

The New Generation of Beta-Cells: Replication, Stem Cell Differentiation, and the Role of Small Molecules

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■ Abstract

Diabetic patients suffer from the loss of insulin-secreting β -cells, or from an improper working β -cell mass. Due to the increasing prevalence of diabetes across the world, there is a compelling need for a renewable source of cells that could replace pancreatic β -cells. In recent years, several promising approaches to the generation of new β -cells have been developed. These include directed differentiation of pluripotent cells such as embryonic stem (ES) cells or induced pluripotent stem (iPS) cells, or reprogramming of mature tissue cells. High yield methods to differentiate cell populations into β -cells, definitive endoderm, and pancreatic progenitors, have been established using growth factors and small molecules. However, the final step of directed differentiation to generate functional, mature β -cells in sufficient quantities

has yet to be achieved *in vitro*. Beside the needs of transplantation medicine, a renewable source of β -cells would also be important in terms of a platform to study the pathogenesis of diabetes, and to seek alternative treatments. Finally, by generating new β -cells, we could learn more details about pancreatic development and β -cell specification. This review gives an overview of pancreas ontogenesis in the perspective of stem cell differentiation, and highlights the critical aspects of small molecules in the generation of a renewable β -cell source. Also, it discusses longer term challenges and opportunities in moving towards a therapeutic goal for diabetes.

Keywords: stem cell · induced pluripotent · embryonic day · transcription factor · definite endoderm · diabetes · transplantation · Pdx1 · beta-cell · growth factor

Introduction

Beta-cells are endocrine cells that reside in the pancreas and secrete insulin in response to glucose. Besides β -cells, the endocrine compartment of the pancreas consists of three other cell types, glucagon-secreting α -cells, somatostatin-secreting δ -cells, and pancreatic polypeptide-secreting (PP) cells. Together, these cells form the islets of Langerhans, which account for ~5% of the pancreatic mass in adults. Loss of β -cells or functional defects result in improper glucose ho-

meostasis, and lead to type 1 or type 2 diabetes, respectively. In type 1 diabetes (T1D), β -cells are almost completely lost as result of a destructive autoimmune process. T1D is one of the most common diseases in childhood, causing significant morbidity and mortality, as well as enormous healthcare costs. Even worse, the incidence is expected to double in children aged under 5 years, by 2020 [1]. Worldwide, the prevalence of all diabetes types is increasing, and the total number of diabetes patients is expected to reach 400 million by 2030.

Autoimmunity and shortage of insulin-producing cells are the main challenges in the quest for a treatment of T1D. To cure T1D in a comprehensive manner, both issues need to be addressed simultaneously. T1D is an excellent candidate for cell replacement therapy as it is caused by the lack of a single, well-defined cell type, the β -cell. Therefore, one approach being considered as a possible cure for T1D, is the combination of β -cell replacement therapy and tolerance induction to the new cells. Cell replacement therapy requires the availability of a renewable source of glucose-responsive, insulin-secreting cells. Promising results have been obtained by transplantation of pancreatic islets of Langerhans or pancreatic tissue [2]. However, this approach is hampered by the limited and irregular supply of cadaveric donor tissue, and the risk of treatment with immunosuppressant drugs. Therefore, new ways have been sought to “generate” the needed cells.

The rationale for a generation of β -cells is driven by the absolute lack of these cells in T1D, and by the deficiency of β -cell mass and function in type 2 diabetes (T2D). There are several approaches being considered for the generation of β -cells, e.g. the use of embryonic stem (ES) cells, adult pancreatic cells, or tissue stem cells. For such stem cells to be used in cell replacement therapy, we need to know how these cells develop. In the next sections, I introduce into pancreas and endoderm development. Then, I review possible approaches to the generation of β -cells from different cell sources.

Embryonic genealogy of β -cells

The pancreas is an organ derived from definitive endoderm (Figure 1). In embryogenesis, endoderm is formed during gastrulation. It is the lineage of origin for liver, lung, thymus, and other respiratory and digestive tract organs. Initially, when endoderm is formed (between embryonic day E6.5 and E7.5 in mouse), it is not committed to specific organ domains. The first specification of definitive endoderm towards pancreatic fate occurs around E8.5 in mice, and at around 3 weeks post-fertilization in humans. Initially, the pancreas forms as a ventral and a dorsal bud. The ventral bud is surrounded by the cardiac mesenchyme, while the dorsal bud is in contact with the notochord, and later the dorsal aorta. All of these mesoderm-derived tissues influence pancreas formation [3].

