1 at 60,000 cells/cm^2 on 100% gelled BME, and cell proliferation was analyzed for 15 days. Data are plotted as the percentage of alamar reduction analyzed at specific time points.



To visualize the internal structure of hAEC spheroids, an immunofluorescence for phalloidin, a marker for f-actin distribution, was performed. **In figure 8** it is possible to observe a representative image that shows a dense core and a well structured spheroid.

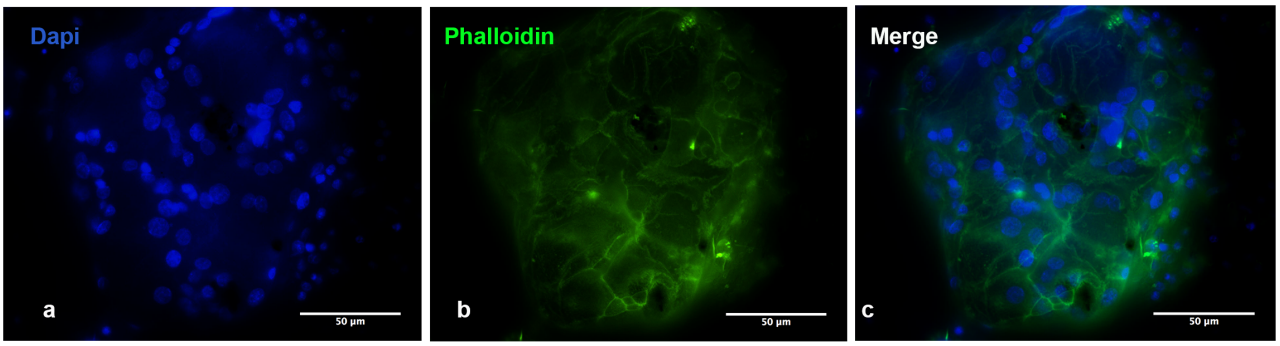


Figure 8. hAECs spheroids structure. Representative image of a cytoskeleton structures evidenced by immunofluorescence assay a) Nuclei counterstained with DAPI (blue). b) Phalloidin staining (green) c) Merge micrograph.

Finally, the mRNA expression of stem cell factors, OCT4, SOX2, and NANOG was checked to determinate their expression in the 3D culture system. **Figure 9** shows a representative image of an RT-PCR that highlight the maintenance of their expression in 3D culture.

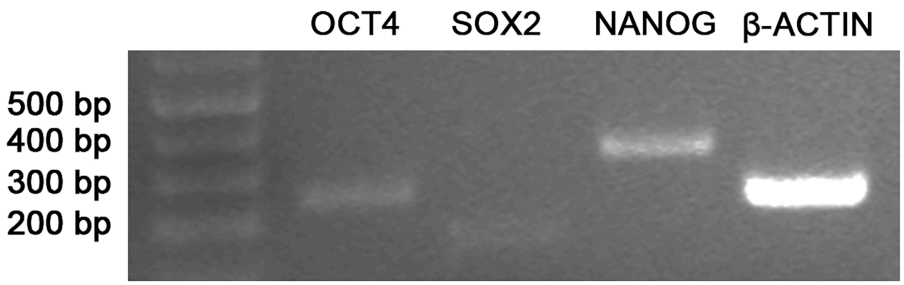
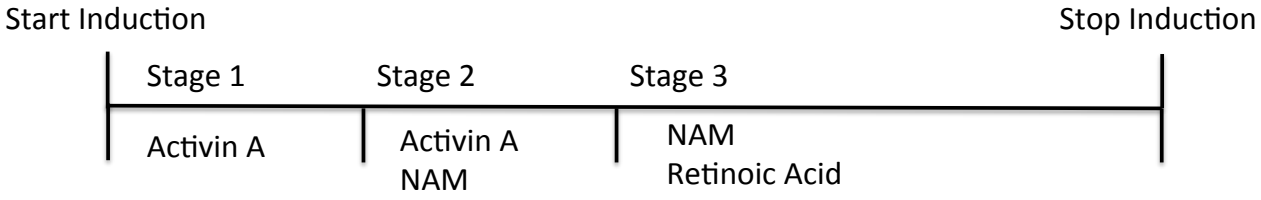


Figure 9. RT-PCR in 3D cultures. Representative image of a RT-PCR for stemness genes OCT4, SOX2 and NANOG in hAECs cultured in a 3D system. β-ACTIN was used as control.

Pancreatic differentiation of hAECs

After the spheroid formation, hAECs were induced for 20 days through the pancreatic endocrine lineage as describe in the materials and methods section.



To determine whether the stepwise differentiation protocol efficiently induced hAEC spheroids into hormone-producing cells in both the two culture media SFM and SRM, we performed an immunofluorescence analysis and determined the presence of pancreatic hormones glucagon and insulin (**figure 10-A**). Basal expression of glucagon was present in undifferentiated control cells (data not shown), but glucagon immunoreactivity became more intense upon differentiation. Instead, basal expression of insulin was absent from undifferentiated hAECs. After 20 days of pancreatic induction in both the conditions, spheroids became insulin-positive. Confocal micrographs revealed the external localization of insulin-positive cells within the spheroids. In particular, merged images of immunofluorescence and phase-contrast micrographs showed the membrane-related position of insulin, while exclusively hAECs localized in the central area of the spheroids expressed glucagon. The peripheral arrangement of insulin is consistent with the exocytosis mechanism for the release of insulin granules in response to high glucose levels. To assess the endogenous production of insulin and verify whether induced hAECs formed mature secretory granules we used transmission electron microscopy (TEM) (**figure 10-B**). TEM ultrastructural analysis of induced hAECs (versus undifferentiated control cells) revealed the high complexity of the cytoplasm and the presence of several vesicles, consistent with intense protein synthesis. In particular, cells contained numerous secretory granules with a bright halo surrounding an electrondense core, which is peculiar to mature insulin containing granules.

Finally, we decided to concentrate our attention to cells cultured only in SFM due to the aim of clinical application. To further confirm the endogenous nature of the detected insulin, C-peptide secretion assay was performed in SFM hAECs differentiated and non-differentiated spheroids (control group) (**figure 11-a**). Basal secretion of C-peptide was measured in control group at 2 mM D-glucose and 20 mM D-glucose concentrations (0.227 ± 0.027 pmol/ng DNA and 0.244 ± 0.033

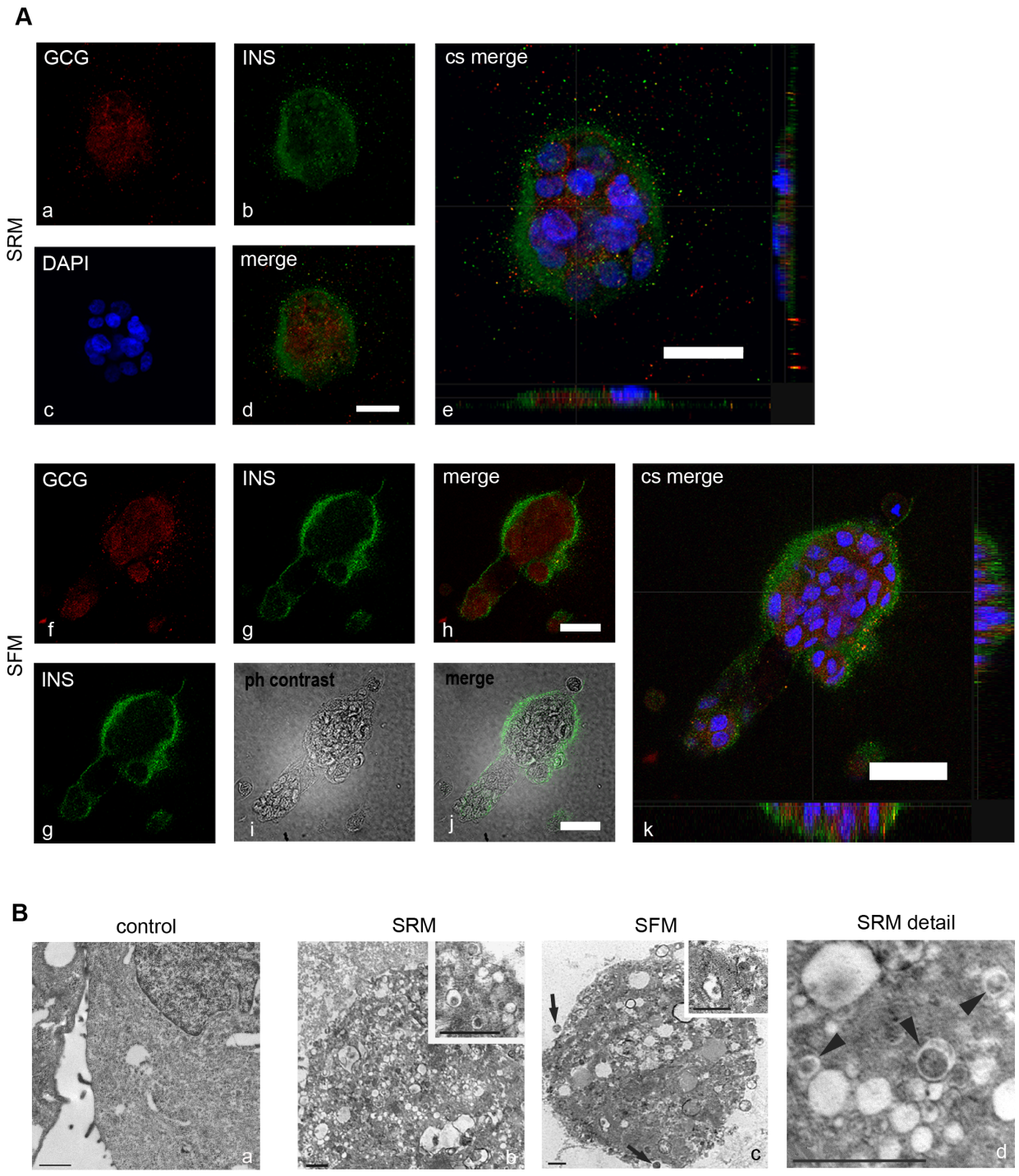


Figure 10. Pancreatic-endocrine induced hAECs express insulin. (A) Confocal immunofluorescence analysis of hormone- expressing cells. Insulin and glucagon staining of hAEC spheroids in SFM (a–d and f-j). Phase-contrast micrograph (i-h) shows the structure of hAEC spheroid. Merge micrograph (e and k) highlights the strict perimembrane localization of insulin. Nuclei are counterstained with DAPI (d). Scale bars = 40 µm. **(B)** Electron micrograph of control hAECs showing normal cytoplasmatic ultrastructure (a) and pancreatic-endocrine induced hAEC spheroid cultured in SRM (b) and in SFM (c) showing granules with crystalline electron-dense cores, reminiscent of beta cell granules (black arrows). Scale bars = 1 µm. Modified from Okere *et al.,* 2015.

pmol/ng DNA, respectively). Pancreatic endocrine differentiated spheroids secreted higher levels (P <0.02) of C-peptide in 20 mM D-Glucose condition (0.697 ± 0.036 pmol/ng DNA), than differentiated spheroids in 2 mM D-glucose condition (0.427 ± 0.050 pmol/ng DNA). Subsequently, immunofluorescence analysis performed on differentiated hAEC spheroids revealed a clear and widespread presence of C-peptide positive cells (**figure 11-b**). These data further support the success of the differentiation protocol and show the acquisition of a glucose-stimulated insulin release mechanism from hAEC spheroids.

