Using stem cells to produce insulin

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USING STEM CELLS TO PRODUCE INSULIN

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Abstract

In the last decade, tremendous progress has been made in generating insulin-producing cells from both mouse and human pluripotent stem cells. Following the principles that guide pancreas embryonic development is a common aspect in all differentiation protocols with considerable success in generating β-like cells in vitro. Greatest outcome of the refined protocols became apparent in the first clinical trial, recently announced by the ViaCyte Company. ViaCyte strategy is based on the implantation of pancreatic progenitors that would further mature into functional insulin-producing cells inside the patient body. In this review we will discuss the state-of-art in β-cell replacement therapies based on the differentiation of embryonic stem cells into glucose-response and insulin-producing cells in a dish. We will also discuss alternative approaches to obtain new sources of insulin-producing cells by enhancing the regeneration of the endogenous β-cell mass.

1. Introduction

Fifteen years ago (November 1999) our group was the first to publish that insulin producing cells may be derived from mouse embryonic stem cells (mESC) and able to normalize blood glucose in a toxic model of diabetes [1]. On October 29, 2014, the company ViaCyte (San Diego, California) announced the first-in-the-world implant of one type 1 diabetic patient with precursor insulin-producing cells derived from human embryonic stem cells (hESC) [2]. In the last twelve months, three studies have come out describing efficient methods to obtain insulin-producing cells from human embryonic stem cells [3-5]. How far have we come along in reaching an effective treatment for type 1 diabetes mellitus (T1DM)?

A scheme of the main contributors to the development of new strategies to generate insulin-producing cells derived from either mice or human embryonic stem cell over the past 15 years is depicted in **Figure 1**. Gene trapping and directed differentiation methods [1, 6, 7] result in cells that contain insulin, express functional markers of the glucose-sensor (eg. glucose blockade of K_{ATP} channels), the exocytotic machinery and insulin gene expression, processing and storage. Progenitors selected by gene-trapping of Nkx6.1 follow maturation after the transplantation under the mouse kidney capsule and display dose-dependent effects[8]. Whilst 1 million cells transiently normalize blood glucose (3-4 days), 5 million cells produce a more permanent effect. In contrast with coaxial methods, other groups succeeded by growth inhibition [9] or

overexpression of Pax4 [10]. Several reviews describe in detail the results of this period [7, 11-13], in which some of the strategies were based in pancreas development (eg. inhibition of sonic hedgehog [6]), whilst others were more empirical (nicotinamide or butirate). It was also clear from the very beginning [11] that in order to translate this mouse knowledge to human embryonic stem cells we would need to faithfully recapitulate the mammalian developmental program. Unfortunately, at that time human embryonic stem cells were not made freely available by the two companies that developed the first lines; Geron in the USA and ESI in Singapore and Australia which imposed very restrictive Material Transfer Agreements. In order to circumvent tight legislative restrictions imposed by Spain and pursue our work on human embryonic stem cells, one of us (Bernat Soria) accepted a Visiting Professorship at the National University of Singapore (from 2002 to 2004) to use the ESI owned human ESC in collaboration with Profs Alan Colman and Sir Roy Y Calne. Although great strives of progress were achieved Confidentiality Agreements signed with ESI limited dissemination of the work. In 2004, the social democrate government of the Region of Andalusia rectified the law offering the opportunity to develop stem cell work in Granada (National Stem Cell Bank) and Seville (CABIMER). In this context, we contributed along with the USA and Sweden to the development of new human ESC lines (HVR1, HVR2 and HVR3). Incidentally, between 2007 and 2009 one of us (BS) was sworn in as Minister of Health of Spain with a mission to rectify the archaic Spanish law on human embryonic stem cells. Interestingly, a recent resolution of the European Patent Office restrict patenting of results obtained using human embryo derived cells, but not on induced pluripotent stem cells (iPSC).

Strategies for in-vitro differentiation of human embryonic stem cells succeeded in better reproducing the transcription factors time-course observed for mammalian pancreas development [14, 15]. The seminal work of the group of D'Amour (Novocell-Viacyte) [16] established some of the key strategies to by-pass the step from ESC to definitive endoderm. Explained below is the work of different groups that drive the long-road to a tentatively non-return point, the implantation of the first patient with beta cell progenitors that may mature inside the patient body and, eventually, control blood glucose.

---------- *Figure 1 near here* ----------

Diabetes describes a group of conditions in which blood glucose is not properly regulated. Diabetes mellitus occurs when β-cells fail to secrete the insulin necessary to maintain the homeostasis of glucose in the blood flow. Most common forms of diabetes are type 1 and type 2 diabetes mellitus. Type 1 results from a cellular-mediated autoimmune destruction of β-cells, whilst in type 2 diabetes mellitus, insulin resistance from peripheral organs is coupled with insulin deficiency resulting from an insufficient β-cell mass or function. Other forms of diabetes include gestational diabetes (glucose intolerance during pregnancy) and monogenic forms, in which mutations in key pancreatic genes are found (for example in *Glucokinase*, *Pdx1*, etc). Over time, diabetes can lead to the rise of different long-term complications such as retinopathy, neuropathy, nephropathy, critical ischaemia of the limbs and other complications. Nowadays, the treatment for diabetes consists in exogenous insulin supply or pancreas/islet transplantation, but the inability to achieve a tight control of glucose regulation by exogenous insulin administration and the shortage of pancreatic islets donors have motivated recent efforts to develop renewable sources of β-cell replacement tissue.

- Why do we need insulin-producing cells?

Pancreatic islet hormones secreted by β-cells in the islets of Langerhans regulate blood glucose levels in adult mammals. These "micro-organs" represent 1-2% of the pancreas and contain several different cell types, including endocrine cells, endothelial cells, and nerve terminals. Pancreatic β-cells, which in humans represent around 50% of the islet mass, synthesize, store, and release insulin in response to nutrients, while α-cells (40%) secrete glucagon and δ-cells (10%) produce somatostatin.

The metabolic interbalance, resulting in blood glucose homeostasis is governed by several hyperglycemic factors (glucacon, growth hormone, glucocorticosteroids, epinephrine) and only one hypoglycemic agonist (insulin). This is the reason why loss of pancreatic β-cells, responsible of the synthesis, storage and release of insulin produces a life threatening disease: diabetes (from the greek "pass through"). High blood glucose saturates renal tubule glucose transporters and glucose appears in urine (glucosuria), the resulting osmotic pressure increase inside the kidney tubules augment urine formation (polyuria) and then patients need to increase liquid intake (polydipsia). Since insulin is needed for glucose to enter into the cells, storage of energy (fat, glycogen) decreases and as a consequence type 1 diabetic patients loose weight. Before the discovery of insulin in the 1920's [17], type 1 diabetes was an acute and lethal disease. Exogenous insulin administration (first purified from porcine and bovine pancreas and later obtained by recombinant methods) allowed blood glucose control but only intensive insulin therapy diminished diabetic complications (retinopathy, nephropathy, myocardial infarction, stroke, diabetic foot, etc), unfortunately with an increased risk for hypoglycemia. In terms of blood glucose regulation pancreatic islets located in the portal vascular bed will do the job better than any option. This is the reason why islets are transplanted into the liver (direct implantation into the exocrine pancreas is not considered because of the risk of pancreatitis). However, islet transplantation as a potential cure for diabetes is limited by scarcity of donors, suboptimal islet procurement techniques and the side effects of long-term immunosuppressive therapy.

