Stem Cells to Pancreatic \( \beta \)-Cells: New Sources for Diabetes Cell Therapy

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The number of patients worldwide suffering from the chronic disease diabetes mellitus is growing at an alarming rate. Insulin-secreting \( \beta \)-cells in the islet of Langerhans are damaged to different extents in diabetic patients, either through an autoimmune reaction present in type 1 diabetic patients or through inherent changes within \( \beta \)-cells that affect their function in patients suffering from type 2 diabetes. Cell replacement strategies via islet transplantation offer potential therapeutic options for diabetic patients. However, the discrepancy between the limited number of donor islets and the high number of patients who could benefit from such a treatment reflects the dire need for renewable sources of high-quality \( \beta \)-cells. Human embryonic stem cells (hESCs) are capable of self-renewal and can differentiate into components of all three germ layers, including all pancreatic lineages. The ability to differentiate hESCs into \( \beta \)-cells highlights a promising strategy to meet the shortage of \( \beta \)-cells. Here, we review the different approaches that have been used to direct differentiation of hESCs into pancreatic and \( \beta \)-cells. We will focus on recent progress in the understanding of signaling pathways and transcription factors during embryonic pancreas development and how this knowledge has helped to improve the methodology for high-efficiency \( \beta \)-cell differentiation in vitro. (Endocrine Reviews 30: 214–227, 2009)

I. Diabetes and Pancreatic \( \beta \)-Cells

A S ONE OF THE MAJOR chronic metabolic diseases, diabetes mellitus affects at least 200 million people worldwide (1). According to the statistics of the American Diabetes Association (http://www.diabetes.org), there are more than 23 million diabetic children and adults in the United States alone. Diabetes results when the body is incapable of producing sufficient quantities of insulin, the hormone that regulates the level of blood glucose. Changes in normoglycemia, i.e. increases as well as decreases, negatively affect human health. Reduction of glucose levels beyond a certain threshold results in hypoglycemic coma, whereas prolonged hyperglycemic episodes lead to long-term complications. Unfortunately, current treatment options are mainly based on the exogenous supply of insulin, an approach not fully capable of mimicking the tight control of endogenously produced insulin released from pancreatic \( \beta \)-cells. Thus, strategies to promote either the expansion of existing \( \beta \)-cells within the body or the supply of stem cell-derived insulin-producing cells are currently debated as future treatment options.

Although the first descriptions of diabetes can be traced back to ancient Egypt, the loss of insulin production in \( \beta \)-cells as the culprit for the disease was not realized before the beginning of the last century. Based on the underlying causes of the disease, diabetes is divided into several categories. The most common form is classified as type 2 diabetes, resulting from impaired \( \beta \)-cell function combined with insulin resistance in peripheral organs (2). In contrast, type 1 diabetes, caused by almost complete elimination of pancreatic \( \beta \)-cells due to autoimmune response, affects only approximately 10% of the diabetic patients (3). Although the current therapy using exogenous insulin has greatly improved the lives of diabetic patients since the 1920s (4), this method is inaccurate and does not completely control for the minute-to-minute fluctuations in systemic blood glucose. Given the fact that \( \beta \)-cell function and insulin release are impaired in both groups, a real cure would have to come from newly formed or replenished \( \beta \)-cells capable of restoring normoglycemia. In fact, over the past four decades whole-pancreas organ transplantation has been the most effective treatment for diabetic patients with serious complications (5).

Recently, cell replacement therapies in which purified islets are transplanted to reconstitute the insulin-secreting cells have also emerged as promising alternatives to whole-organ transplantation (6, 7). Islet transplantation carries the advantage of being less invasive and results in fewer complications compared with the traditional pancreas or pancreas-kidney transplantation. The clinical outcomes of islet transplanta-
tions are encouraging. In its 2008 annual report, the Collaborative Islet Transplantation Registry (http://www.citregistry.org) stated that about 23% of the 279 patients who received islet-alone transplantation from 1999–2007 achieved insulin independence for 3 yr. The 5-yr follow-up report from Shapiro and colleagues (8) showed that 10% of the 65 patients enrolled in the Edmonton Center study remained insulin-free for 5 yr after the islet transplantation. Although long-term insulin independence was not achieved in the majority of the patients, islet transplantation did help control blood glucose level more easily when combined with exogenous insulin injections.

Like other organ transplantations, one major hurdle that prevents islet transplantation from being widely applied is the lack of adequate sources of donor islets and β-cells due to the limited availability of cadaveric tissue (5). The discrepancy between the effectiveness of cell therapy and the limited amount of transplantable material has urged researchers to seek for new sources of β-cells. The most prominent and promising cell source for β-cell progenitors is embryonic stem cells (ESCs) derived from the inner cell mass of blastocysts during early stages of embryogenesis (9, 10). ESCs differ from others in that, under the right growth and differentiation conditions, they carry the potential to give rise to any cell type in the body. The substantial advances in studies on human ESC (hESC) differentiation have raised the vision for new strategies aimed at generating large amounts of glucose-responsive, insulin-producing β-cells for therapeutic purposes. However, critical aspects of β-cell differentiation have not yet been replicated in cell culture, indicating a more thorough understanding of pancreas and β-cell development as the key for the successful generation of fully functional β-cells from hESCs. To understand better the current obstacles in guiding β-cell differentiation in vitro, we will briefly discuss the information we have gained from studies of embryonic pancreas development.