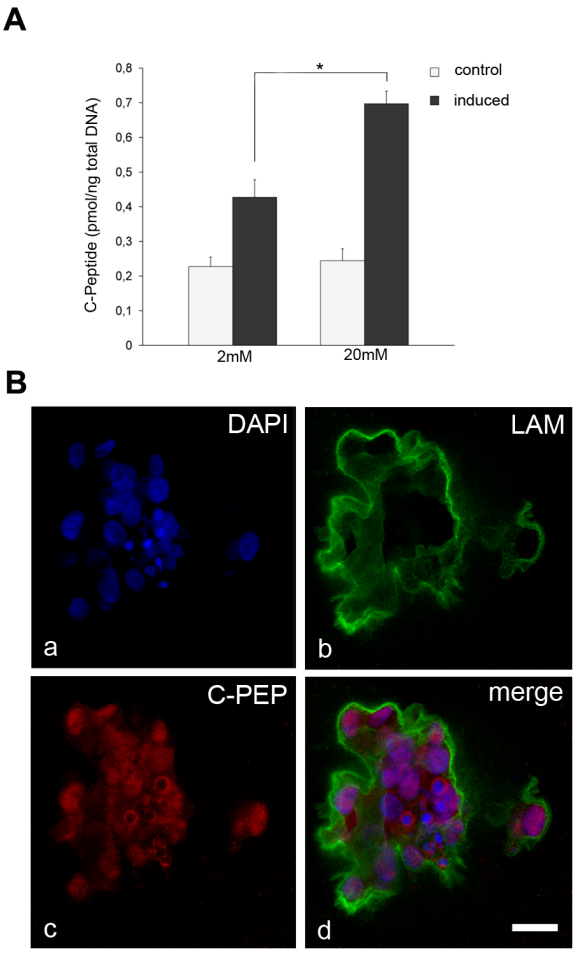


Figure 11. Pancreatic differentiation and c-peptide release. (A) C-peptide release by hAEC spheroids in SFM in response to hypoglycemic (2 mM D-Glucose) and hyperglycemic (20 mM D-Glucose) conditions. C-peptide levels were measured by ELISA and normalized to total DNA content. **(B)** Confocal immunofluorescence analysis of hAEC spheroids. Differentiated cells exposed to 20 mM D-Glucose show a widespread C-peptide (c) expression. Laminin (b) staining highlights its enveloping function at the spheroids perimeter. Merge micrograph is shown (d). Nuclei are counterstained with DAPI (a). Scale bar = 20 µm. Okere *et al.,* 2015.

Pancreatic Islets Mesenchymal Stromal cells (PI-MSCs)

Isolation and culture

Pancreatic Islets (PI) were isolated from cadaveric donor pancreas following the Ricordi method. After the isolation procedure, pancreatic islets (PI) purity was analyzed with trypan blue, and viability was analyzed with dithizone staining respectively (data not shown). PI were then seeded in DMEM L. Glucose with 10% FBS in tissue cultured treated plate. Whitin a week, PI cells adhere to plastic plates (**figure 12-a**).

During the following week cells with fibroblastic morphology grow out of pancreatic islets structures (**figure 12-b**). Within two weeks in culture, cell cultures reached the confluence and could be expanded *in vitro* for different passages (**figure 12-c**). Isolated PI-MSCs isolated were expanded for at least for 5 passages. Culture-expansion in DMEM L glucose + 10% FBS and cryopreservation determine the loss of islet endocrine cells, and the emerging mesenchymal population results homogeneous, without endocrine or exocrine contaminants.

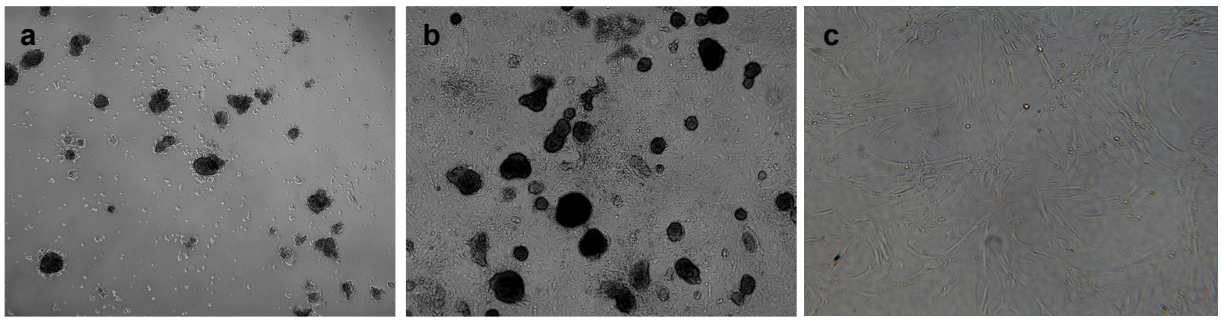
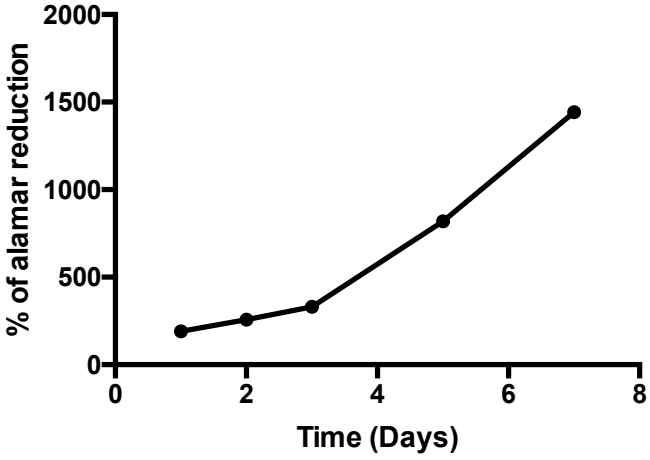


Figure 12. PI-MSCs isolation: a) Pancreatic islets after three days of culture. b) MSCs growing from pancreatic islets after one week. c) PI-MSCs at passage five.

Figure 13. PI-MSCs growth kinetics.
In vitro expansion of PI-MSCs. Cells were isolated and seeded at passage 5 at 5,000 cells/cm^2, and cell proliferation was analyzed for 7 days. Data are plotted as the percentage of alamar reduction analyzed at specific time points.



At passage 5 a cell growth assay was performed for 7 days. PI-MSCs showed a higher proliferation rate compare to hAECs (**figure 13**).

An immunophenotypic cytofluorimetric analysis of isolated cells was conducted to confirm their mesenchymal origin. The immunophenotypic characterization (**figure 14**) shows the absence or the low expression of two hemopoietic markers CD34 and CD45 and one classical epithelial marker, PanCitokeratin. Instead, PI-MSCs show a high expression of specific mesenchymal markers as CD44, CD73, CD90 and CD105. This characterization confirms the mesenchymal nature of the isolated cells. Moreover, PI-MSCs do not express the HLA-DR, a major histocompatibility complex (MHC) class II cell surface receptor. This suggests the ability of PI-MSCs to escape the immune system, an important characteristic for clinical application.

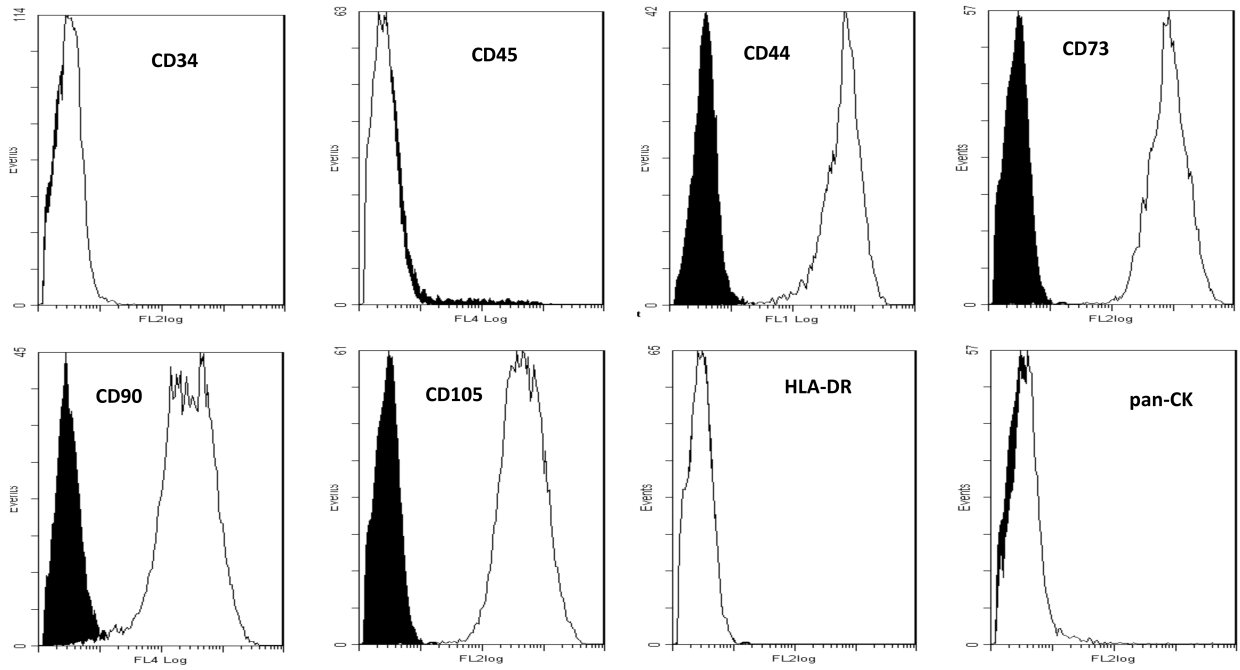


Figure 14. Pi-MSCs immunophenotypic characterization. Cytofluorimetric analysis of the expression of hemopoietic markers (CD34 and CD45). of mesenchymal markers (CD44, CD73, CD90 and CD105), HLA-DR and epithelial marker Pan-CK. Unstained controls are presented as filled black histograms , the specific cell markers as white histograms.