Glucose-induced insulin release needs a glucose-sensing mechanism (the "glucose-sensor"), a response element (the "exocytotic machinery") and a regulated gene expression, processing and storage of insulin (transcription factors, miRNAs, Golgi apparatus, secretory granules) (**Figure 2**). Although pancreatic β-cells share many properties with peptide secretory nerve terminals, some of the mechanisms are unique for the β-cell. Glucose-sensing mechanisms need a high capacity low affinity glucose transporter (Glut-2) that equilibrates glucose concentration at both sides of the plasma membrane, a kinase that works at physiological blood glucose concentrations (hexokinase IV or glucokinase) and a potassium channel, which is blocked by the ATP and diadenosine polyphosphates formed during glucose metabolism (ATP-dependent K-channels). Additionally, β-cells in order to sense glucose lack the Pasteur effect, then glucose degradation is not stopped by augmentation of the ATP/ADP ratio, which in turn increases ROS formation with subsequent β-cell damage. Exocytosis is initiated by the blockade of ATP-dependent K-channels depolarizing the β-cell, opening voltage-activated Ca^{2+} channels and increasing Ca^{2+} in the submembrane region [18] creating a $[Ca^{2+}]_i$ code that do not desensitize and correlates with insulin secretion. Pancreatic β-cells initiate an oscillatory and widespread bursting activity throughout the whole islet due to the gap-junction conductance oscillations [19, 20]. Ca^{2+}-sensing proteins of the exocytotic machinery promote the fusion of the granule with the membrane and release insulin to the extracellular space. All these "fast" effects of glucose on the β-cell operate, provided that β-cells are ready to be stimulated. Insulin gene expression is finely tuned by nutrients as is processing in the Golgi apparatus and storage in secretory granules (together with C-peptide and Zn^{2+}). In contrast with the detailed study of the transcription factors involved (PDX1, Nkx6.1, CREB, etc), the biophysical processes governing these "slow" regulatory effects of glucose have been less studied [21]. Glucose is considered to be an "initiator" of insulin release, whilst other metabolic signals (amino acids)

or neural and paracrine factors (acetylcholine, colecystokinin), are unable to induce insulin release by their own, and rather "potentiate" the effect of glucose using mainly the release of Ca^{2+} by intracellular stores.

---------- *Figure 2, near here* ----------

In summary, a long list of proteins (transporters, ion channels, connexins, $[Ca^{2+}]_i$ sensors, etc) are needed for glucose sensing and insulin release. Furthermore, another long-list of transcriptions factors and miRNAs, regulate insulin gene expression and processing. A proper characterization and identification of a surrogate β-cells should not only identify the presence of these components but also their ability to work and respond to glucose (initiator of insulin release) and to potentiate -agonists- the effect of glucose (potentiators), and thus, to normalize blood glucose levels in diabetic animal models.

2. From ESC to Insulin-producing cells: The challenge of making a β-cell

Stem cells are non-specialized clonogenic cells defined by two important characteristics: *self - renewal* and *pluripotency*. These paradigmatic properties depend on transcription factors (Nanog, Oct4, etc), ion channel activity [21, 22] and cell-to-cell communication [23]. Proliferation will permit the expansion of millions of cells from a native undifferentiated cell. In terms of functional substitution in type 1 diabetes an estimate of 10^9 β-cells are needed to control blood glucose in an adult. Subsequent changes in cellular phenotype may be transiently reversible [24], however epigenetic modifications driving cell lineage differentiation may permanently lock the new entity into an adult post-mitotic cell or a committed progenitor. In terms of proliferation and differentiation capabilities embryonic stem cells are the most attractive, then multiple efforts reported multistep differentiation procedures, which end with insulin-producing cells, therefore, the human embryonic stem cells hold the promise of an un-limited, but allogenic, source of cells for this propose. On the other hand, the discovery of the induced pluripotent cells (iPSC) by Yamanaka's team [25] made possible the use of patient own-cells and in both cases to build a bank of HLA compatible cells.

After the proof-of-concept was established in rodent [1] and human [26] ESCs, more than one hundred papers reported the conversion of different stem cells and progenitors into insulin-producing cells either from monocytes [27], hematopoietic stem cells or mesenchymal stromal cells [28]. Transdifferentiation from the three germ layers into insulin-producing cells exceeds the aim of this review. Here we will discuss the consolidated knowledge using mammalian embryonic stem cells as well as innovative concept of islet cell regeneration giving support to a new era in the treatment of diabetes mellitus.

a. Lessons learned from islet development

The current success in generating pancreatic cell lineages from human ESCs relies on recapitulating the key events that regulate pancreatic lineage commitment in the embryo. The advances in our understanding of the key transcription factors and signaling pathways that govern pancreas development and β-cell formation have been crucial for the design of new protocols for generation of in vitro insulin-producing cells from ESCs. Our knowledge of human pancreas development derived largely from animal models, such as rats, chicks, fish and mainly mice, and it is based on the assumption that the molecular and cellular aspects of pancreas development are conserved, although some aspects of the mouse ESCs differentiation protocols may differ from those applied to human ESCs differentiation protocols.

Tremendous progress has been made in the field of pancreas development and it has been extensively reviewed [29, 30]. In this review, we will focus on the main transcription factors that govern different stages of islet development and β-cell fate.

- Pancreas Organogenesis and Islet Development

After gastrulation, the definitive endoderm is specified by the expression of endodermal markers including FoxA2, Sox17 and GATA4 and GATA6 transcription factors [31-33]. The development of this germ layer involves highly orchestrated morphogenetic events, reciprocal interactions with the adjacent mesoderm and ectoderm, and cell determination and differentiation [34-38]. The result of these patterning events is a gut tube in which budding organs are defined in determined regions along the tube [39]. Each stage of pancreas development is achieved by combinatorial of signals that activate in a specific spatial and temporal way a set of transcription factors. The region of the gut tube that will acquire a pancreatic fate requires retinoic acid signal and the exclusion of both Sonic hedgehog (Shh) and Bone Morphogenic Proteins (BMPs) [40-43]. The combined actions of activation and inhibition of these signaling pathways will result in the expression of *Pdx1, Ptf1a, Sox9 and Nkx6.1,* which are known as multipotent pancreatic progenitor markers [44, 45]. The homedomain transcription factor PDX1 is one of the first markers of the developing pancreas. Lack of PDX1 function leads to pancreatic agenesis in mouse and mutations in human *PDX1* are associated to hypoplastic or absence of pancreatic tissue, indicating the importance of this transcription factor in pancreas development [45-47]. Similarly, mutations in *PTF1a* also have been linked to pancreatic agenesis in both mice and humans [48, 49]. More recently, genomic sequencing of neonatal diabetes patients associated to pancreas agenesis has revealed a new gene responsible for this pancreatic disease, *GATA6* [50]. Studies in mice have shown that GATA4 and GATA6 are required to maintain the number and identity of pancreatic progenitor pool to allow the normal progression of pancreas development [51, 52].

Around embryonic stage (e) 9.5 in the mouse, epithelial buds undergo branching morphogenesis invading the surrounding mesenchyme resulting in the formation of small ductules, which contain the precursor cells of the acini, ducts, and islets of Langerhans [53]. The most important of the transcription factors that have been identified as specific for endocrine development is the bHLH transcription factor *Neurogenin3* (*Ngn3*). *Ngn3* expression is first observed at e9.5 in the mouse, and its expression peaks around e15.5, a stage that corresponds to the endocrine differentiation wave [54-56]. Lineage tracing experiments have shown that *Ngn3* expressing-cells (Ngn3^{+}) function as endocrine precursor cells and give rise to all hormone-secreting pancreatic cells; α (glucagon-secreting), β (insulin-secreting), δ (somatostatin-secreting), PP (pancreatic peptide-producing) and ε (ghrelin-secreting) cells [57]. That observation agrees with the phenotype of *Ngn3* knockout mice, which lack all endocrine cells types [58]. Ngn3-positive cells undergoe dynamic changes in gene expression, resulting in the activation of Ngn3 targets (Pax4, Arx4, Rfx6, NeuroD1, Pax6, Isl1) [59, 60].