Lineage tracing studies have determined the presence of Pdx1⁺ multipotent progenitors in the

pancreas, which give rise to all pancreatic cell types [4, 5]. In humans, heterozygous mutations in Pdx1 highly predispose to adult-onset of diabetes, while absence of Pdx1 results in pancreatic aplasia [6].

Abbreviations:

AA - activin A
 BETA2 - see NeuroD
 bHLH - basic helix-loop-helix
 cdk4 - cyclin-dependent kinase 4
 c-Myc - v-myc myelocytomatosis viral oncogene homolog (avian, homo sapiens)
 CpA1 - carboxypeptidase A1 (transcription factor important in pancreas development)
 DiPS - diabetes (T1D)-specific pluripotent stem
 ES - embryonic stem
 E - embryonic day
 FDA - Food and Drug Administration
 FGF - fibroblast growth factor
 FoxA2 - forkhead-box protein A2 (also termed Hnf3 β)
 Hnf3 β - hepatocyte nuclear factor 3 beta
 iPS - induced pluripotent stem
 MafA - v-maf musculoaponeurotic fibrosarcoma oncogene homolog A (transcription factor necessary for beta-cell maturation)
 MafB - v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (transcription factor important for alpha- and beta-cell development and mature alpha-cell function)
 MODY6 - maturity onset diabetes of the young type 6 (mutations of the gene for the transcription factor NeuroD1 i.e., neurogenic differentiation 1)
 mRNA - messenger ribonucleic acid
 NeuroD - neurogenic differentiation (also known as BETA2; transcription factor expressed in pancreatic cells)
 Ngn3 - neurogenin 3 (member of the bHLH family of transcription factors expressed in the nervous system)
 Nkx6.1 - Nk6 homeobox protein 1 (required for β -cell development)
 NOD - non-obese diabetic
 Pax4 - paired box gene 4 (transcription factor involved in fetal and pancreas development)
 Pdx1 - pancreatic and duodenal homeobox 1 (transcription factor necessary for pancreas development)
 PP - pancreatic polypeptide
 Ptf1a - pancreas transcription factor 1 subunit alpha (important in pancreatic development)
 RA - retinoic acid
 T1D - type 1 diabetes
 T2D - type 2 diabetes
 VEGF-A - vascular endothelial growth factor A

Pdx1 is expressed at different stages of pancreatic development (from E8.5 until adult). The Pdx1⁺ cell potential to generate different cell lineages is temporally regulated. Pdx1 expression starts in the ventral pancreas at E8.5, and about 12 hours later in the dorsal pancreas, as well as in the caudal stomach and proximal duodenum. By E16.5, Pdx1 expression diminishes in exocrine lineages, and from E19 onwards, it is restricted to



β -cells and to 20% of δ -cells. After E12.5, the Pdx1⁺ cell potential becomes more restricted. The majority of duct cells are born before E12.5. Also, there is a spatial regulation of Pdx1⁺ cells and their developmental potential. Cells at the distal tip domain of the pancreatic epithelium, which express Pdx1, Ptf1a, c-Myc, and CpA1, are multipotent progenitors, and contribute to all cells within the pancreas [7]. These tip cells are in close contact with the mesenchyme. Perhaps a yet unidentified factor secreted by the mesenchyme is influencing the potential of these tip progenitors. These pro-

genitors largely disappear after E14.0. During this time window (E10.0-14.5), there is significant expansion and branching of the pancreatic epithelium. Thereafter at E15.5, the endocrine precursors delaminate from the pancreatic epithelium, and cells that remain within the epithelium differentiate to form the exocrine compartment of the pancreas (Figure 1) [3, 8].

Expression of the basic helix-loop-helix transcription factor Ngn3 in the pancreas marks endocrine progenitors that form all endocrine cell types. This finding was established by the loss-of-function phenotype of Ngn3⁺ cells. This phenotype leads to the specific and complete loss of all pancreatic endocrine cells, while exocrine and duct compartments remain unaffected [9, 10]. In addition, genetic lineage tracing studies show that Ngn3⁺ cells exclusively give rise to pancreatic endocrine cells [11]. Most endocrine cells do not start differentiating until the secondary transition is completed. Moreover, ectopic Ngn3 expression converts early endodermal progenitor cells into endocrine islet cells [12], and Ngn3 controls the expression of multiple genes that influence endocrine differentiation and function [13]. There is some evidence suggesting that Ngn3 mRNA and protein can also be detected in adult mice [14]. The function of Ngn3 at later stages, when the endocrine differentiation is completed, is not well understood, but could be related to islet cell maturation and maintenance.