Mesenchymal differentiation

In order to further confirm the mesenchymal origin and the multi-differentiation potential of the PI-MSCs, the differentiation through the three classical mesodermal lineages was stimulated. Adipogenic, osteogenic and chondrogenic differentiation ability was tested with *in vitro* protocols. Adipogenic differentiation was analyzed via Oil Red O staining, a dye that stains neutral triglycerides and lipids. Only a few Pi-MSCs matured into cells containing multiple lipid-rich

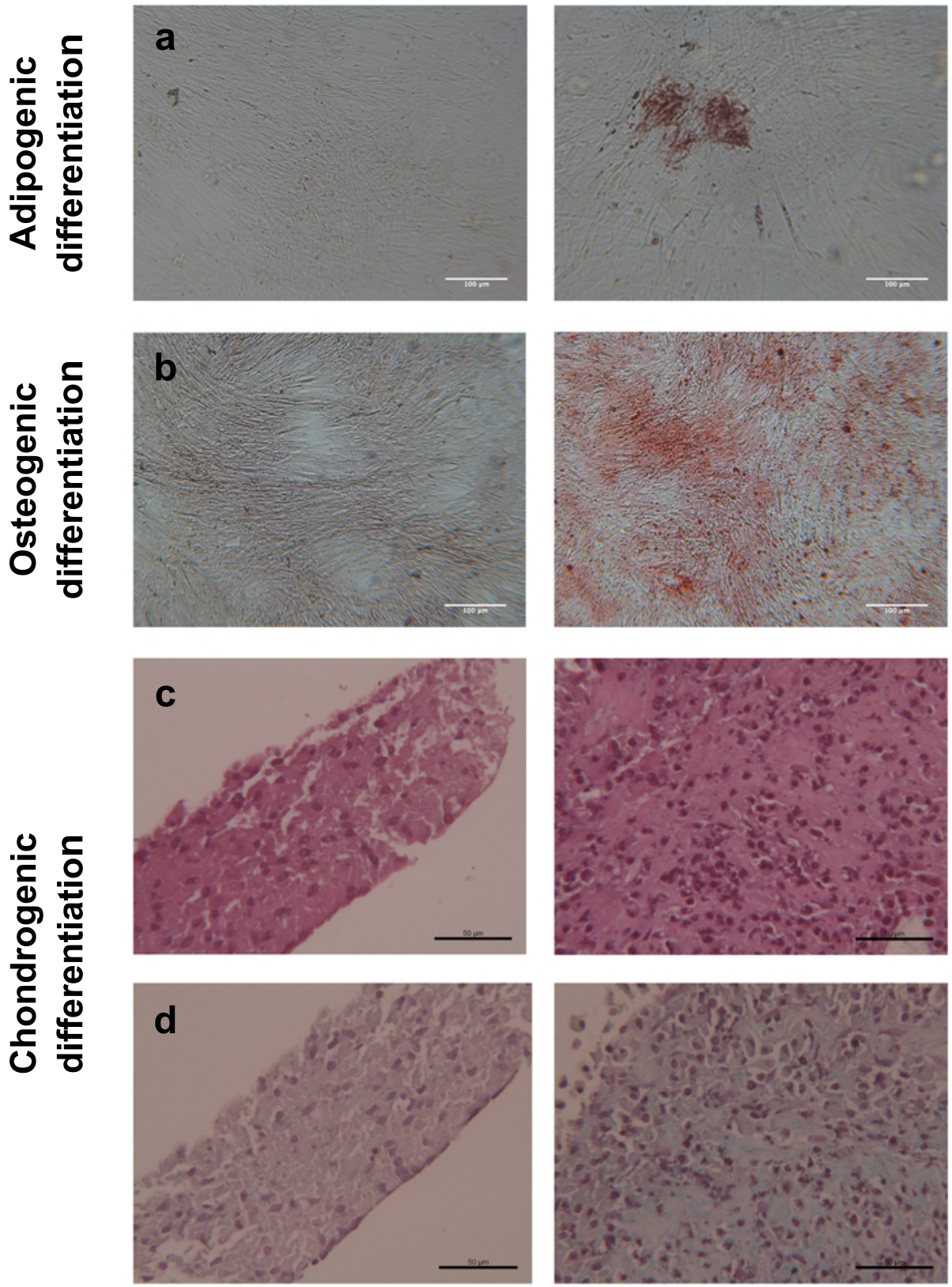


Figure 15. Mesenchymal lineages differentiation. a) Oil red O staining on control and induced cells after adipogenic differentiation. 10X Magnification. b) Alizarin Red staining on control and induced cells after osteogenic differentiation. c) hematoxylin eosin staining on control and induced cells after chondrogenic differentiation. d) Alcian blue staining on control and induced cells after chondrogenic differentiation.

vacuoles in the cytoplasm, vacuoles that increased in size and number during the three weeks of induction and were intensely stained red with Oil Red O (**figure 15-a**). Osteogenic differentiation was tested after two weeks of induction with Alizarin Red staining, a staining for calcium accumulation. Intense red staining was observed in induced cells indicating extracellular mineralization, a key step toward the formation of calcified matrix. Uninduced cells do not show Alizarin Red positive deposits **(figure 15-b)**. Chondrogenic differentiation was performed in a pellet culture system in order to create a 3D structure. **Figure 15-c** shows the formation of a cell pellet, stained with hematoxylin eosin, more compact and well structured in induced cells. The chondrogenic differentiation was further documented by Alcian Blue staining (**figure 15-d**). Differentiated cells were embedded in an abundant extracellular matrix that proved highly positive for the presence of a proteoglycan component as evidenced by Alcian Blue staining in the induced cells, but not in the control cells.

Immunomodulation assay

Immunomodulation assay on hAECs

For clinical applications, immunomodulatory properties are pivotal to avoid rejection after the transplant. In this contest, the capability of hAECs to interact with the immune system was analyzed. A co-culture of hAECs cultured in SRM and SFM with PHA-activated PBMCs was performed. PBMCs were activated with PHA in order to recreate an inflammatory condition. **In figure 16-a** it is shown how after 3 days of co-culture hAECs are able to inhibit the proliferation of the activated PBMCs. In particular, a significantly lower BrdU incorporation of activated PBMCs was observed when co-cultured with hAECs (SRM 15,0 ± 11,9% and SFM 17,1 ± 10,4%) compared to the one cultured in standard conditions (100 ± 9,7%), p<0,0001. hAECs are also able to arrest PBMC in the G0-G1 phase decreasing the number of cells in the S-G2M (**figure 16-b**). Non-activated PBMCs were all in G0-G1 phase (96,4 ± 6,3%), while activated PBMCs in the absence of hAECs significantly decreased in the G0-G1 phase (68,7 ± 3,3%) and increased in the

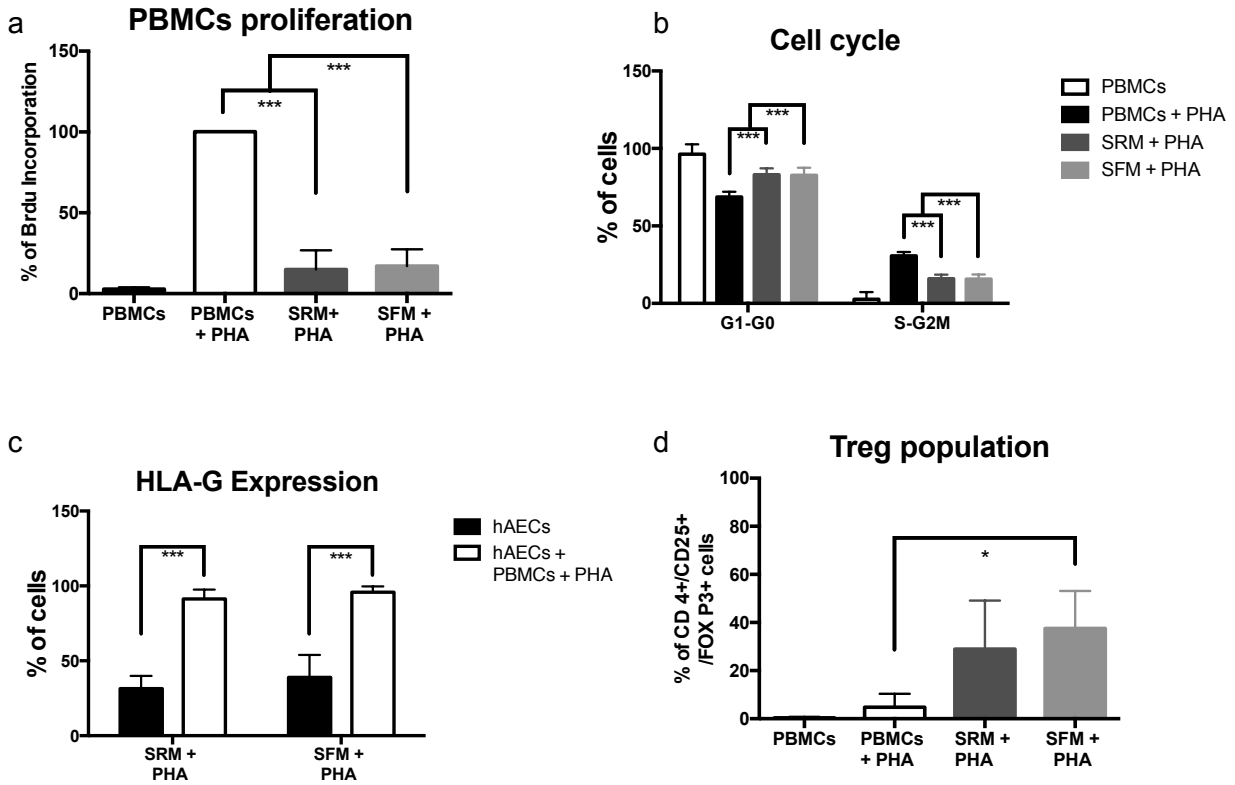


Figure 16. hAECS immunomodulation. (a) PBMCs BrdU incorporation, p<0,0001. (b) PBMCs cell cycle analysis, P <0.005. (c) hAECs HLA-G expression alone (black bars) and after co-culture with active PBMCs (white bars), p< 0,0001 (d) Treg subpopulation pre and after co-culture with hAECs, p<0,01 only in SFM. All data are expressed as means ± SD.

S-G2M phase (30,7 ± 2,5%), P <0.0001. After co-culture with hAECs in SRM and SFM, we observed a significant increase of PBMCs in G0-G1 (SRM 83,0 ± 4,1% and SFM 82,7 ± 4,9%) and an equally reduction of PBMCs in the S-G2M phase (SRM 15,9 ± 2,6% and SFM 15,7 ± 2,9%), P <0.005. Following this observation, we may assume that hAECs cultured in either SRM or SFM, similarly reduce the proliferation of stimulated PBMCs. In order to study possible mechanisms of these properties, we have analyzed the HLA-G expression and T lymphocyte subpopulations. **In figure 16-c** it is shown how the hAECs' HLA-G expression increase after the co-culture in SRM from 33,3 ± 8,5% to 91,3 ± 7,2% and in SFM from 41,5% ± 16% to 95,4% ± 4,4%, p< 0,0001. Finally, it is also possible to observe an increase of the Treg subpopulation analyzed as CD4, CD25, and FOXP3 positive PBMCs (**figure 16-d)**. The percent value increase from 0,4 ± 0,5% in the negative control (PBMCs) and to 4,8 ± 5,6% in the positive control (PBMCs + PHA). It increases more after the co-culture in both SRM (28,9 ± 20,2%) and in SFM (37,5 ± 15,7%), p<0,01 only for SFM.

3D immunomodulation assay

The hAECs immunomodulatory properties were analyzed not only in 2D but also in the 3D culture system. **In figure 17** it is shown a representative experiment of the same hAECs sample, cultured in SRM in both the culture system. This experiment shows how hAEC spheroids are able to inhibit the activated PBMCs proliferation. The percent of BrdU incorporation decrease in SRM from 100% in positive control (PBMCs + PHA) to 19,3 ± 5% in 2D and 12,3 ± 0,4% in 3D culture. In SFM the incorporation decrease from 100% to 19,2 ± 4,1% in 2D and 43,5 ± 0,6% in 3D culture.

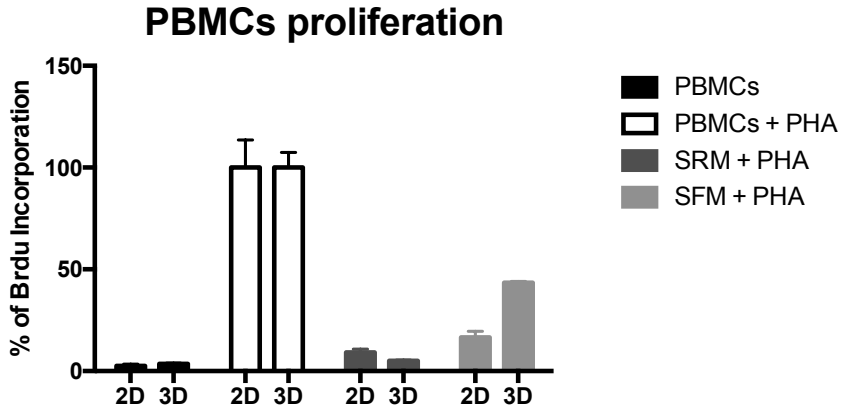


Figure 17. 2D and 3D hAECs immunomodulation. Comparative experiment of PBMCs BrdU incorporation after co-culture with hAECs in SRM cultured in 2D and in 3D system. Data are expressed as a percentage of BrdU incorporation. Means ± SD.