The hormone-expressing cells become apparent around e13.5. By this time, the gut tube rotates to bring both buds into proximity and dramatic changes occur in the cellular architecture of the pancreas. Similarly, a rapid branching morphogenesis and acinar cell differentiation occurs [53]. There is a major amplification of endocrine cell numbers, mainly β-cells, which organize into islets clusters. Choice between α- and β-cells fate rely on the mutual repression of lineage-specific transcription factors. The transcription factor Pax4, Pdx1 and Nkx6.1 are critical for β-cell commitment, whereas Arx4 determines α-cell fate [61-65].

In addition to its role in early pancreas development discussed above, *Pdx1* is expressed in β-cells at later stages of pancreas development and persist in the adult life. Its activity is required to activate important genes for β-cells function, including *Glut2* and *Glucokinase* [66-68]. Cell maturity is also achieved by the cooperation of MafA and NeuroD and Pdx1 in the transcriptional activation of the *Insulin* gene [69-71].

b. In-vitro differentiation

As mentioned before, the common approach to differentiate human embryonic stem cells and human induced pluripotent cells towards β-cells is based on a multi-stages protocol attempting to reproduce *in vivo* pancreas development. Protocols aim to induce hESCs and hiPS to follow a sequential transition through mesendoderm, definitive endoderm, gut-tube endoderm, pancreatic endoderm and endocrine precursor stages, to finally obtaining functional insulin-expressing cells [3-5, 72]. Reported signaling pathways and factors required to direct pluripotent stem cells differentiation towards functional insulin-secreting cells are the result of years of investigation. However, due to the complexity of the aim, a highly efficient step-wise differentiation protocol is still missing.

The major problems in directing hESCs and iPS differentiation to β-cell-like cells are:

- The low reproducibility of the current differentiation protocols and
- The low amount of insulin-secreting cells produced at the end of the differentiation processes, which indicates that we are still far away from obtaining an optimal β-cell mass ($1x10^9$ cells) that could be used for cell therapy.

Protocols described so far generate *PDX1* and/or insulin positive cells, which need further maturation when transplanted into immunocompromised mice [73-75].

---------- *Figure 3 near here* ----------

- Obtaining definitive endoderm

In order to drive human ESCs differentiation towards insulin producing cells, the first goal is the efficiently generate definitive endoderm, which is the first step towards commitment into pancreatic fate. Whereas Activin A and Wnt activators are commonly used for DE induction, Noggin supplementation is justified by the requirement for low BMP signalling to direct the mesendoderm towards anterior primitive streak derivatives [16, 76, 77]. This first step of differentiation has been readily achieved by D'Amour et al. [16] using a combination of TGFβ family member, Activin A, to activate Nodal signaling, and low serum concentration of media to avoid the activation of PI3K. Furthermore, to improve the yield of definitive endoderm cells, the activity of PI3K could be inhibited using two different inhibitors, LY 294002 or wortmannin. Wnt3a-mediated Brachyury expression is also important for the migration of precursors cells through the anterior region of the primitive streak (PS) and the formation of a mesendoderm population from which both endoderm and mesoderm will generate depending on the magnitude and duration of Nodal signalling. Hence, the efficiency of definitive endoderm generation further improve with exposure of human ESCs to a combination of Activin A and Wint3a in the absence of serum on the first day, followed by one day of culture in medium supplemented with Activin A and 0,2% of serum and 3 days in medium supplemented with

Activin A and 2% of serum [26]. In contrast to Wnts, BMPs inhibit endoderm induction. Therefore, inhibition of BMP signalling using the BMP antagonist, Noggin, resulted in increased expression of PS/endoderm markers and in a rapid reduced expression of PS/mesoderm markers, thus demonstrating the cooperatively intertalk of canonical Wnt/β-catenin, Activin/Nodal and BMP signaling pathways during ESCs specification of PS, mesoderm and endoderm [76]. A different approach to induce definitive endoderm has been recently published [78], consisting in the use of two small molecules identified as endoderm inducers (IDE1 and IDE2) with efficiency similar to that obtained with Activin A treatment.

- Pancreatic progenitors and late maturation

Maturating endocrine precursors toward specialized and functional hormone-secreting cells is still the most problematic step to direct pluripotent stem cells differentiation to insulin-producing cells [79, 80]. Despite the great number of biologically active compounds that have been already tested for this purpose, none of them has successfully worked [81, 82]. D'Amour et al. [26] used a mix of different "maturation factors" such as IGF1, Exendin-4, HGF and B27 supplement during terminal differentiation stages, but observed only minor effects on differentiation when these factors were omitted. On the other hand Cho et al. [83] demonstrated that the application of betacellulin and nicotinamide to D'Amour's protocol resulted in sustained *Pdx1* expression and led to subsequent insulin production. Nevertheless, cells obtained from *in vitro* differentiation strategies are not mature enough to be completely functional; although they express different markers of β-cells, such as insulin, GLUT2 or Glucokinase (GK), they display functional defects in the glucose sensing pathway or the exocytotic machinery [84-87]. Hence, strategies to improve the *in vitro* maturation process of endocrine precursors are needed and until quite recently has been achieved [3-5].

- Other approches to take into account for maturation

All strategies describe so far for pluripotent stem cells differentiation to obtain functional insulin secreting cells are the result of a decade of research and the fact that it has not been still achieved demonstrate the complexity of reaching this aim. New factors and different culture conditions are mandatory to induce a complete differentiation and maturation of pluripotent stem cells-derived β-cells. Here we mention some novel approaches that could be useful to improve definitive endoderm generation and final maturation of the endocrine precursors, resulting in a more efficient insulin-secreting cells differentiation strategy, below some of these strategies are overviewed.

i. Effects of soluble factors in the maturation process

Screening for new active molecules to be used as "maturation factors" could be helpful. In this context, a previous study described fetal soluble factor, released by pancreatic buds, that has been used to induce *in vitro* endocrine pancreatic differentiation from mouse ESCs [7]. Subsequent proteomic studies (unpublished data) have demonstrated that one of the most abundant proteins present in the soluble factors released by pancreatic buds was Regenerating 1 (Reg-1). Reg-1 is normally induced in pancreatic β-cells and acts as an autocrine/paracrine growth factor for β-cell regeneration [88, 89]. Based on this information, Reg-1 could be used in differentiation protocols to induce human ESCs-derived β-cells maturation.

ii. Nitric oxide and definitive endoderm induction

The relevant role of Nitric Oxide (NO) in developmental processes in the embryo has been previously described [90, 91], including the induction of ESCs differentiation into cardiomyocytes [92, 93]. Short time exposure of ESCs to exogenous donors of NO like diethylenetriamine/nitric oxide (DETA-NO) induces early differentiation towards a definitive endoderm phenotype. Treatment of ESCs with DETA-NO for only 19h induces the expression of endodermal markers Pdx1 and GATA4, which represents an attractive alternative to the classical treatment with Activin/Wnt3a for 3-5 days to direct the cells towards a endodermal fate [24, 94]. During pluripotent stem cells differentiation, lineage commitment is controlled not only by a gamut of transcription factors and soluble factors, but also by epigenetic events [24, 95]. In this regard, NO, which is a messenger molecule involved in a wide variety of pathophysiological processes, has been shown to have a direct effect on gene expression through epigenetic events [24, 96]. The link between NO-dependent early stem cells differentiation and its epigenetic effects opens a new field of investigation aimed at defining the possibility to *ex vivo* prime stem cells with NO donors and/or more classical epigenetic drugs as a strategy to obtain specialized cell populations.