Within the endocrine compartment, glucagon-expressing cells, and double glucagon- and insulin-positive cells, appear very early during pancreas development, at approximately E9.5 [15]. These cells are limited in number. Lineage tracing studies suggest that these cells do not contribute to mature islets of adult animals [11]. Also, the function of these early endocrine cells is not clear. True hormone-secreting cells including β -cells become apparent around E13.5. At E14.5, dramatic changes in pancreatic development, termed as secondary transition, lead to the generation of numerous insulin- and glucagon-expressing cells [16].

In rodents, the most abundant cell population (60-80% of total cell number) in the islet are β -cells located in the core, surrounded by a mantle composed by the three other endocrine cell types: α -, δ - and PP cells. Recent studies documented the existence of another, fifth type of endocrine cells, namely ghrelin-secreting ϵ -cells. Ghrelin-positive cells are rare (<1%), and disappear after birth [17]. Human endocrine islets are composed of 40-60% β -cells and 30% α -cells. The β -cells are mixed

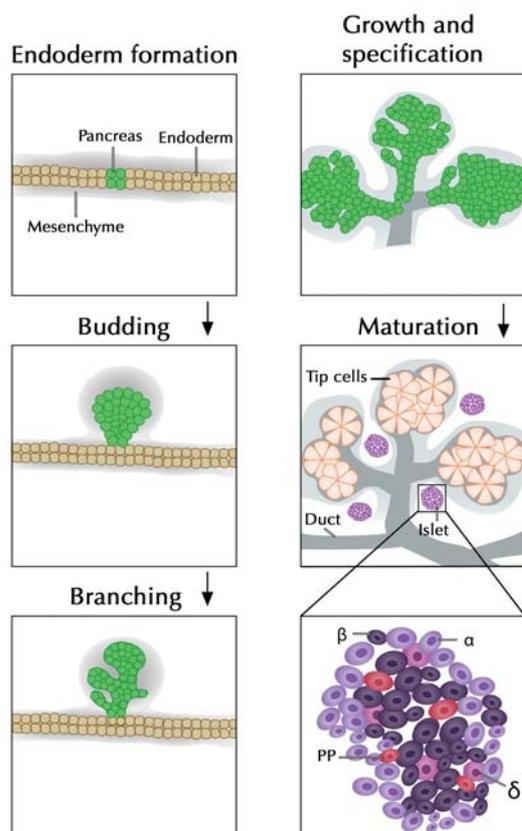


Figure 1. Overview of pancreas ontogenesis in the perspective of stepwise differentiation of ES cells to β -cells. In principle, pancreas development is similar in mouse and human. Differences were only reported in the final stage, the β -cell maturation. During embryonic development, the pancreas is generated from the portion of definitive endoderm pattern that gives rise to the gut tube. Endoderm forms two pancreatic buds (dorsal and ventral). Subsequently, pancreatic epithelium is induced and expanded. Pancreatic epithelium gives rise to endocrine progenitors which differentiate into β -cells and other islet components; among the latter are α -cells, δ -cells, pancreatic polypeptide (PP)-cells, as well as the exocrine and duct cells.



with other endocrine cells, and are distributed evenly throughout the islet structure [18]. The final architecture of Langerhans islets is not formed until 2-3 weeks after birth.

Many of the studies that used markers or growth factors for directed differentiation of ES cells took years of work on embryonic development, and they used various animal model systems in the pancreatic field. Despite significant progress achieved in understanding pancreatic development, many aspects of pancreatic organogenesis are still a mystery. Therefore, studies addressing progressive lineage commitment mechanisms, and cell fate in organogenesis, are of great value and significance for understanding cell fate in pancreas development and directed differentiation.

β -cells are not alone: signals between endothelium and tissues accompany the differentiation and maintenance of β -cells

In organ development, endothelial cells and progenitor populations are co-localized with cells from diverse tissues such as pancreas, heart, brain, liver, adrenal, bone, and blood. This "disorder" reflects the need for regulatory signals generated by endothelium, or nascent vasculature, to control primitive cell fate. Endothelial cells are important during pancreas development. Key events of pancreatic development occur in close proximity to endothelial cells. Pancreatic buds form precisely at the place where endoderm is in close contact with large vessels, the aorta dorsally, and vitelline vessels ventrally. Also, endocrine differentiation of the first insulin- and glucagon-expressing cells occurs in areas close to the overlying endothelium, as pancreatic cells delaminate from the budding endodermal epithelium [19-21]. There is strong evidence that endothelium and surrounding tissues maintain cross talk. It is well known that tissues signal to endothelial cells to provide clues for the patterning of vessels. Also, there are identified reciprocal signals from endothelial cells back to surrounding tissues. Overexpression of vascular endothelial growth factor A (VEGF-A) in mice leads to hypervascularization in the pancreas and hyperplasia of islets [22]. Insulin-expressing cells appear in the hypervascularized regions of stomach and duodenum which also express the transgene. When development is complete, vascularization and endothelial cells are essential for proper β -cell function.