Proved this, we studied if the immunomodulation properties of the hAEC spheroids are also maintained after the pancreatic differentiation (**Figure 18**). PBMCs do not incorporate BrdU (1.2 ± 3.2%), while PHA-activated PBMCs regularly incorporate high levels of BrdU (100 ± 4.1%). Moreover, BrdU incorporation levels significantly decreased after co-culture with induced (SRM 21,4 ± 16,3% and SFM 8.1 ± 5.7%) or non-induced (SRM 23 ± 11,7% and SFM 8.5 ± 4.4%) hAEC spheroids (P<0.0001). To conclude, no significant differences in inhibition activity on PBMCs proliferation were observed between differentiated and non-differentiated hAECs spheroids.

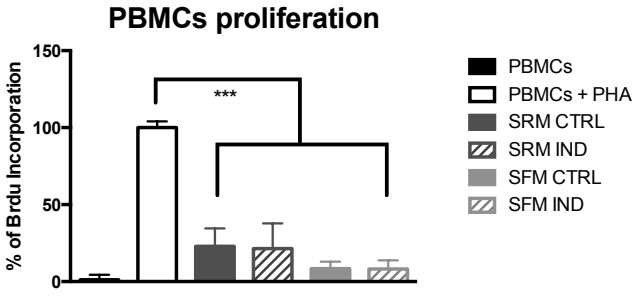


Figure 18. 3D immunomodulation after pancreatic differentiation. Co-culture of PHA-activated PBMCs with control and differentiated hAEC spheroids in SRM and SFM led to significant reduction in PBMC proliferation assayed by BrdU incorporation levels. Data are expressed as a percentage of BrdU incorporation. Means ± SD; P <0.0001. Modified from Okere *et al.,* 2015.

Immunomodulation assay on PI-MSCs

Moreover, the immunomodulation properties of MSCs isolated from pancreatic islet were tested (**figure 19**). **In figure 19-a** it is shown how after five days of co-culture PI-MSCs are able to inhibit the proliferation of the PBMCs activated with PHA. In particular, activated PBMCs show a significantly lower BrdU incorporation when co-cultured with PI-MSCs (19,0 ± 1,7%) compared to the one cultured under standard conditions (100 ± 2%). PI-MSCs are also able to arrest PBMCs in the G0-G1 phase decreasing the number of cells in the S-G2M phase (**figure 19-b**). Non-activated PBMCs were all in GO-G1 phase, while activated PBMCs in the absence of PI-Non-activated

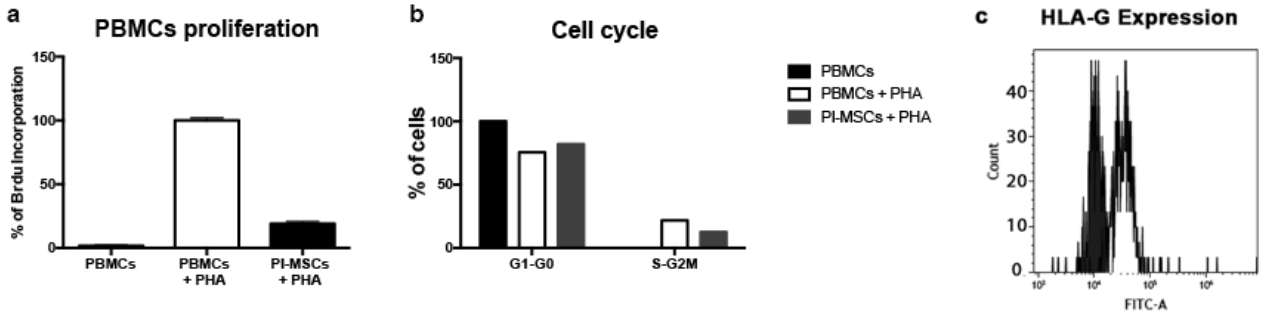


Figure 19. PI-MSCs immunomodulation. a) PBMCs BrdU incorporation. (b) PBMCs cell cycle analysis. Data are expressed as means ± SD. (c) PI-MSCs HLA-G basal expression (filled black histograms) and after co-culture with active PBMCs (white histograms).

PBMCs were all in GO-G1 phase, while activated PBMCs in the absence of PI-MSCs significantly decreased in the G0-G1 phase to 77,6% and increased in the S-G2M phase 22.4%. After co-culture with PI-MSCs, we observed a significant increase of PBMCs in G0-G1 phase to 86,6% and an equally reduction of PBMCs in the S-G2M phase, 13,4%. Finally, HLA-G expression was analyzed and **in figure 19-c** it is shown an HLA-G increase from 25,8 to 83,3% after co-culture with PI-MSCs.

Isolation of endodermal cells

Main Pancreatic Duct

The main pancreatic duct was obtained from cadaveric donor pancreas through Organ Procurement Organization. The tissue was conserved in RPMI with 10% FBS and trypsin inhibitor until further manipulation to avoid autodigestion from the exocrine component. Once in the laboratory all the pancreas, exocrine and endocrine components, was removed to prevent endocrine contamination in culture. Tissue was then minced and digested with collagenase II. Isolated cells were grown in Kubota Medium (KM) with 10% FBS and trypsin inhibitors in collagen IV coated plates for several days **(figure 20)**. Collage IV is one the component of the pancreas ECM *in vivo,* and it could boost the pancreatic differentiation *in vitro*. After few days in culture, it was possible to appreciate the formation of “carpet” like colonies of tightly packed, uniformly cuboidal shaped cells.

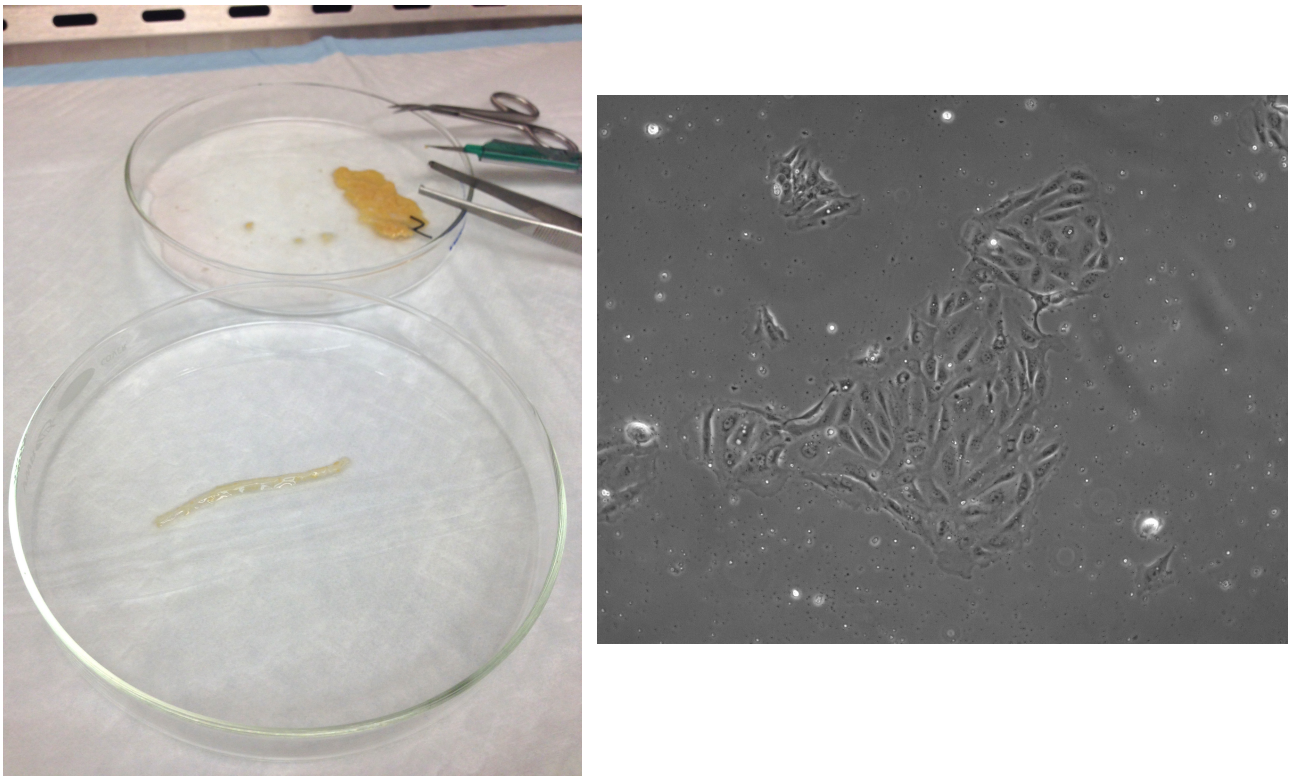


Figure 20. Representative images of the Main Pancreatic Duct. On the left isolation of the main pancreatic duct and on the right a representative light microscopy image of the MPD “carpet” like colonies *in vitro*.

Biliary tree

The duodenum was obtained from cadaveric donors pancreas and conserved in RPMI 10% until further manipulation. Once in the laboratory the hepatopancreatic duct was identified in the internal part of the duodenum and cut paying attention to avoid any incisions on the inner membrane that could cause contaminations. Tissue was then minced and digested with collagenase II. Cells, as obtained, were cultured in KM 10% FBS for different days on collagen IV coated plates (**figure 21**). After few days in culture, two type of colonies were observed in culture. Type 1 formed “carpet” like colonies of tightly packed, uniformly cuboidal shaped cells. Type 2 colonies are composed of cells with an undulation, swirling morphology.