iii. Micro-RNAs

Emerging evidence indicates that micro-RNAs (miRs) a group of small non-coding RNAs are prime candidates to fine-tune signaling pathways and gene expression and therefore able to control a variety of physiological processes, including glucose homeostasis [97]. Several miRs are expressed at high levels during human pancreatic islet development and are known to have a functional role in pancreatic β-cell development and function. Among the pancreatic miRs identified, *miR-15a* has been shown to induce insulin biosynthesis by inhibiting UCP-2 gene expression [98]. *miR-30d* has an important role in the regulation of insulin gene transcription by glucose through negative regulators of insulin gene expression [99]. *miR-124a* regulates *Foxa2* gene expression and preproinsulin [100]. *miR-9* is a key factor in the modulation of Sirt1 expression, and therefore modulates insulin secretion because by regulating exocytosis [101]. overexpression of *miR-373* leads to differentiation towards the mesendodermal lineage [102], *miR-148* involved in regulating insulin synthesis via upregulation of insulin transcription [103], *miR-375* has been implicated not only in pancreatic islet development but also in mature islet function because is required for normal glucose homeostasis [104, 105], *miR-7* is the most abundant endocrine miR and is expressed at high levels also during human pancreatic islet development [106], and inhibition of *miR-7* results in decreased β-cell numbers and glucose intolerance in the postnatal period [107]. Previous studies have shown that overexpression of *miR-375* promotes differentiation of ESCs to pancreatic endocrine, and provide evidence that constitutive *miR-375* expression in ESCs led to the expression of beta cell markers as well as the production of insulin in reponse to glucose in islet-like clusters [108]. Furthermore, expression of *miR-7* in human fetal pancreas increase at weeks 14-18 that correspond to induction of PDX-1 and other genes required for endocrine cells fate specification [107]. Altogether suggests a novel mechanism in the control of endocrine cell differentiation and consequentially miR-7 could be considered as an important player for the achievement of a complete differentiated human pluripotent stem cells-derived β-cells. The mechanisms by which miRs regulate this process remain poorly understood.

- Recent achievements

As previously mentioned, insulin-producing cells obtained by numerous *in vitro* differentiation protocols published so far are commonly immature and non-functionally glucose-responsive. As a consequence, many research groups omitted the late *in vitro* differentiation steps, and allowed pancreatic progenitors to specialize into functional β-cells by *in vivo* maturation after transplantation in STZ-induced hyperglycaemic mice [5, 73-75, 109] or included small molecules and

growth factors to medium formulation in the last stage of cell differentiation. IGF1, Exendin-4, HGF and B27-supplement were used as a "maturation factors" during late differentiation stages, but only minor effects were observed [26], fibronectin and insulin-transferrin-selenium (ITS) were successfully used during the suspension culture step [110], and recently, R428 (a selective small-molecule inhibitor of the tyrosine kinase receptor AXL), Alk5 receptor inhibitor (Alk5i), N-acetyl cysteine (N-Cys) and thyroid hormone T3 were successfully used to come up with highly differentiated cells quite similar to mature β-cells [3-5].

i. Impact of Resveratrol on stem cells-derived endocrine precursors maduration:

Several studies reported the beneficial impact of resveratrol (RSV) on insulin secretion and how this compound potentiates glucose-stimulated insulin secretion (GSIS), not only in rat insulinoma cell lines (INS-1E), but also in isolated human islets [111]. Based on this knowledge, we investigated whether RSV could improve the final maturation step of hESCs differentiation towards β-cells. RSV (3,5,4'-trihydroxy-trans-stilbene) is a polyphenol that has been shown to activate SIRT1, a NAD^+-dependent sirtuins or class III histone deacetylase (HDACs) [112, 113]. We have shown that SIRT1 contributes to the establishment of specific developmental/differentiation programs of hESCs [114]. SIRT1 in combination with antagonists (nicotinamide) [1, 83, 127] promotes stem cells differentiation and in combination with agonists (RSV) [4] promotes maturation. Other studies demonstrated the effect of RSV on insulin secretion using INS-1E and human islet [111, 115]. SIRT1 represses mitochondrial uncoupling protein-2 (*Ucp2*) transcription by binding directly to its promoter [116] resulting in increased ATP production and insulin secretion in INS-1E and in BESTO mice islets [117, 118]. Additionally, RSV induced an up-regulation of key genes for β-cell function such as *Pdx1*, *Glut2*, *Gk*, *Hnf1α* and *Tfam* in both INS-1E cells and human islets [111], this up-regulation has been described as a possible mechanism by which RSV potentiates metabolism-secretion coupling in β-cells and interestingly for the maintenance of the β-cell identity [119, 120]. We have shown for the first time that RSV is a critical compound improving the maturation of hESCs-derived endocrine precursors towards insulin-secreting cells, thus proposing its use for a more efficient insulin-secreting cells differentiation strategy [4].

ii. Two independent works, same achievement

Using high-throughput screening techniques, Kieffer's team [5] improved considerably their original protocol, which -for the first time- showed that fully functional beta-like cells could be generated *in vitro* and were able to permanently reverse hyperglycemia when transplanted into diabetic mice. It is a seven-stage in vitro differentiation protocol that builds upon protocols previously used to specify pancreatic progenitors. The addition of vitamin C at early stages of differentiation results in production of $PDX1^+/NKX6.1^+$ pancreatic progenitors with low expression of NGN3 and its downstream targets. Further differentiation of pancreatic progenitors using a combination of reagents including an ALK5 inhibitor, BMP receptor inhibitor and thyroid hormone T3, wich results in the increase of *NGN3* expression and a substantial fraction of $PDX1^+/NKX6.1^+/NEUROD1^+$cell populations. The continued exposure to ALK5 inhibitor, BMP receptor inhibitor, thyroid hormone T3 and Notch inhibitor results in the generation of $NKX6.1^+/insulin^+$ cell populations that express insulin but not glucagon or somatostatin. Finally, the screening of a number of additional reagents (a library of >40 small molecules and growth factors) identified R428, an inhibitor of AXL, which, in combination with ALK5 inhibitor and T3, potently induces MAFA expression in $PDX1^+/NKX6.1^+/NEUROD1^+$ cells that are $insulin^+/glucagon^-/somatostatin^-$. The resulting highly differentiated cells display key characteristics of mature beta cells, including glucose-induced insulin secretion, and rapidly reverse diabetes after transplantation in mice.

A 70 kg diabetic patient may need about 0,5-1 x 10^9 insulin-producing cells in order to normalize blood glucose. To obtain such a critical mass of pancreatic progenitors committed to be differentiated into insulin-producing cells or a similar number of surrogate β-cells, it is required to expand the population of pancreatic precursors. Melton's group [3] follows a very similar protocol to that of Kieffer's, with common elements but slightly simpler, and report a strategy for large-scale production of functional human β cells from human pluripotent stem cells. By using sequential modulation of multiple signalling pathways in a three-dimensional cell culture system, without any transgenes or genetic modification, they succeed to generate glucose-responsive, monohormonal insulin-producing cells that show key features of a bona fide β-cell, including coexpression of key β-cell markers and ultrastructure. Furthermore, these cells mimic the function of human islets both in vitro and in vivo.