Maturation of β -cells

In the field of directed differentiation, maturation of β -cells has emerged as an important, yet unsolved, question. So far, all differentiation protocols and efforts have failed to produce β -cells that secrete insulin in response to glucose at levels comparable to endogenous β -cells. Several transcription factors have been implicated in β -cell maturation *in vivo*. The bHLH transcription factor BETA2/NeuroD is a potent activator of insulin mRNA. During development, NeuroD is expressed starting at E9.5 in scattered cells in the pancreatic epithelium, and later (from E14.5 onwards) in Ngn3⁺ endocrine progenitors. After birth, NeuroD expression is restricted to β -cells. In humans, mutations in NeuroD expression can predispose individuals to maturity onset diabetes of the young (MODY6), suggesting a role of NeuroD in β -cell function. Mice lacking NeuroD expression in insulin-producing cells respond poorly to glucose, and show a metabolic profile similar to that of immature β -cells [23].

Other candidate factors implicated in β -cell maturation are those of the Maf transcription factor family. The expression of two members of this family, MafA and MafB, have been shown to change from immature to mature β -cells [24, 25]. MafB is expressed earlier than MafA in pancreatic development. It regulates the expression of genes required for the specification of α - and β -cells. The appearance of MafA is concomitant with the ability of β -cells to sense glucose. It is related to other features of the mature β -cell phenotype. Finally, the permanent expression of functional maturation markers is also necessary for the maintenance of islet cell identity.

Maintenance of β -cell mass *in vivo*

Genetic studies have shown that β -cells do not come from multipotent progenitors in adulthood [26, 27]. Conversely, the major homeostatic regulation of β -cell mass occurs via β -cell replication. Pancreatic organ size appears to be constrained by the initial progenitor population, and there is little cellular compensation [28]. However, β -cell mass is dynamic, and under specific circumstances, it undergoes significant changes.

Normally, adult β -cells have a slow growth rate. However, β -cell mass can expand extensively during pregnancy [29]. This phenomenon occurs in mice, rats, and humans. Rates of β -cell replication in pregnancy increase up to factor 1.5. There is evidence that circulating maternal hormones in