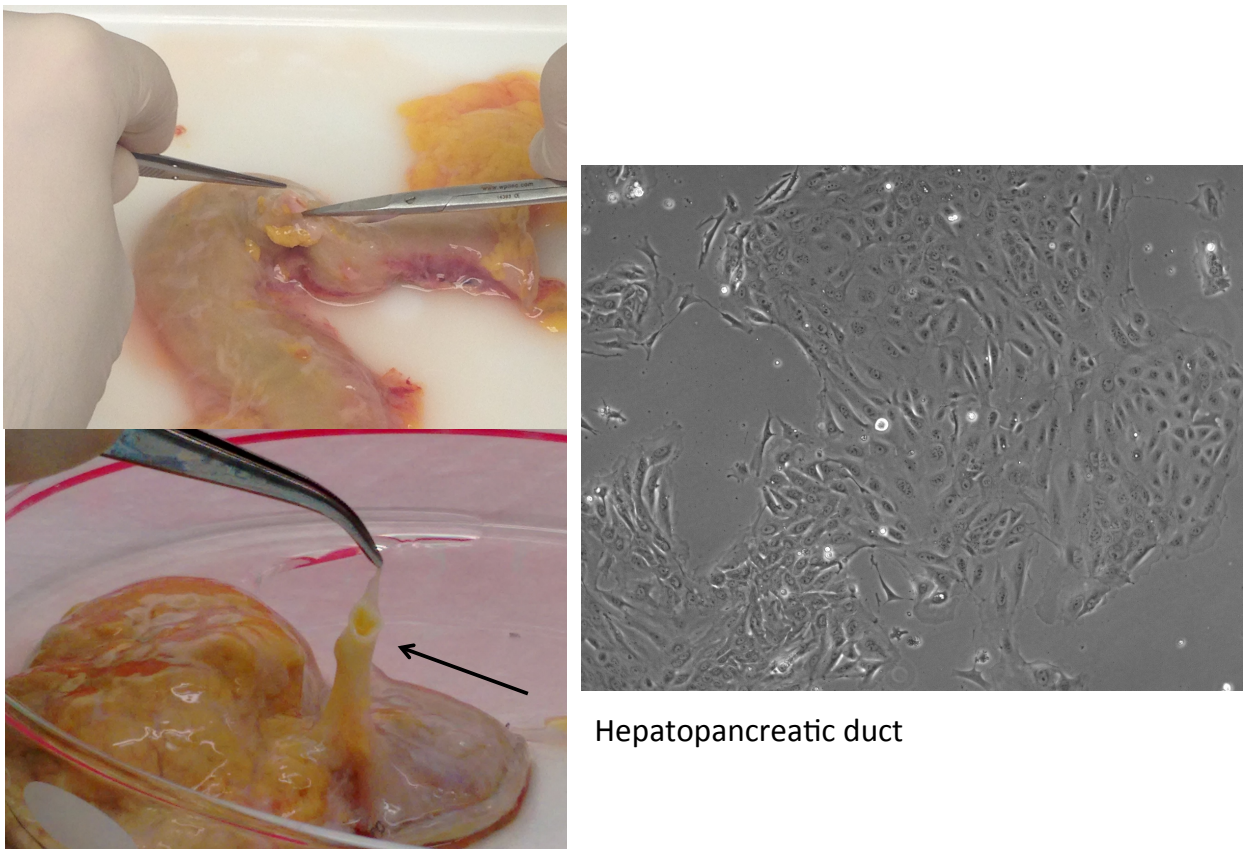


Figure 21. Representative images of the Biliary Tree. On the left an image of the procedure for the separation of the biliary tree from the duodenum. On the right a light microscopy image of BT cells in culture.

Brunner's glands

Once in the laboratory, the duodenum was emptied and then washed to remove all the internal mucous. After that, the mucosa was decellularized and peeled to get access to the submucosa where are located the Brunner's glands. Subsequently, the tissue was minced and digested with collagenase II. Cells, as obtained, were cultured in KM 10% FBS for different days in collagen IV coated plates (**figure 22**). After few days in culture, it is possible to appreciate the formation of colonies with cells of endodermal swirling morphology.

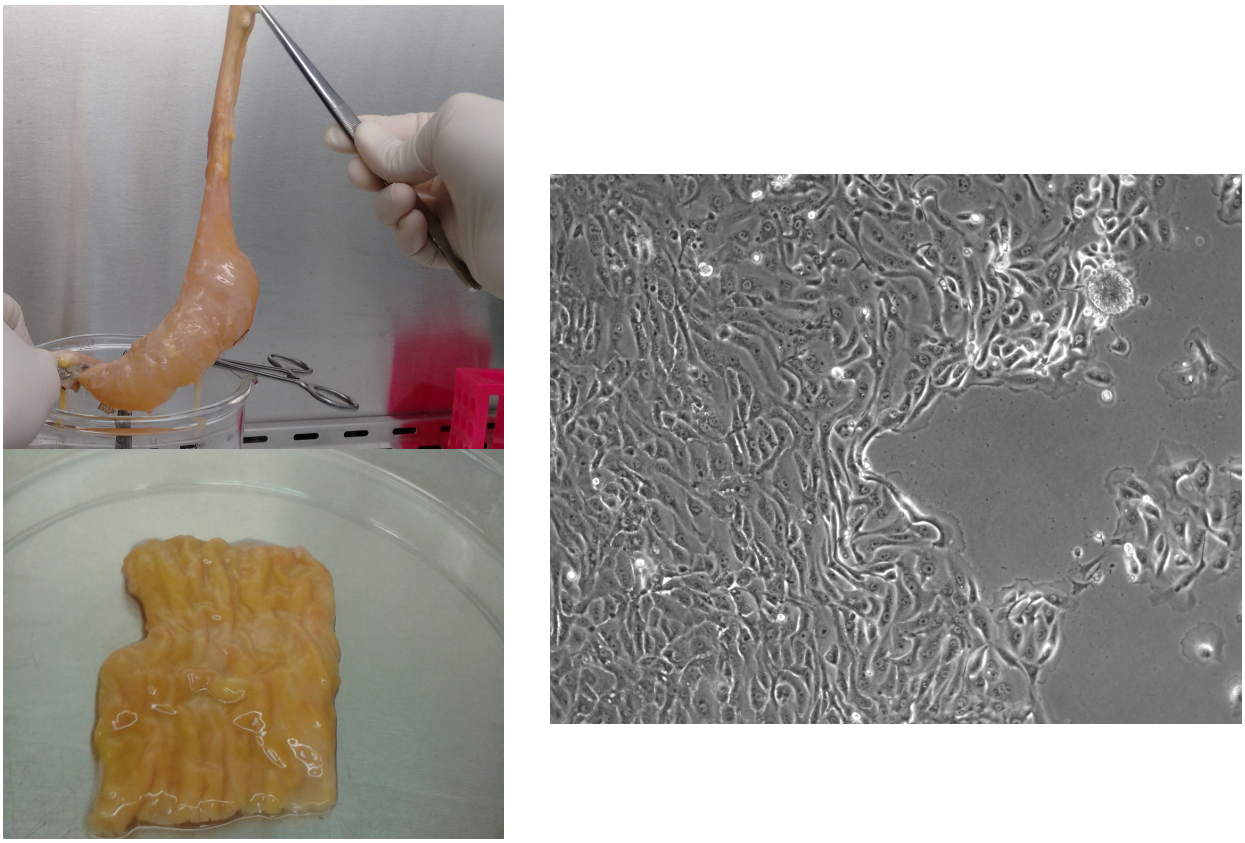


Figure 22. Representative images of Brunner's glands. On the left a representative image of the procedure for cells isolation from the duodenum. On the right a light microscopy image of BG cells in culture.

Pancreatic differentiation

After the establishment of cells cultured from all the three endodermal origin and PI-MSCs were induced to mature towards the pancreatic endocrine lineage as previously described (See Materials and Methods section). Briefly, control cells were maintained in serum-free KM, while induced cells were cultured in HDM-P+. Cells from endodermal origin were treated for five days with one medium change, while PI-MSCs were induced only for two days. Cells were induced with the combination of different factors. One of the most important is the cyclopamine, a steroid alkaloid, able to inhibit Sonic Hedgehog and consequently induce the expression of PDX1. Another important factor is Exendin 4, a GLP1 agonist that binding GLP1 receptor could increase insulin secretion. After the induction, RNA extraction was performed and the expression of specific pancreatic genes was analyzed. Pancreatic islets were used as positive control, and all data were shown as fold increase compared to uninduced cells. **In figure 23** it is possible to observe an increasing trend of different genes involved in the pancreatic differentiation.

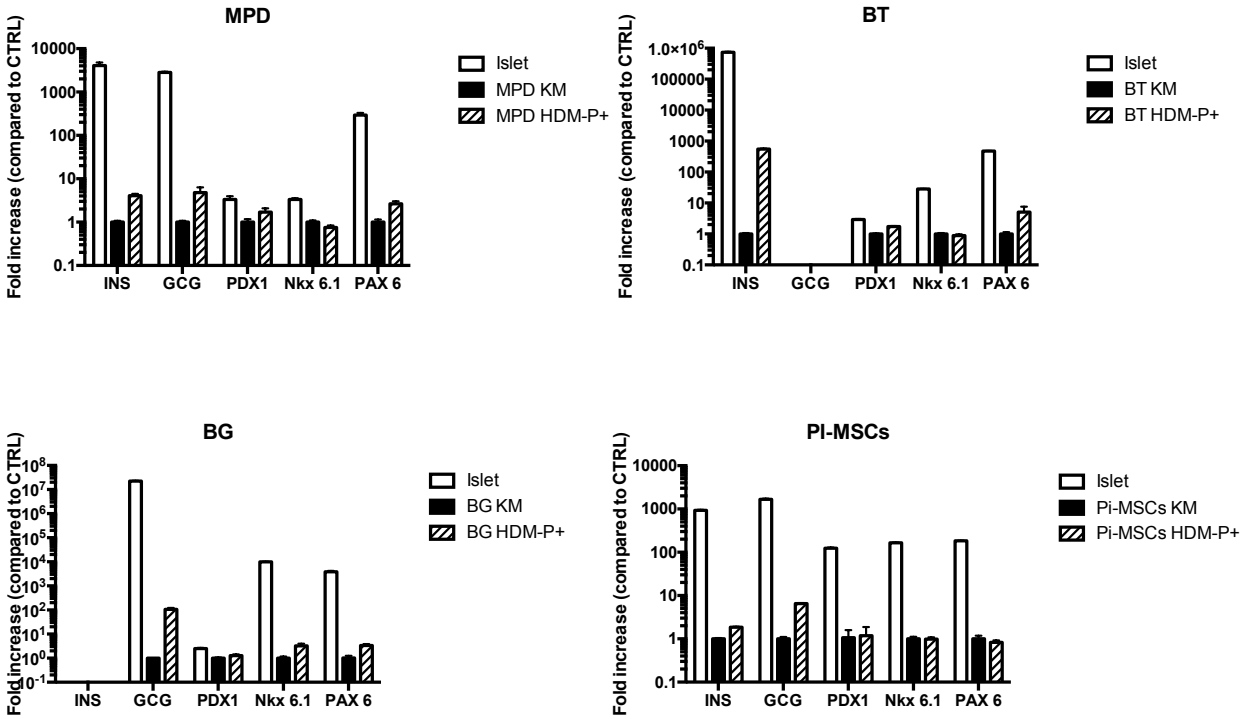
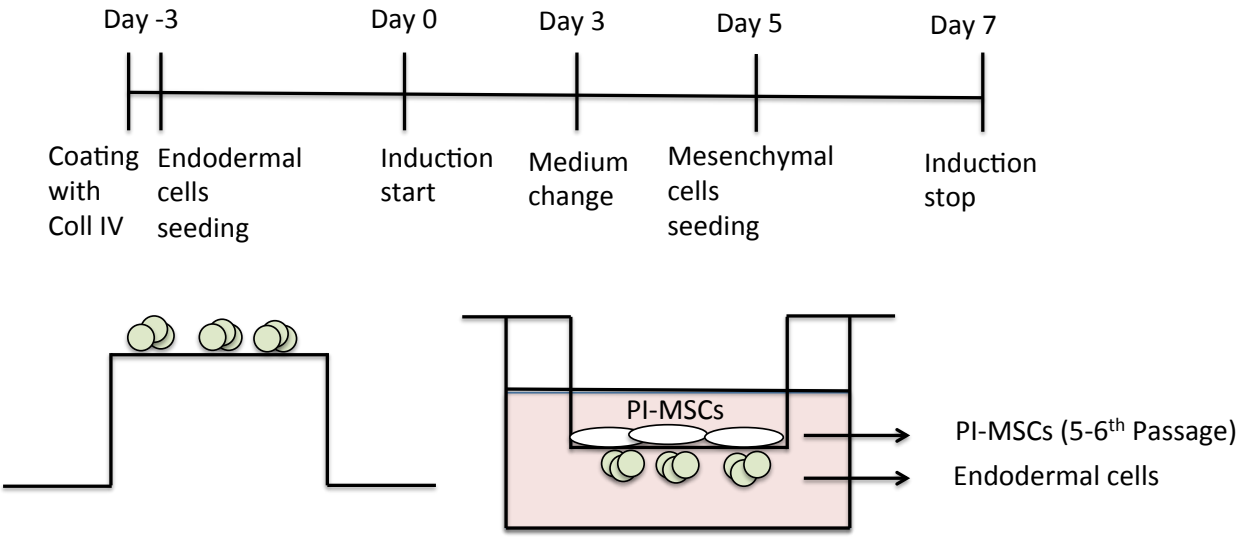


Figure 23. Gene expression analysis. RNA expression of Insulin (INS), Glucagon (GCG), PDX1, Nkx 6.1 and PAX6 in MPD, BT, BG and PI-MSCs. Data are shown as fold increase compared to not induced cells. Pancreatic islets are used as positive control.