The recent success in generating more mature beta cells from human pluripotent stem cells is very encouraging and although more basic research is needed, the field has taken important steps towards using human pluripotent stem cells-derived β-cells in cell therapy in diabetes [3-5]. Actually, the first and generally accepted initiative of obtaining pluripotent stem cells-based therapy is that headed by ViaCyte Inc. Looking ahead, it is likely that phase 1 clinical trials based on using insulin-producing β-cells derived from human pluripotent stem cells, promoted by ViaCyte Inc. Company, will soon follow. ViaCyte strategy consists in implanting non-mature progenitors that may thereby follow the maturation process inside a device (VC-1) inside patient body and follow the maturation process by measuring insulin and human C-peptide production in parallel with exogenous needs for blood glucose regulation.

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3. Cell selection strategies

In-vitro directed differentiation methods have been successfully applied to generate hESC-derived β-cells and β-progenitor cells. However, many of these methods yield different islet-like cell enrichment. Thus, independently of the percentage of cells, which are positive for human C-peptide (20 to 60%), Pdx-1 (aprox 100%) and other markers, currently, there is not a single method that generates a completely pure post-mitotic human ESC-derived β-like cell culture. This generates several problems: *i)* it is important to enrich first the population of differentiated cells and then they could be expanded and *ii)* the risk for undifferentiated cells inducing teratoma formation exists [73, 121]. Thus, without a well-designed method of selection of tissue-specific precursors, the cells obtained after the different differentiation protocols are not yet scalable for clinical application.

A variety of methods could be used or have been developed to select islet-cell differentiated cells. For example, the uses of lineage-specific cell surface markers. In the case of β-cells it would be the glucose transporter 2 (Glut-2), that it is expressed in the cellular membrane of β-cells [122]. However, Glut-2 could not be enough specific because is also expressed in the liver [123] also coming from endoderm. Another authors [124] were able to enrich pancreatic endoderm cells and endocrine cells, derived from hES cells, by using CD142, CD200 and CD318 cell-surface markers. Nowadays, there are still large gaps in our ability to select islet-cell differentiated cells on the basis of surface marker expression. In addition, the use of fluorescent reporters of gene expression has been used as an approach to select differentiated islet-cells. Then, the differentiated cells can be selected by using fluorescent-activated cell sorting. In this regard, Shiraki et al. [125] were able to select endoderm and Pdx1-positive pancreatic progenitors from ES cells. Alternatively, cell-trapping methods using antibiotic resistant genes coupled to the insulin gene promoter [1, 126, 127] or the Nkx6.1 gene promoter [6]

has also been used. This technology consists on the use of reporters that activate expression of a selectable marker that can drive differentiated islet-cells to almost 100% homogeneity by restrictive survival using antibiotics. The generation of reporter lines that carries an eGFP reporter gene under the control of insulin or any transcription factor needed for islet-cell differentiation, such as Pdx1 is another strategy [128] employed. However, random genomic integration and unregulated transgene expression are limitations to this approach. To address these limitations, homologous recombination to "knock-in" a fluorescent protein into a specific genetic locus can be used. Currently there exist hESC reporter lines that have been used to sort neuronal [129], cardiac precursors [130] and β-cell precursors [131, 132].

Actually, new alternatives to avoid the use of genetically modified stem cell lines for isolating tissue-specific progenitors are being explored. In this regard, King et al. [133] modified dual-fluorescence resonance energy transfer (FRET) "molecular beacon" technology, using fluorescence-activated cell sorting (FACS). This new technology allows the isolation of live differentiated ESCs based on expression of intracellular proteins and leaving the stem cell genome intact.

In conclusion, the ability to select specific differentiated stem cells with tissue-specific properties is a key aspect that will need to be overcome for clinical application of stem cells.

- Role of non β-cell partners:

Intra-islet interactions have shown that β-cells act as a functional syncytium and that other endocrine (alfa-, delta- and PP-cells) and non-endocrine (endothelial) cells play a relevant role in the construction of an integrated response to nutrient signals. Although isolated β-cells keep the whole machinery to respond to nutrients, its efficiency increases substantially when couples other β-cells [134]. Minimal size aggregates that better mimic islet behavior were estimated in 10-15 β-cells. More recently, a role for α-cells has been reported [135].

Moreover successful engraftment may be improved by cotransplantation of mesenchymal stromal cells that will also protect from immune attack (ESC and banks of HLA compatible cells are allogenic in nature).

This new approach is be based on the ability of MSCs to secrete many cytokines and growth factors that both provide an *in vivo* favorable microenvironment supporting engrafment of insulin-producing cells, angiogenesis and immunomodulation

Renewal of β-cells may depend on replication of differentiated β-cells and/or ductal progenitors, then it is tempting to speculate that an in-vitro obtained progenitor of ductal and endocrine cells which differentiate and maturate after implantation may be closer to endocrine pancreas repair. Most efforts were focused on the post-mitotic β-cell whilst, in theory, a ductal-endocrine progenitor will be better in order to keep tissue homeostasis. Then, efforts to better control developmental decisions ending with ductal, endocrine or exocrine in-vitro differentiation [136] are needed.

4. Minimal standards to accept a β-cell surrogate

As described above a pancreatic β-cell gathers together three main features: glucose-sensing, exocytotic machinery and insulin gene expression, processing and storage complexes. Whilst early and late progenitors could be better characterized the transcription factors governing the

process (**Figure 3**) to characterize a postmitotic β-cell we should test the presence and function of the 3 complexes. Tables I and II summarize these in insulin-producing cells derived from mouse and human embryonic stem cells. The aim is not a full descriptive report but a summary of the breakthrough published papers with an historical perspective, which drive this research.

Tables I and II

5. From adult progenitors to Insulin producing cells

a. Defining the Holy Grail of Islet Re-Juvenescence

In recent years, the concept of β-cell regeneration has come into the limelight as a potential complementary approach to stem cell therapy for future treatment of Type 1 Diabetes Mellitus (T1DM) [137]. These two approaches or not mutually exclusive as on one hand lessons learned from *in vivo* regeneration may aid in the development of improved *in vitro* differentiation protocols while on the hand characterization of novel differentiating genes highlighted in *in vitro* studies may become useful markers to identify a *bona fide* re-juvenescence cell source within the pancreas. The notion of regeneration stemmed from studies revealing that individuals with long standing T1DM were found to retain a residual and functional β-cell mass, which persisted in a steady state turnover within the hostile autoimmune environment [138-140]. In agreement with these human data, studies performed in mouse model of experimental autoimmune diabetes, revealed that the immunological destruction of islet β-cells was associated with enhanced β-cell regeneration [141, 142]. Consistent with an intimate dialogue between immunity and islets to promote β-cell regeneration, immunosuppressive therapy using anti-CD3 monoclonal antibodies impeded β-cell replenishment [141]. These studies highlight a fundamental paradigm, if ever an *in vivo* regenerative approach to treat T1DM is to be implemented: A non-mutually exclusive strategy in which the immune response as well as β-cell regeneration and function are exquisitely fine tune is essential in order to successfully regain an optimally performing β-cell mass and maintain normoglycemia.