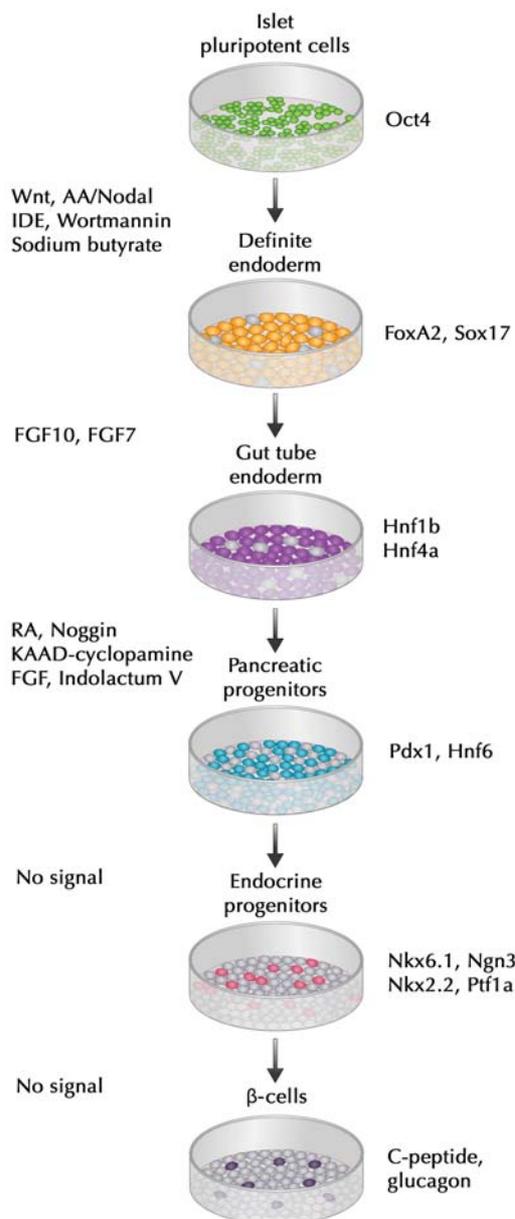


Figure 2. Differentiation of embryonic stem cells and/or induced pluripotent stem cells towards β -cells. Transition between each step is achieved by the modulation of signaling pathways by the use of proteins, or small molecules. Stages of differentiation are monitored by the expression of the key transcription factors, or C-peptide, in the last stage. During the first step, a vast majority of undifferentiated ES cells become definitive endoderm. However, the signals and culture conditions controlling later steps of differentiation are less well understood, and efficiency is decreasing. The final product, mature β -cells, is rare at the end of nearly 3 weeks *in vitro* differentiation process. AA: activin A. FGF: fibroblast growth factor. IDE: inducer of definite endoderm. RA: retinoic acid.

the bloodstream (prolactin, placental lactogen) can directly regulate β -cell proliferation in pregnancy through the intracellular factor menin [30-32]. Over the first 10 days postpartum, the increased β -cell mass regresses as a result of reduced β -cell proliferation and increased apoptosis [33].

β -cell mass increases also with age to compensate for increasing metabolic needs. In rodents, the β -cell number increases particularly in the first year of life [34]. The rate of β -cell replication changes significantly during the life of mice and rats: from ~20% per day in pups over ~10% per day at weaning, to 2-5% per day in young adults, and ~0.07% per day in aged 1-year-old mice [35]. The mechanisms of this change are unknown. In particular, it is not well understood what slows down β -cell replication with aging. The current assumption is that it is due to the dilution of growth factors in the bloodstream. At molecular level, there is strong evidence that the cyclin-dependent kinase 4 (cdk4)-cyclin D complex is responsible for β -cell replication during neonatal and adult growth [36, 37]. Finally, β -cell mass fluctuates in response to environmental signals. For example, increased glucose levels and insulin resistance can cause a higher β -cell replication rate [38].

Advancement of β -cell replication by small molecules

Most diabetic patients, even those with long-standing T1D, have a small number of β -cells that continually replicate and undergo destruction [39]. Ironically, this unfortunate cycle gives hope that one day T1D may be conquered by preventing autoreactive β -cell destruction, and by supporting replication of the surviving β -cells [40]. Therefore, screening for factors that increase β -cell replication *in vivo* may have direct therapeutic benefits to diabetic patients. Recently, a high-throughput screening of a chemical library for inducers of β -cell proliferation has been done by Wang *et al.* [41]. The group used growth-arrested, reversibly immortalized mouse β -cells, and found a number of diverse molecules that promoted beta-cell replication. Among them was a novel Wnt signaling agonists and L-type calcium channel (LTCC) agonist. The LTCC agonist 2a was found to induce β -cell replication by activating Ras signaling. Co-treatment of β -cells with LTCC agonist 2a and exendin-4 showed an extended effect on β -cell replication.

Promotion of endogenous β -cell replication by small molecules, or biological signals, is a promis-



ing avenue, but it is not yet ready for clinical application. For success, putative molecules would need to show β -cell specificity. Also, these molecules would need to be tested for their effect on other cell types within the body, to exclude unwanted neoplastic effects. If we can find a way to harness endogenous β -cell replication, and align it with effective prevention of autoimmune responses in humans, then we might develop a new therapy for T1D.

Differentiation from pluripotent cells and the role of small molecules

In recent years, many studies have been carried out to generate β -cells through differentiation of ES cells. These studies have been based on the premises that ES cells can give rise to any cell type in the body including β -cells, and that the key events of embryonic development can be recapitulated *in vitro*. The use of ES cells as starting population has several advantages: *i*) the relatively easy accessibility, *ii*) the unlimited capacity to proliferate *in vitro*, and *iii*) the uniformity of a pluripotent cell population.

Good progress has been made in differentiating human ES cells into β -cells [42-46]. All these investigations rely on the assumption that the differentiation from ES cells to β -cells cannot be achieved in a single step, but requires a series of transition steps which represent key events of pancreatic embryonic development (Figure 2). Several signaling pathways, including those activated by retinoic acid [47, 48], bone morphogenetic protein [49], fibroblast growth factor (FGF) [50-53], Wnt [54, 55], Hedgehog [56] and activin [56, 57] families have been implicated in pancreas organ formation. Clearly, modulators of the signaling cascades have been applied for ES to β -cell differentiation. By applying a cocktail of growth factors, human or mouse ES cells move through the key events of pancreatic development. Specifically, ES cells are coaxed first to generate definitive endoderm, then gut tube endoderm, and pancreatic progenitors. Finally, they turn into insulin-expressing cells *in vitro*. Each step can be characterized by the expression of prominent transcription factors.