Co-culture and Transwell protocol

Once confirmed the capability of these cells to express specific pancreatic genes to enhance the efficiency of differentiation, we investigated whether the co-culture of endodermal cells with pancreatic islets stromal cells could improve the differentiation yield. To recreate the *in vivo* conditions, we combined endodermal cells, stromal cells, and extracellular matrix component, (collagen IV) *in vitro*.



Furthermore, we have cultured cells in a transwell system with the aim of analyze gene expression of the two populations. The porosity of the transwell membrane was 0,4 μm that do not allow cells to permeate but only contacts between them. The mRNA expression of MPD cells was analyzed and **figure 24** shows after this co-culture strategy the up-regulation of all the pancreatic genes, Insulin, Glucagon and also the intermediate genes PDX1, Nkx6.1, and PAX6.

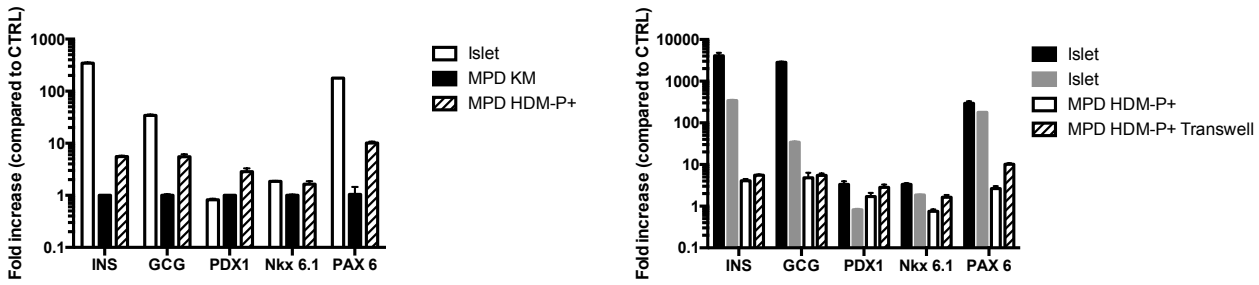


Figure 24. Gene expression PCR MPD cultured in a Transwell system. On the left, MPD gene expression level for Insulin Glucagon PDX1, Nkx 6.1 and PAX6. Cells were cultured in the transwell system and the gene expression is shown as fold increase compared to uninduced cells. On the left the same data are compared to the one of MPD cultured in the classic 2D system.

C-peptide quantification

To complete the characterization of induced cells, we analyzed the beta cell-specific function of glucose-responsive c-peptide release in the supernatant. F**igure 25** shows the spontaneous release of c-peptide in the medium during the differentiation protocols. The c-peptide release was markedly increased after the co-culture with PI-MSCs in both the cells population. This support the idea that the environment is crucial to enhance the pancreatic differentiation.

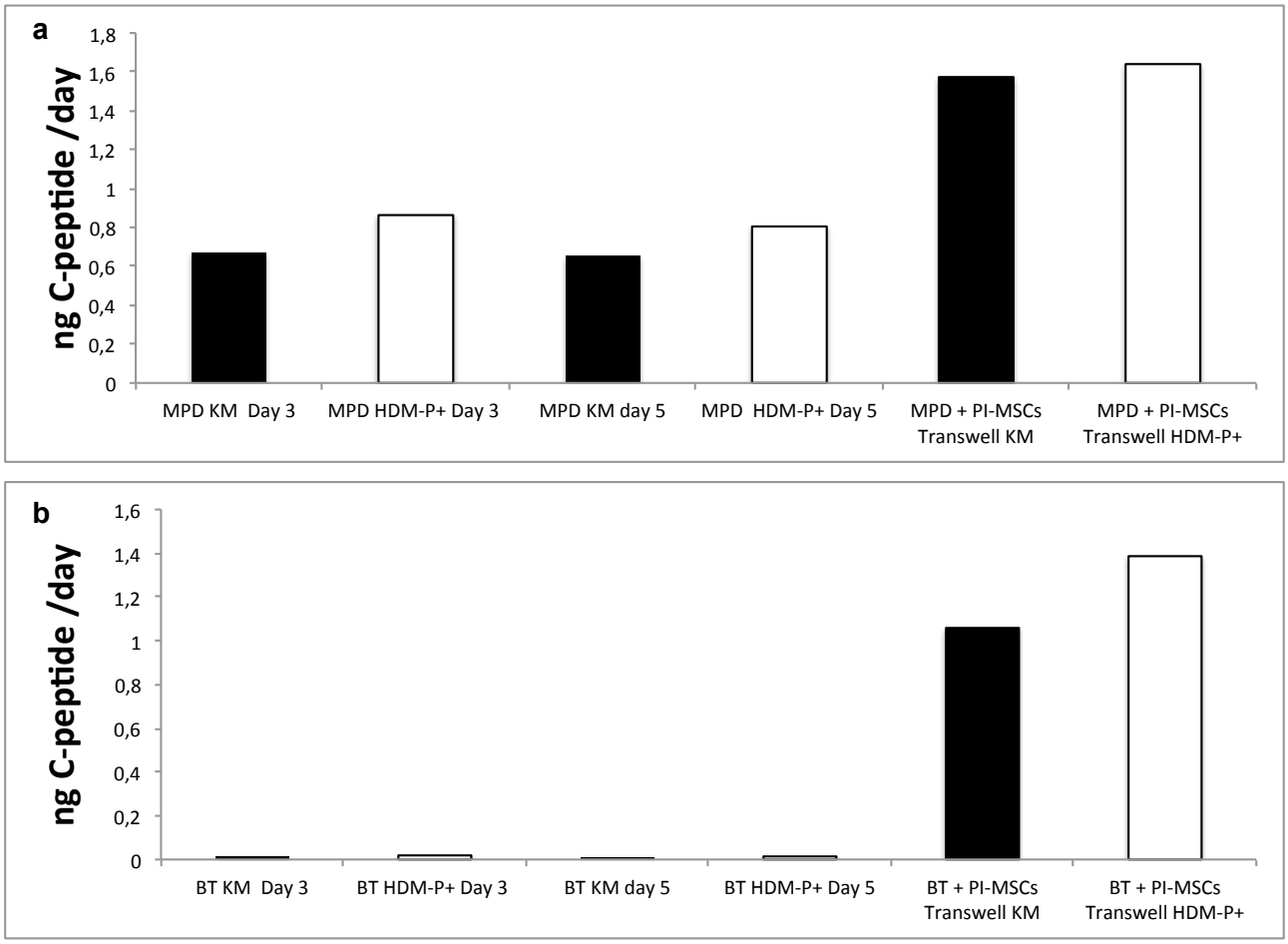


Figure 25. C-peptide release. a) MPD c-peptide release during differentiation protocol, after 3 and 5 days and at the end for induction. b) BT c-peptide release during differentiation protocol, after 3 and 5 days and at the end for induction. Data are presented as ng C-peptide/day.

In order to verify the terminal maturation of the differentiated cells a static incubation was performed. Cells were subsequently incubated with different concentration of glucose 2,5 and 20 mM respectively lower and higher concentration. By the data of static incubation analysis we observed in same case (notably BGs+PI-MSCs and BT+PI-MSCs) the development of functional glucose responsive beta-like cells (**Figure 26**) .

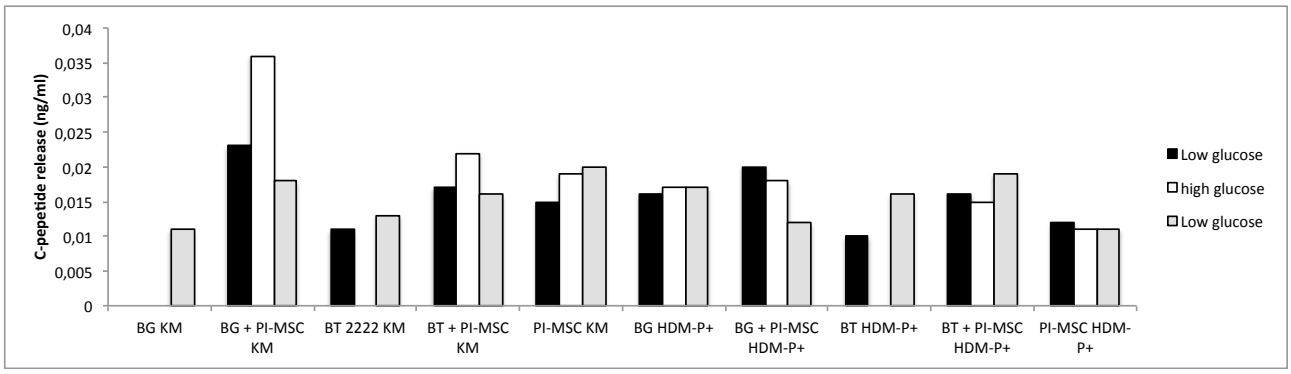


Figure 26. Static Incubation. C-peptide releases after incubation with different glucose concentration. Low glucose 2,5 mM and 20 mM for high glucose. Data are presented as ng/mL.

hAECs and PI-MSCs

Co-Culture system

Endodermal cells are a fascinating model for pancreatic endocrine differentiation studies, but their use is not sustainable for clinical application. This led us to establish a co-culture system of hAECs and PI-MSC in order to improve hAEC pancreatic endocrine differentiation and generate islet-like spheroids. First of all, we had co-culture together the two cell populations (**figure 27**). Pi-MSCs were seeded in culture and treated with mitomycin C to create a cell feeders layers. The days after hAECs cultured in ultra low attachment plates for 72 hours were seeded on the top of the PI-MSCs layers. Cells were then cultured for different days in DMEM high glucose added with 10% FBS and EGF 10 ng/mL. hAECs created compact colonies and PI-MSCs constitute as a barrier around (**figure 27-a**). A phalloidin immunofluorescence was performed in order to study the cytoskeleton