Notwithstanding this complex crosstalk that likely triggers the regeneration process, the subsequent target cells and mechanisms that leads to β-replenishment remains a matter of controversy [143, 144]. Indeed, depending on the experimental mouse model and degree of injury inflicted to the pancreas, new β-cells were shown to be generated by neogenesis of ductal epithelium cells [145, 146], by trans-differentiation of α- and δ-cells to β-cells [147-150] and from rare pancreas-derived multipotent precursor cells [151, 152]. Interestingly, in young animals trans-differentiation after extreme β-cell loss was shown to arise from δ-cell conversion while in older mice replenishment was predominantly through α-cell trans-differentiation [147, 148]. These findings indicate that islets retain an age-dependent specific cell plasticity challenging the current perception that regeneration is gradually lost with age [153, 154]. Although exocrine acinar cells can be reprogramed to insulin-producing cells *in vivo* via the combined viral mediated ectopic expression of PDX1, NGN3 and MAFA [155], this conversion does not appear to occur spontaneously in either mice or human, excluding these cells as a likely source of *in vivo* regeneration. On the hand, bone marrow stem cells also appear to indirectly contribute to islet regeneration by promoting proliferation of resident islet cells [156, 157]. Alternatively, lineage-tracing studies demonstrated that pre-existing mouse adult pancreatic β-cells were the major source of new insulin-producing cells during adult life and also after pancreatectomy [158-160].

Furthermore, it was shown that all β-cells could contribute to islet growth and maintenance [161]. Albeit the compelling evidence that β-cell replication is the main mechanism of β-cell regeneration in rodents, neogenesis and trans-differentiation were also reported in pancreas of T1DM patients providing the proof-of- concept that all three processes independent of their contribution are important for β-cell regeneration [162-165]. Identifying factors or signals regulating these processes potentially triggered and sustained by the autoimmune environment will be the key to harnessing a successful *in vivo* regenerative therapy.

b. Pax4 and Arx, master regulators of β-cell regeneration

Lessons learnt from pancreas development studies have clearly demonstrated an antagonistic role of the transcription factors Pax4 and Arx in islet β and α cell faith decision from an early Ngn3-positive endocrine progenitor [166]. High expression levels of Pax4 relative to Arx were shown to favor a β-cell phenotype while low levels relative to Arx result in a α cell phenotype [62]. Seminal work performed by Collombat and colleagues demonstrated that forced expression of Pax4 in endocrine precursors as well as in mature α-cells in mice impelled the conversion of these cells into insulin-producing cells resulting in enlarge islets. In parallel, a continuous replenishment of α-cells was detected through neogenesis of an Ngn3-positive progenitor subpopulation located in the ductal epithelium vicinity [167]. Similarly, the selective inhibition of the Arx gene in α-cells through conditional loss-of-function in mice resulted in the efficient conversion of adult α-cells into β-like cells at any age [168]. More importantly both transgenic animal models were rescued from hyperglycemia subsequent to toxin-induced β-cell destruction as a result of constant β-cell renewal through α- cell transdifferentiation [167, 168]. Independently, we demonstrated that conditional overexpression of Pax4 in adult β-cells protected transgenic animals against streptozotocin-induced hyperglycemia [169]. More recently, we have also validated this protection in an animal model of experimental autoimmune diabetes (manuscript in preparation). Interestingly, long-term expression of Pax4 *in vivo* also resulted in loss of islet insulin secretion with the concomitant appearance of a $Pdx1^{+}$/$insulin^{-}$ /$BrdU^{+}$ cell subpopulation suggesting a de-differentiation of β-cells that potentially acquire a proliferative phenotype [169]. Interestingly, these cells are reminiscent of the sub-population reported by the group of van der Kooy [151, 152]. Substantiating this notion, we demonstrated using a transgenic mouse model in which EGFP along with the CRE recombinase expression is under the transcriptional control of the Pax4 promoter (pPAX4/EGFP-CRE) the existence of a Pax4/EGFP-enriched β-cell subpopulation. More importantly, mitogens such as activin A, betacellulin and GLP-1 increased Pax4 mRNA levels specifically in EGFP-positive β-cells [170, 171]. These results indicate the potential co-existence of two β-cell subpopulations within islets: A predominant Pax4-negative subpopulation which is functionally active in maintaining glycaemia and a Pax4-expressing subpopulation prone to proliferation that adapts the β-cell mass in response to physiological cues. These studies combined with the work of Collombat clearly define both Pax4 and Arx as master regulators of β-cell regeneration through the concerted processes of trans-differentiation, neogenesis and replication.

c. Pax4 and Arx as 'druggable' targets: Reality or fiction

The next challenge is now to seek naturally occurring or chemical factors which temporally induced Pax4 expression or repress Arx levels in order to promote regeneration within the autoimmune environment. Recently Dirice and colleagues reported that soluble factors such as IL-2, -6 and 10 secreted by invading immune T-cells stimulated β-cell regeneration [172]. Whether these cytokines have an impact on either Pax4 or Arx expression remains to be established. Glucagon-like peptide-1 (GLP-1) was also proposed to potentially promote α- to β-cell trans-differentiation through activation of Pax4 [173]. The feasibility of targeting transcription

factors with small therapeutic molecules was recently validated for the islet-enriched transcription factor Pdx1 [174]. These exciting results open a new era towards a potential regenerative therapy for the treatment of T1DM by targeting transcription factors such as Pax4 and Arx.

6. Expert Opinion

The last 15 years have witnessed different strategies to obtain insulin-producing cells from stem cells. Since the pioneer "proof-of-concept" with mouse embryonic stem cells it was crystal clear that developmental biology-based approaches were instrumental in the challenge of making a pancreatic β-cells. ViaCyte Inc. group succeeded in translating mouse knowledge into human embryonic stem cells. Whilst recently several groups, including ours, have came out with new strategies to generate functional β-cells from human stem cells, ViaCyte Inc. took a step ahead and start a clinical trial with 4 diabetic patients transplanted with pancreatic progenitors derived from stem cells. The maturation of pancreatic progenitors are expected inside the ViaCyte device implanted in patient's body, which could be very promising in the finding of a cure for type 1 diabetic people. Undoubtedly the recent events prelude the beginning of a new era in diabetes therapy. In spite of that, in our opinion there are still so many unanswered questions. While the generation of pancreatic progenitors from stem cells seems to be easier to accomplish than obtaining glucose-responding and insulin-producing cells, should we then focus in the in the β-cell maturation process? Are the pancreatic β-cells interconnected with other non-β endocrine cells, and if so, should we generate whole islets from stem cells? Should they be vascularized? Could we find a "miraculous" small molecule or cellular treatment that promote β-cells regeneration? Would cell-selection be needed to avoid remaining non-differentiated cells? Would these cells be tolerated by immune system?

Successful projects on making β-cells from stem cells have been funded by public systems in Europe and USA. However, it is a private company who is taking the control of such success, Johnson & Johnson Group. In order to get the best cost-effective treatment so every diabetic person could benefit of the in vitro generated β-cells, we think that the combination of private and public systems should control the production with its risks and benefits. That will definitively accelerate the process. We keep the hope that a century after the discovery of insulin we are now close to a cure for diabetes rather than for new treatment.