The first steps of pancreatic differentiation, are achieved with high efficiency, 60-80% for definitive endoderm and 40-50% for pancreatic progenitors. Importantly, the ES cell-derived population can be subsequently differentiated into cells of the next pancreatic development stage. For instance,

definitive endoderm can give rise to pancreatic progenitors, with sufficient identity and specification. However, the final population of insulin-positive cells corresponds to immature β -cells, as these cells present low insulin content, co-express multiple hormones (insulin and glucagon) in the same cell, or show little response to glucose stimulation [44, 46].

An alternative approach involves the combination of *in vitro* ES cell differentiation, followed by *in vivo* differentiation, and maturation into glucose-responsive β -cells. It was shown that committed pancreatic progenitors, marked by the expression of FoxA2, Pdx1, and Nkx6.1, when injected into mice tissue can give rise to glucose-responsive, insulin-secreting cells after several weeks of *in vivo* maturation [44, 46]. However, the time necessary for the appearance of insulin-secreting cells (measured by the levels of human C-peptide in mouse blood) is unexpectedly long, and the origin of these cells is unclear. Also, the nature of the maturation process is unknown. It is not clear whether it requires signals from host environment or from co-transplanted non- β -cells.

Many protocols to generate insulin-producing cells from ES cells utilize the potential of small peptides, cytokines, and proteins to coax ES cells towards pancreatic fate. Several studies reported that small molecules can facilitate this process, and could substitute recombinant proteins [58-60]. Small molecules can even be more efficient, less expensive, more stable, and more easily controlled than growth factors. Furthermore, some chemical libraries for these molecules include FDA-approved drugs. These drugs are relatively well characterized such that their function, downstream targets, and toxicity is well known. This means that molecules identified in the abovementioned differentiation experiments could be clinically applied in their own right. Given the increasing number of libraries containing diverse chemical compounds, and the technical advances that allow rapid screening of these libraries, we may expect that the identification of specific molecules that guide ES cells to differentiate into β -cell is close to reality. β -cells proliferate *in vivo* only under certain circumstances such as pregnancy or metabolic demand increase. Therefore, expansion of mature β -cells and application of pancreatic progenitors should be considered as approaches to the generation of new β -cells.

In recent years, one of the major scientific breakthroughs has been the discovery that adult cells such as skin fibroblast and others can be re-



programmed to the pluripotent state by virus-mediated, ectopic expression of just a few transcription factors [61-63]. The cells generated by reprogramming, termed as induced pluripotent stem (iPS) cells, share many features with ES cells, including the ability to generate β -cells and other cell types. Similar to ES cells, sequential application of growth factors, or small molecules, leads to the generation of some insulin/C-peptide-positive cells from iPS cells [64].

Generally, the characterization of obtained populations at each of the steps should be considered with caution. It is critical to know, how close the cellular phenotype of ES-derived cell populations is to that of the naïve embryonic population. This knowledge is likely to be a key aspect for the generation of functional mature β -cells *in vitro*. However, it is technically challenging to characterize human populations in this way.

Transferring ES-derived β -cells to clinical application

There is a great hope that stem cell research can make determining discoveries for regenerative medicine. However, some obstacles need to be overcome before stem cell-derived β -cells can be transferred to the clinic.

Safety is the supreme concern. Any of the prospective cellular therapies will require rigorous evaluation, in particular to eliminate the risk of tumorigenic growth. Teratoma appear when ES cells, and their early derivatives that still possess some multipotency, are injected into mice [65]. Beside the safety concerns, it is unlikely that one protocol, or method, will enable complete and efficient conversion of the starting cell population into β -cells. Several strategies have been developed and tested. The options for this purpose are either to purify β -cells, or alternatively to remove undifferentiated cells and non- β -cell populations from the culture.

The generation of a human ES cell line carrying fluorescent reporters under the β -cell-specific promoter, such as insulin, could enable the separation of these cells by flow cytometry. These lines cannot be considered for direct clinical application, but they could lead to the identification of cell surface and reporter molecules, uniquely labeling β -cells. Alternatively, negative selection can be applied to eliminate unwanted cells from the cultures, at early stages, and at final differentiation. The same can be applied to iPS cells, especially during the process of iPS cell generation, when on-

cogenes, or viral sequences, might be introduced. There are alternative strategies used to generate iPS without viral sequence. They are based on the delivery of modified RNA encoding the gene of interest [66], or a serial protein transduction with recombinant protein-incorporating peptide moieties [67].