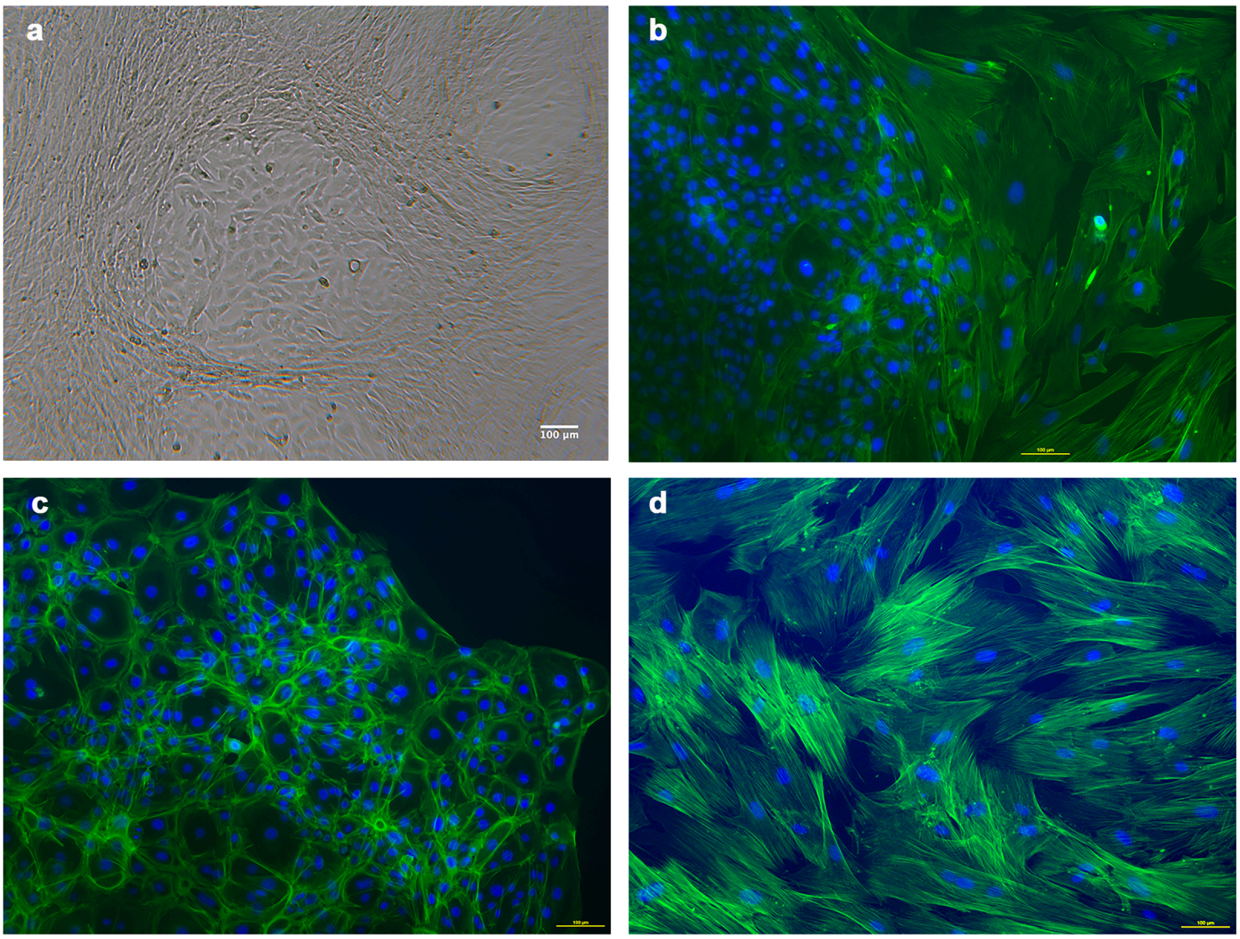


Figure 27. hAECs and PI-MSCs co-culture. (a) Light microscope image of co-culture system. (b) Immunofluorescence for phalloidin of co-culture system. (c) Immunofluorescence for phalloidin of hAECs. (d) Immunofluorescence for phalloidin of PI-MSCs.

cells conformation. hAECs and PI-MSCs show a different peculiar cytoskeleton structure. In hAECs, the F-actin filament is distributed predominantly at the plasma membrane while PI-MSCs showed a dense network of F-actin bundles with tight, parallel stress fibers (**figure 27-c and d**). This classical and peculiar conformation is also preserved in the co-culture system (**figure 27-b**)

Creation of cell spheroids in Ultra Low Attachment

In order to recreate a complete pancreatic islet, with both epithelial and stromal component, was tested the possibility to combine the two cell populations in a three-dimensional system. hAECs were firstly cultured in ultra-low attachment T25 flasks designed to avoid the cell contact with the plastic and boost the cell auto aggregation. As shown **in figure 28-a** hAECs were seeded in a single cell suspension, and after 48-72 hours it is possible to appreciate a spheroid formation

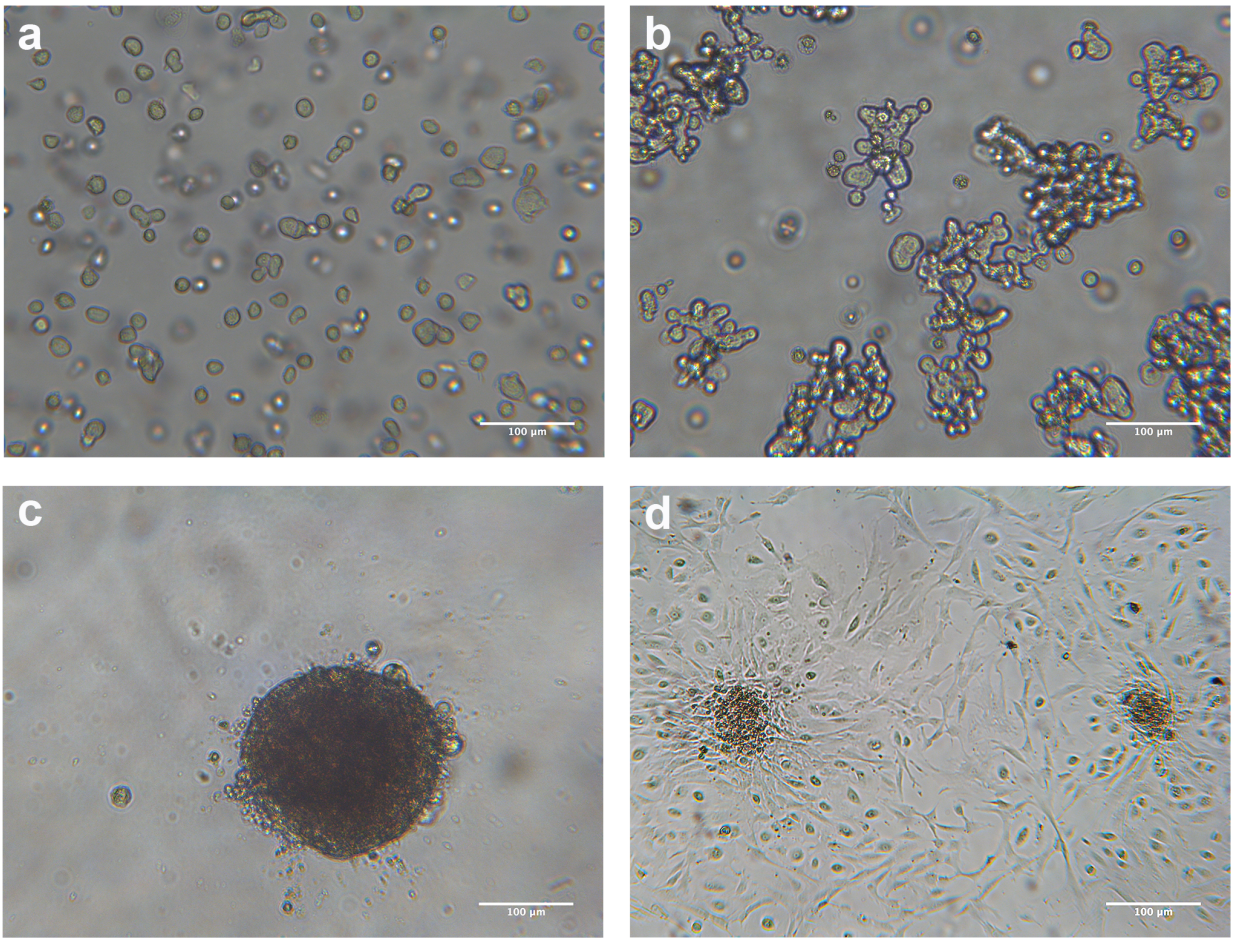


Figure 28. Cluster formation. (a) light microscopy image of hAECs in Ultra Low Attachment flask at time 0. 10X magnification. (b) light microscopy image of hAECs in Ultra Low Attachment flask after 48 h. 10X Magnification. (c) light microscopy image of hAECs cluster after one day of co-culture with PI-MSCs in Ultra Low Attachment flask. 10X Magnification. (d) light microscopy image of hAECs/PI-MSCs after three days after re-plating in tissue culture flask. 4X Magnification.

(**figure 28-b**). hAECs are epithelial cells which need to grow in a mutual contact. Consequently, an ultra low attachment system is not the ideal one, intact it led the formation of irregular and uncompacted spheroids. After 72 hours PI-MSCs were added in a ratio of 1:4 at the cell culture. The day after, it is possible to observe the presence of very compact and well structured spheroids (**figure 28- c,e**) with a diameter size in a range of 50-100 μm. To assay the viability and to test if spheroids were miscellaneous, they were transferred again in a tissue treated culture plate. **In figure 28-d** it is shown how spheroids are able to adhere to the plastic once in culture and after few days is possible to appreciate the presence of both hAECs and PI-MSCs.

Spheroid separation

For clinical application, it is critical the standardization of every steps of cell preparation process. In particular, to improve the chance of engraftment after transplantation, it is pivotal the dimension of islet-like spheroids. Significantly larger spheroids do not allow gas exchanges and re-vascularization processes that are crucial for the insulin release into the bloodstream. For this reason, we applied a tag-less sorter (Celector) to collect spheroids with dimension suitable for transplantation. **In figure 29** it is shown a representative fractogram of an hAEC spheroids injection. The fractogram shows a main band distribution (**figure 29-b**). In this study, three different fractions were collected, F1 from 0 to 2 minutes, F2 from 2 to 4 minutes and F3 from 4 to 10 minutes. Cells were then collected and plated in culture. As is possible to observe in **figure 29-c**, in F1 are present only spheroids with a diameter of 50-100 μm for the most part. F2 is characterized by spheroids with a lower dimension (diameter <50 μm) and single cells alike. Finally, in F3 only single cells or very small aggregate of four/five cells are present. The same process was performed with hAEC spheroids and also mixed spheroids (hAECs/PI-MSCs) (**figure 29-d**).