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Figure legends

Figure 1. From Embryonic Stem Cells to Insulin Producing cells: a summary of progress. Schematic representation of the contributors to the development of differentiation strategies to generate insulin producing cells from mouse and human pluripotent cells. ViaCyte Inc. signed rights agreement with Janssen Research & Development LLC. BetaLogics is a subsidiary of Janssen Research & Development LLC a Pharmaceutical company of the Johnson & Johnson Group. Contributions identified by the first author (see Reference section)

Figure 2. The Challenge of making a β-cell: **A.** Simplified scheme of glucose-sensing mechanism, exocytotic machinery and gene expression, processing and storage of insulin. *G: Glucose; Glut-2: Glucose transporter; GK: Glucokinase; G-6-P: glucose-6-phosphate; ATP: adenosinetriphosphate, DPs: diadenosinepolyphosphates;* K_{ATP}*: ATP-regulated potassium channels; T. Factors: transcription factors.* **B.** Intraperitoneal glucose-tolerance test in overnight fasted non-diabetic mice (o) and in streptozotocin-diabetic mice transplanted with insulin-producing cells under the kidney capsule *(taken from: Vaca P. et al. Stem Cells 2006; 24:258-265)*. **C.** Blood glucose after transplantation of insulin producing cells under the kidney

capsule. *TX: Transplantation; GR: graft removal (from León-Quinto T. et al. Diabetologia 2004; 47:1442-1451)*. **D.** Dose-dependent effect on blood glucose. *(o) Control STZ-diabetic mice; (Δ) Transplanted with 1 x 10^6 cells or with (•) 5 x 10^6 cells under the kidney capsule (from Roche E. et al. Handb Exp Pharmacol 2006; 174:147-167)*. **E.** Blockade of ATP-dependent K currents by ATP. Inside-out patch recoding at membrane potential = 0 mV *(taken from Vaca P. et al. Stem Cells 2006; 24:258-265)*. **F.** Graft removed 3 weeks after transplantation *removal (from León-Quinto T. et al. Diabetologia 2004; 47:1442-1451)*.

Figure 3. Summary of Pancreas Organogenesis and Differentiation and Maturation strategies to obtain a β-cell fate. *Protocol Stages: ESC: embryonic stem cells; iPS: induced pluripotent cells; ME: mesendoderm; DE: definitive endoderm; PG: primitive gut; PF: posterior foregut; PE: pancreatic endoderm; EP: endocrine precursors. Transcription Factors and Signaling Pathways: FoxA2: Forkhead box A2; GATA4: GATA binding protein 4; Sox17: SRY (sex determining region Y)-box 17; PDX1: Pancreatic and duodenal homeobox 1; PTF1a: pancreas specific transcription factor 1a; Nkx2.2: NK2 homeobox 2; Sox9: SRY (sex determining region Y)-box 9; Ngn3: Neurogenin 3; NeuroD: Neuronal differentiation; MafA: V-maf avian musculoaponeurotic fibrosarcoma oncogene homolog A; Wnt: Wnt signaling pathway; PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase; Shh: sonic hedgehog. Soluble Factors and culture media inducing in-vitro differentiation: FBS: fetal bovine serum; Wnt3a: Wingless-type MMTV integration site family, member 3A; Sirt-1: Sirtuin 1; bFGF:* Basic fibroblast growth factor*; RA: retinoic acid; ITS: insulin-transferrin-selenium. New Factors: R288: small-molecule inhibitor of the tyrosine kinase receptor AXL; Alk5i: Alk5 receptor inhibitor; T3: thyroid hormone T3; N-Cys: N-acetyl cysteine. Potential factors: Reg-1: regenerating islet-derived 1; mir7: mir-7 microRNA precursor; mir375: miR-375 microRNA.*

Figure 4. Effect of late maturation on hESCs-derived insulin-secreting cells. Upper: In-vitro staining for human C-peptide and insulin in hESC-derived insulin containing cells which followed a conventional protocol. Lower: hESC-derived cells exposed to late maturation factors.

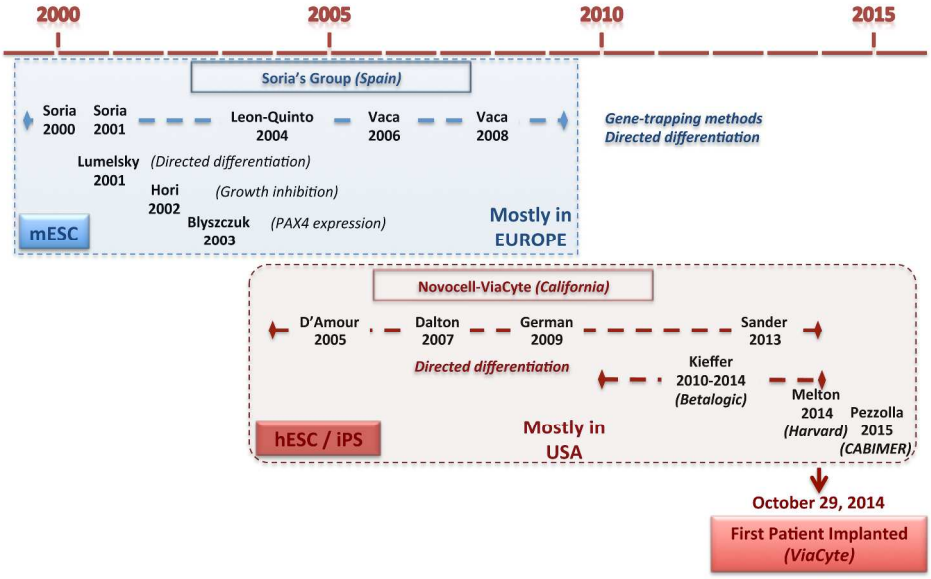

Figure 1. Schematic representation of the contributors to the development of differentiation strategies to generate insulin-producing cells from mouse and human pluripotent cells.
297x192mm (300 x 300 DPI)

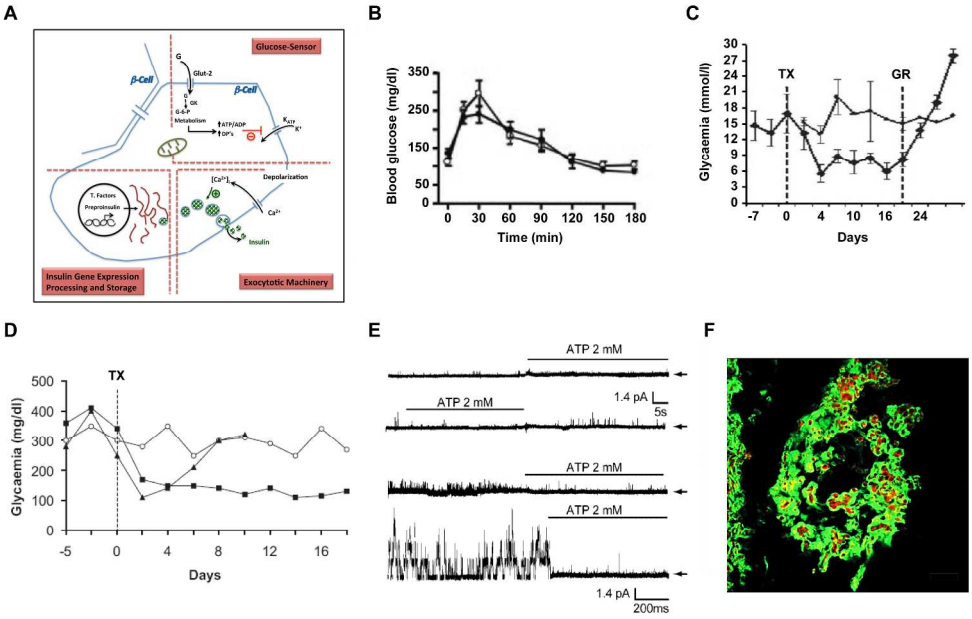

Figure 2. Pancreatic β-cell: A representation of glucose-sensing mechanism, exocytotic machinery and gene expression, processing and storage of insulin.
289x187mm (300 x 300 DPI)