Another critical issue is the scaling-up of β -cell generation from ES or iPS cells. Approximately 6,000-10,000 islets/kg body weight are necessary for successful β -cell transplantation, which roughly corresponds to 420,000 islets per person. This number accounts for 40-50% of islet number in non-diabetic humans [2]. We know that these needs cannot be met by using cadaveric pancreatic organs. Based on the current efficiency of β -cell differentiation from ES/iPS cells with an increase of about 10-20%, this strategy requires a substantially large starting population. Up to 50% of β -cells gets lost during purification and preparation for the transplantation. Therefore, strategies are needed to scale up the process to yield a realistic therapy option. Changes in culture formats, e.g. monolayer culture vs. floating culture, could aid the scaling-up process. This might require modifications or even replacement of differentiation protocols.

In 1970, successful pancreatic islet transplantation was established using rodents [68]. By 1980, it became reality for humans. The application of this technology to patients with T1D has proved to be difficult. For various reasons, it lagged as a successful procedure until 2000, when the Edmonoton protocol was performed [2]. Since the first report of successful pancreatic islet transplantation to reverse diabetes in rodents, there has been a great interest in determining the optimal site for implantation. The majority of clinical islet transplantations have been performed into the portal vein. Islets were removed from a donated pancreas by collagenase digestion, followed by purification to separate the islets from exocrine tissue. Islets were then infused by gravity into a catheter lodged in the hepatic portal vein. In most cases, this procedure has been proved successful. Recipients of transplants have achieved normal blood glucose levels without extraneous insulin injections. However, this benefit was not permanent. In all cases, β -cell function declined early or after some years of transplantation. Other putative transplantation sites include: kidney capsule, omental pouch, subcutaneous, and intraperitoneal sites.

As an endocrine tissue, pancreatic islets require additional environmental factors if they are



to carry out their work of sensing and responding to glucose. From an immunological perspective, islets should be transplanted at a site where the inflammatory reaction is prevented, or at least minimized, such that long-term survival is ensured. Islet encapsulation could obviate the need for using anti-rejection drugs, but there is still lack of models to enable studying the fate of microencapsulated islets close to the human T1D situation [69].

***In vitro* maintenance of β -cells**

β -cells are highly specialized and rarely proliferate *in vivo* under normal conditions. Therefore, it is not surprising that long-term culture of β -cells, and *in vitro* expansion, appears to be difficult. In many cases, cultures of purified islets are overrun by insulin-negative cells, within a few days after plating. Some reports suggested that β -cells can dedifferentiate *in vitro*. These cells could then be expanded through epidermal-mesenchymal transition, and finally be directed towards β -cells again. However, the β -cell phenotype was not fully recovered; the new β -cells expressed only a fraction of their original insulin levels. Moreover, the origin of the new β -cells derived in this way was not clear due to possible contamination by other pancreatic cells which are mitotic active, e.g. duct, acinar, stromal, and endothelial cells. Finally, it is not clear what caused the dedifferentiation of β -cells *in vitro*. The loss of key β -cell features might result from the change in the cellular environment. *In vivo*, β -cells reside in a 3-dimensional islet structure, and interact with exocrine, endothelial, and neuronal cells. Most of the culture for β -cell expansion is based on monolayer of purified β -cells. It might be beneficial to co-culture primary β -cells with exocrine or duct cell lines, and/or pancreatic mesenchyme, to facilitate maintenance of insulin expression *in vitro*. Screening of bioactive chemicals and co-culture substrates may elucidate the conditions for *in vitro* β -cell expansion more precisely.

Alternative pathways to β -cells: reprogramming of mature cells

In rare cases, cells of one lineage can be converted into cells of another lineage [70]. For instance, intestinal cells can be converted into esophagus, and embryonic dermal fibroblasts.