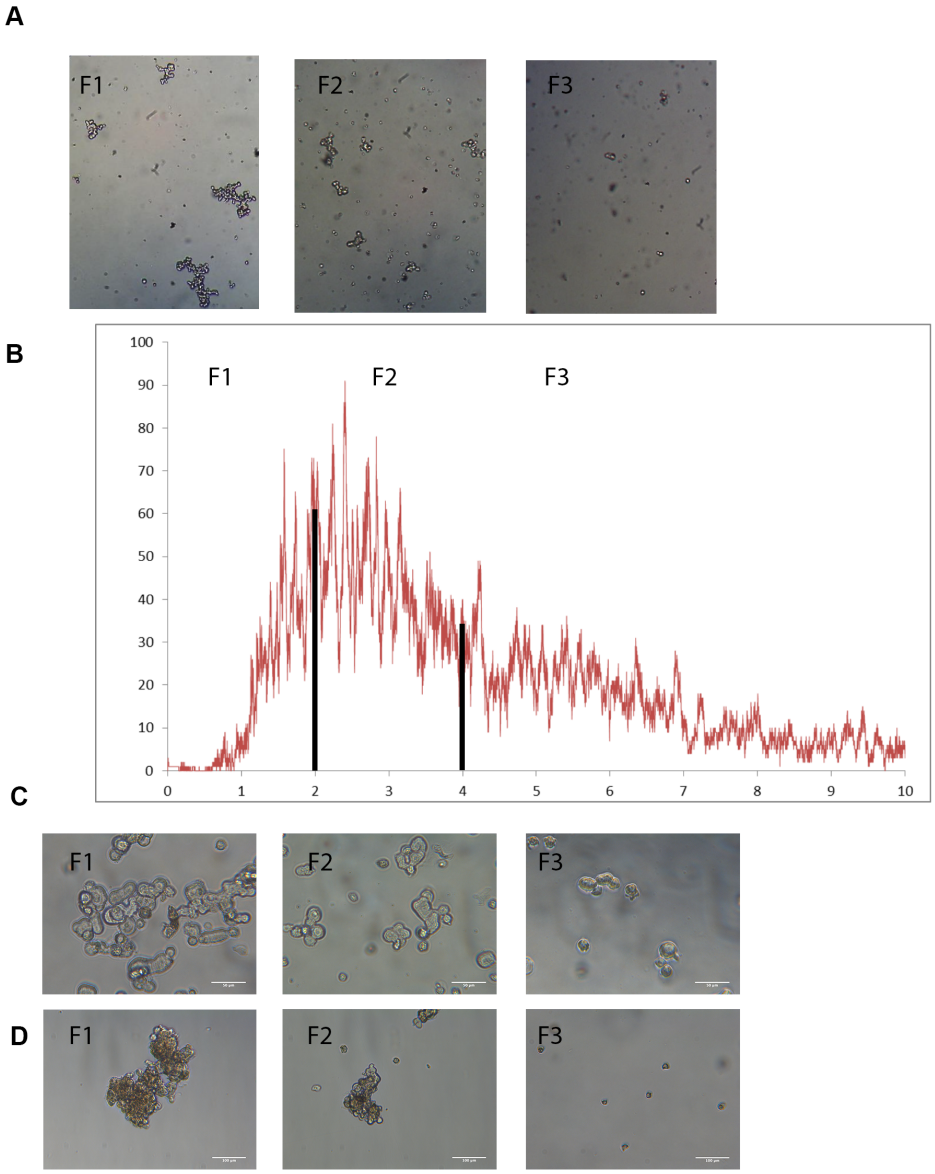


Figure 29. hAEC spheroids fractionation. a) images of cells during fractionation process. b) Representative fractogram with F1, F2 and F3 as collected fractions. c) light microscopy images of hAEC spheroids after the fractionation process. 10X magnification. d) light microscopy images of mixed hAEC/Pi-MSCs spheroids after the fractionation process. 10X magnification.

I tell young people: Do not think of yourself, think of others. Think of the future that awaits you, think about what you can do and do not fear anything.

Rita Levi Montalcini

Conclusion

Type 1 diabetes (T1D) is an autoimmune disease that affects insulin-producing beta cells in the pancreas. In patients affected by T1D, the immune system mistakenly attacks beta cells, and the loss of these cells determines a loss of the ability to control the metabolism of carbohydrates, leading to glucose accumulation in the blood and toxicity to multiple organs. Chronic exogenous insulin administration is a life-saving intervention and represents the standard of care for T1D patients. Over time, complications occur in a large subset of patients. The most severe cases of T1D are poorly managed with insulin administration. These cases can be treated with transplantation of pancreas or pancreatic islets from cadaveric donors. Transplantation strategies can control complications and even revert the symptoms of diabetes. A large number of patients could benefit from transplantation, but unfortunately several factors limit the widespread application of transplants. First and foremost, there is shortage of cadaveric pancreata. Moreover, engraftment and long-term function are suboptimal. Furthermore, patients need to be treated with immunosuppression, with severe side effects, and autoimmunity can recur even in the presence of immunosuppression. Nevertheless, pancreas and islet transplantation served as proofs of concept that beta cell replacement can treat T1D very effectively. In recent years, multiple stem cell-based strategies have been developed for patients with T1D. Certain types of stem and progenitor cells can be expanded to large numbers, and can be stimulated to mature into insulin-producing β-like cells. Other types of stem cells have the ability to modulate the immune system, a feature that could be exploited to facilitate beta cell survival and inhibit recurrence of autoimmunity.

In this contest, we have concentrated our attention on human Amniotic Epithelial Cells (hAECs). The amniotic epithelium develops from epiblast before the gastrulation process. hAECs could have escaped the trilaminar lineage commitment, maintaining characteristics similar to the embryonic stem cells. hAECs could also be isolated in a clinically revenant number for application in cell therapies. Their isolation and use does not pose ethical concern. hAECs are endowed with the potential to commit toward multiple cell types. Moreover, hAECs (along with the amniotic membrane) possess anti-inflammatory and immunomodulatory properties [119,121,123]. These features are of great interest for the design of cell based strategies for T1D.

In the first part of this study, we have demonstrated the possibility to isolate and culture hAECs both in Serum Rich Medium (SRM) and in Q282 medium, a Serum Free Medium (SFM). Q286 is a

further developed DMEM enriched with selected serum components and growth enhancers, tailor made for epithelial cells. We have studied Q286 because of the advantages for clinical applications in terms of reproducibility, standardization, and for the absence of ethical concerns. hAECs characteristics, as reported in literature [106,107], seem not to be affected by the culture in a SFM. Cell morphology, proliferation, immunophenotypic profile and stemness markers are all maintained. Interestingly, AECs cultured in SFM show an increased expression of stemness markers that characterize embryonic stem cells (OCT4, SOX2, NANOG). The second part of the study was concentrated on the development of a cell culture model and a protocol to enhance the pancreatic endocrine differentiation. We hypothesized that the assembly of hAECs in 3D structures could be important to facilitate differentiation. The organization in 3D structures enables the establishment of additional cell-cell and cell-matrix contacts and closer interactions among cells. It can determine the acquisition of polarization and patterning features, ultimately affecting fate determination due to differences in the microenvironment. Therefore, cells were cultured on a thick layer of 100% reduced growth factor BME, that allow the formation of multicellular structures. hAECs were then inducted along endocrine pancreatic lineage with a step-wise protocol adapted from previous reports [53,54,113]. In our step-wise protocol only three induction molecules were employed. In the first step, Activin A is added to the medium to impart the commitment of hAECs towards definitive endoderm [56]. In the second step, Nicotinamide (NAM) and Activin A are additioned to the medium, to promote the pancreatic-endocrine differentiation [128]. In the final step, Retinoic Acid (RA) and NAM are added to induce the maturation of insulin-producing cells [129]. At the end of the induction protocols, insulin and C-peptide (a peptide cleaved from proinsulin) were analyzed. To test the complete maturation of hAECs into beta-like cells, we tested C-peptide release by sequentially culturing cells in media with different concentrations of glucose. hAEC cultured as 3D spheroids showed potential to differentiate towards pancreatic endocrine cell types and to give rise to functional beta-like cells that release C-peptide in a glucose-responsive way. As previously discussed, beta cell replenishment alone would be insufficient for T1D patients: there is an unmet need to effectively inhibit the immune system in order to facilitate transplant survival and to avoid recurrence of autoimmunity. For these reasons, we have analyzed the immunomodulatory properties of hAECs. These cells are able to inhibit the proliferation of peripheral blood mononuclear cells (PBMCs) activated with phytohemagglutinin (PHA) *in vitro*. We have observed that HLA-G increases when hAECs are co-cultured with activated PBMCs. HLA-G is a potent

immunoregulatory molecule involved in the inhibition of immune cells in the placenta environment. Moreover, we have shown the increase of T-regulatory (T-reg) cells increase in co-culture. Not stopping at this we have also demonstrated how hAECs spheroids in 3D culture are able to inhibit PBMCs proliferation before and after pancreatic differentiation. Furthermore, we have shown that hAECs spheroids in 3D culture conditions are able to inhibit PBMCs proliferation before and after pancreatic differentiation. This is of great interest for the development of strategies for T1D.

The efficiency of differentiation towards beta-like cells was not satisfactory. This stimulated us to redesign the differentiation protocol based on knowledge recently acquired in the field of pancreas progenitor cells. Until few years ago, the vast majority of investigators did not believe in the existence of progenitor cells in the adult pancreas. More recently, multiple studies have reported their existence, and described putative niches inside and close to the organ. Stem/progenitor cells were found in the pancreatic duct glands (PDGs), located mainly in the main pancreatic duct, and in the peribiliary glands (PBGs), which are located along the biliary tree [130,131]. Tracing back the embryological development of pancreas and liver, we hypothesized that more ancestral progenitor could be present in the Brunner's Glands (BGs). BGs are mainly located in the submucosa of the proximal duodenum, more specifically in the region of the ampulla of Vater where biliary three, liver and pancreas developed [88]. We established cultures of endodermal cells from adult human MPD, BT, and BD. Isolation was based on culture selection with Kubota Medium (KM). KM has been shown previously to select for early endodermal stem/progenitors [82,84]. Cells were cultured on Collagen IV coated plates. We have also isolated Mesenchymal Stromal Cells from adult human Pancreatic Islets (PI-MSCs). In order to confirm the mesenchymal origin was performed a characterization in accordance with the minimal criteria of the international society of cellular therapy [69]. Also PI-MSCs showed the capacity to modulate immune cells, as they inhibit PBMCs proliferation and express the HLA-G after co-culture *in vitro*.

Cells were stimulated to differentiate towards pancreatic endocrine cell types by using a Hormonally Defined Medium for Pancreatic differentiation (HDM-P+), a medium able to stimulate pancreatic islet commitment in endodermal cells isolates from Biliary Tree [84]. We established co-cultures of endodermal progenitor cells and PI-MSCs in an attempt to improve the efficiency of islet differentiation. Our results suggest that the cell populations that we isolated from adult tissues have the potential to commit towards islet cell fates, but the efficiency of differentiation differs significantly among them. When endodermal cells were co-cultured with PI-MSCs we observed a

marked increase of C-peptide release, and in some cases (notably BGs+PI-MSCs and BT+PI-MSCs) we observed the development of functional glucose responsive beta-like cells. The co-culture protocol proved useful to further stimulate beta cell differentiation. Such protocol enables studies on the mechanisms of islet cell differentiation.

In the last portion of the study, we established a co-culture system of hAECs and PI-MSC in order to improve hAEC pancreatic endocrine differentiation and generate islet-like spheroids. We observed that the two cell populations can be co-cultured in 2D system with no signs of loss of viability. We subsequently designed a three dimensional co-culture method that could enhance the differentiation process and that could yield transplantable islet-like clusters. We started by culturing hAECs in ultra low attachment plates (ULA), which are designed to avoid the cell contact with the plastic facilitating self-aggregation. After 2-3 days in culture, hAECs did not develop compact aggregates. The addition of PI-MSCs in co-culture yielded compact and well structured aggregates. To improve the chance of engraftment after transplantation, the diameter of islet-like spheroids should be lower than 300 μm: spheroids with a larger diameter do not allow gas exchanges and re-vascularization processes, crucial for the release of insulin into the bloodstream. Moreover, clusters of dead cells or debris of the same size range would be deleterious for the engraftment yield. Therefore, in order to select viable aggregates with dimensions and features suitable for transplantation we have tested a proprietary tag less sorting device, the Celector. This device allows to sort and collect cell samples without any manipulation. Cells are separated due to their physical interaction with a flow of liquid in the presence of gravity. The instrument does not employ antibodies and does not interact with samples in any way. With this tool we were able to sort spheroids with diameters between 50 and 100 μm. After the collection, spheroids could be cultured again in plastic plates and we observed viable cells from both populations in culture. Future steps will be aimed at testing the pancreatic differentiation protocol in these 3D cell cultures and will test combination with MSCs from other high-yield sources.

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