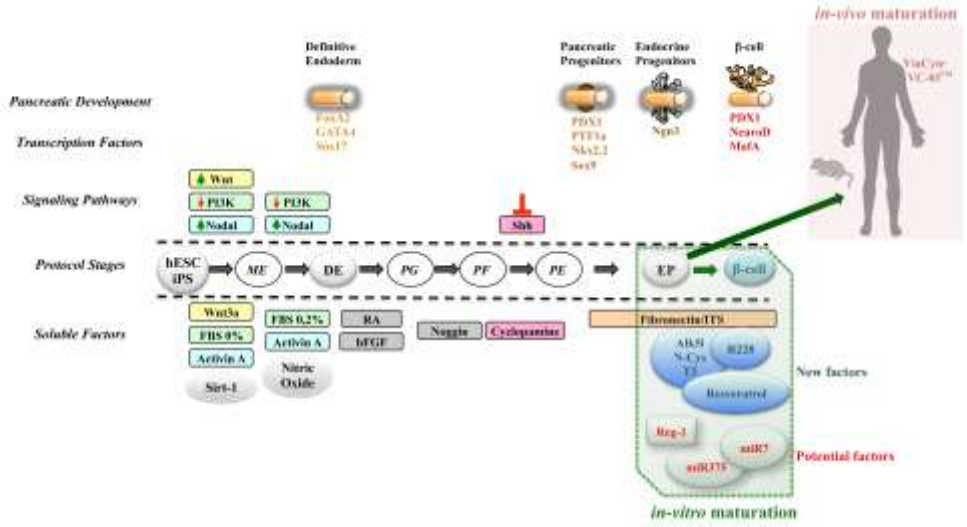

Figure 3. Pancreas organogenesis summary, differentiation strategies and maturation processes used to obtain a β-cell fate.

hESC, human embryonic stem cells; iPS: induced pluripotent cells; ME, mesendoderm; DE, definitive endoderm; PG, primitive gut; PF, posterior foregut; PE, pancreatic endoderm; EP, endocrine precursors; RA, retinoic acid; ITS, insulin-transferrin-selenium; R288, small-molecule inhibitor of the tyrosine kinase receptor AXL; Alk5i, Alk5 receptor inhibitor; T3, thyroid hormone T3; N-Cys, N-acetyl cysteine.

352x193mm (300 x 300 DPI)

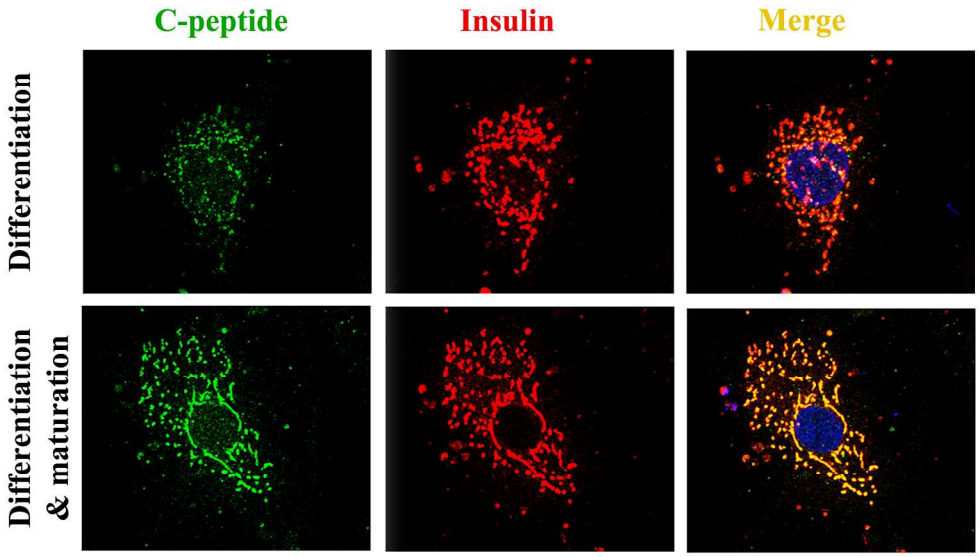

Figure 4. Maturation promotes hESCs-derived insulin-secreting cells obtention.
178x104mm (300 x 300 DPI)

MINIMAL CRITERIA FOR Mouse PANCREATIC BETA CELL MARKERS
(Adult & Progenitors)

	Soria (2000-2008)	Lumelsky (2001)	Hori (2002)	Blyszuck (2003)
1. Glucose-sensor				
Glut-2	+	+		+
Glucokinase	+			
Kir6.2 & functional K_{ATP} channels	+			
2. Exocytotic machinery				
EM granules				+
3. Gene Expresión, Processing and Storage				
mRNA				
Insulin	+			+
Transcription Factors				
Progenitors				
Pdx1	+			+
PTF1a				
NKx2.2				
Nkx6.1	+			
Sox 9				
Ngn3	+		+	+
GATA4			+	
Pax-4				+
Isl-1			+	
Adult				
Pdx1	+			
NeuroD	+			
MafA				

Protein (Western, ELISA,Immunosteaining, Cytometry) *Insulin* *Dithizone*	+ 	+ 	+ +	
4. Insulin and C-peptide content	+	+	+	+
5. Stimulus-secretion coupling (Glucose-induced insulin release) *In-vitro* *Basal vs Stimulated*	 +	 +	 	
6. Blood glucose normalization *Rodent models*	 +	 +	 +	 +
7. Preclinical Studies *In vivo maturation* *Teratoma formation*	 + -	 + +	 	

MINIMAL CRITERIA FOR Human PANCREATIC BETA CELL MARKERS
(Adult & Progenitors)

	Novocell-Viacyte (2006-2011)	Kieffer (2011-2014)	Melton (2014)	Pezzolla (2015)
1. Glucose-sensor				
Glut-2				+
Glucokinase				+
Kir6.2 & functional K_{ATP} channels				+
2. Exocytotic machinery				
EM granules	+/-	+	+	
Synaptophysin		+		
3. Gene Expresión, Processing and Storage				
mRNA				
Insulin	+	+	+	+
Transcription Factors				
Progenitors				
Pdx1	+	+		+
PTF1a				
NKx2.2		+		+
Nkx6.1		+	+	
Sox 9	+	+		+
Ngn3		+		+
HNF6	+			
HNF1B	+			+
HNF1A	+			+
Adult				
Pdx1	+	+	+	+
NeuroD		+	+	+
MafA		+	+	+

Protein (Western, ELISA,Immunosteaining, Cytometry)	+	+	+	+
Insulin		+		
Preproinsulin		+	+	+
C-peptide				
Transcription Factors				
Progenitors				
Pdx1	+	+	+	+
PTF1a				
NKx2.2	+	+		
Nkx6,1	+	+	+	
Sox 9	+			
Ngn3		+		
Pax4				
Pax6	+	+		
Isl-1	+	+		
Adult			+	+
PDX-1	+	+	+	
NEURO D		+	+	
MAFA	+	+		
Pax 4				
Pax6				
VCN-3				
4. Insulin and C-peptide content		+	+	+
5. Stimulus-secretion coupling (Glucose-induced insulin release)				
$[Ca^{2+}]_i$ signals		+		+
Glucose-induced insulin release				
In-vitro				
Basal vs Stimulated			+	+
Depolarization-induced		+	+	

In vivo *Human C-peptide/insulin* *Glucose challenge*	+ +		+ +	+
6. Blood glucose normalization *Rodent models*	+	+	+	+
7. Preclinical Studies *In vivo maturation* *GLP studies*	+ +	+	+	