Pigmented epithelial cells can be converted into beating cardiomyocytes, and human epidermis can be converted into pancreatic endoderm [71]. This research led to attempts to generate β -cells from various mature starting cells types, including pancreatic exocrine, duct, and liver cells. Expression of just three transcription factors (MafA, Ngn3, and Pdx1) enables the conversion of exocrine pancreatic cells into β -cells [72]. These cells express markers of mature β -cells, ultra-structurally closely resemble β -cells, and contain insulin-positive secretory granules. Alternatively, liver cells are often selected as starting population for transdifferentiation into pancreatic β -cells, as they share a common embryonic progenitor. Also, ectopic expression of Ngn3 or NeuroD has been reported to induce liver to pancreas conversion [73, 74]. Another example involves overexpression of transcription factors Pdx1 and Pax4 in the liver cells. Insulin-positive cells induced in this way can revert hyperglycemia in streptozotocin-induced diabetic mice.

The reprogramming approach requires the right combination of transcription factors that are overexpressed, and a suitable environment such as the "open genome structure", or conditions supporting the survival of induced cell types. To increase the probability of this rare phenomenon, some studies combine the overexpression of master genes with injury stimuli to activate progenitor cells. The molecular mechanism of this phenomenon remains largely unknown. However, we can imagine the possibility of deliberately reprogramming cells from one tissue type to another by manipulating the expression of transcription factors. This approach could generate new therapies for diabetes and many other human diseases. However, this approach is also afflicted with several obstacles. For instance, viral delivery of transcription factors is afflicted with safety concerns. Also, similar to the ES cell differentiation approach, insulin-positive cells obtained through reprogramming often express low insulin levels. Another challenge is that induced β -cells persist as individual cells and do not form islet-like clusters.

A more direct genetic engineering approach is nuclear reprogramming. This approach has also been examined by constitutively expressing, critical transcription factors in ES cells. Early reports have shown that more robust pancreatic differentiation could be achieved when ES cells express high levels of ectopic Pdx1, Ngn3, and Pax4; although no functional β -cells were generated from these protocols. The combination of genes is criti-



cal for the reprogramming outcome. The level of expression, the time window, and the choice of the starting cell population can change the outcome and efficiency of reprogramming.

***In vitro* modeling of T1D using patient-specific β -cells**

Progress towards finding a cure for diabetes is hindered by the lack of sufficient understanding of the disease itself. The best characterized animal model of T1D is the non-obese diabetic (NOD) mouse [75, 76]. The recent derivation of ES cell lines from NOD mice may facilitate the identification of genes involved in the T1D [77]. However, ideally we would like to study the disease directly in humans, as the outcomes predicted by murine studies do not match the outcomes of clinical trials. This especially applies in case of clinical trials for T1D prevention and treatment [78]. Using three transcription factors (Oct4, Sox2, and KLF4), T1D patient-specific pluripotent cell lines have been generated [64, 79], termed as DiPS. Originally, the authors reported the generation of two DiPS from skin fibroblast biopsied from T1D patients, but at the moment, the number of the DiPS lines continues to increase.

The DiPS cell lines have the hallmarks of pluripotency, and accordingly were shown to differentiate into pancreatic lineage. These patient-specific pluripotent cell lines could provide a model to investigate the disease and its etiology. Also, they may serve as platform to seek novel prophylactic, and therapeutic, treatment strategies. Generation of immune cell types (T cells, macrophages, and dendritic cells) from hematopoietic stem cells may assist to develop a model of cellular interaction in T1D. This would facilitate detailed study, and follow-up, of the disease. T cells are educated on thymic epithelium such that they can distinguish between self and foreign molecules. With all these cells available, the disease could be recreated either *in vitro* or *in vivo* using humanized mouse models [78]. All these studies might be instrumental in the generation of β -cells

resistant to autoimmune attack. A regenerative source of mature human β -cells (ideally patient-specific) would allow examination of many questions relating to type 1 and type 2 diabetes.

Conclusion and future perspective

Insulin is an essential hormone for the control of glucose levels in the blood. Severe complications ensue in the absence of this protein. In T1D patients, pancreatic β -cells are almost completely destroyed as a result of an autoimmune process. However, it has been found that β -cells can replicate and regenerate *in vivo* by differentiation from adult progenitors in the pancreas. In T1D, β -cell mass cannot regenerate as it is exposed to the recurring or continuing process of autoreactive destruction. Therefore, a new source of β -cells protected against immune reactions could escape the destructive process and reveal protective mechanisms. This strategy could afford prevention and even reversal of the disease.

In recent years, impressive progress has been made in various approaches to the generation of β -cells. The discovery of human pluripotent stem cells has sparked new interest by academia, and by the general public, to see these cells utilized as a potential source for β -cells. There is the great promise that some successful route will one day enable the generation of β -cells. Hopefully, the renewable source of functional β -cells could directly be used as a treatment for diabetic patients, or indirectly provide a platform for finding a cure.

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