II. Pancreas and β-Cell Development

A. Embryonic development

One of the main interests in the studies of embryonic pancreas development is to deepen our understanding of how β-cells are generated under normal conditions. This requires not only dissecting the numerous cascades of signaling pathways and transcription factors that regulate cell autonomous differentiation, but also gathering knowledge about epithelial-mesenchymal interactions and factors secreted from surrounding tissues that instruct endocrine and β-cell development. It is the hope that once all this information has been collected, it can be combined to reconstruct the embryonic differentiation program for ex vivo generation of therapeutic β-cells.

The pancreas is a complex endoderm-derived organ. It consists of multiple cell types that conduct both endocrine and exocrine functions. The exocrine compartment accounts for more than 90–95% of the pancreatic mass and harbors the acinar cells that secret digestive enzymes such as lipases, carbohydrases, and amylases, as well as ducal cells that transport these enzymes into the duodenum (reviewed in Ref. 11). Although the hormone-secreting endocrine cells make up only 1–2% of the pancreatic cell population (12), they play an essential role in establishing euglycemia in the body. Within the pancreas, the islets of Langerhans harbor five different endocrine cell types (reviewed in Ref. 13). The insulin-producing β-cell is the dominant endocrine cell type and makes up 60–80% of the islet. In rodents, and to a lesser extent in humans, the β-cells are located within the center of the islets, surrounded by other endocrine cell types (14). This cellular proportion and organization in the adult pancreas and the morphological changes that occur during pancreas development have been studied in great detail over the last century. More recently, initiated by the advent of transgenic mouse technology, tremendous information has been gathered about the molecular mechanisms that regulate pancreas organogenesis and epithelial cell differentiation (reviewed in Refs. 11, 15, and 16).

During vertebrate embryogenesis, the three principle germ layers, ectoderm, mesoderm, and endoderm, are formed through intensive cell migration during gastrulation. In mouse, the favorite mammalian model system to study embryogenesis, a thin cup-shaped sheet of embryonic endoderm further develops into the primitive gut tube that can be divided into several regions along the anterior-posterior axis (reviewed in Ref. 17). Each region has a distinct developmental potential and normally gives rise to different endodermal organs, including liver, lung, stomach, and pancreas (reviewed in Ref. 17) (Fig. 1, upper panel). Specification of the pancreatic field occurs around embryonic day 8.5 (E8.5) in mouse and 3 wk in human. Three pancreatic primordia initially bud out from the definitive gut epithelium, the first one from the dorsal side, followed by two primordia on the ventral side (18). Because the ventral and dorsal pancreatic buds originate independently and from distinct regions of the primitive gut tube, there are noted differences with regard to the surrounding environment, the timing and specificity of signaling pathways, as well as gene expression profiles that guide these processes (reviewed in Ref. 19). Soon after forming, one of the ventral buds regresses, whereas the remaining ventral bud is brought together and later fuses with the dorsal evagination during the rotation of the gut tube around E12.5 (see Ref. 18; and for review, see Ref. 15). Subsequently, the pancreatic epithelium grows significantly in size and branches into the surrounding mesenchyme (Fig. 1, upper panel). Although glucagon-producing cells and a few cells coexpressing insulin and glucagon can be detected as early as E9.5 (20–22), fully differentiated β-cells and other hormone-secreting cells become first evident at E13 (22). At this stage, termed the secondary transition (23), endocrine cell number increases significantly through proliferation and subsequent differentiation of pancreatic progenitors (24, 25).

Interestingly, recent work has shown that the development of the endocrine compartment is directly connected to the formation of exocrine tissue. Cells positive for the acinar marker carboxypeptidase A are located at the tip of the branching pancreatic epithelium and display properties of multipotent progenitors that develop into acinar, duct, and endocrine lineages (25). Toward the end of gestation, endocrine cells that have delaminated from the duct-like epithelium aggregate into clusters dispersed in between acini and...
Embryonic pancreas development

In vitro ESC differentiation

B. Extracellular signals and transcription factors

Like other organs, pancreas development is guided by signals received from the surrounding environment. Many of these signals are diffusible factors secreted from adjacent germ layers (30, 31). During specification of pancreatic primordia, extracellular molecules secreted by the surrounding mesodermal tissues, such as notochord and dorsal aortas, have been shown to play critical roles in the induction of pancreatic epithelium (30–32). The cross-talk mediated by soluble factors between pancreatic mesenchyme and epithelium is also necessary for endocrine/exocrine lineage spec-
ification and differentiation (30). Another indispensable source of inductive molecules is the vascular endothelium, comprised of cells lining the interior part of blood vessels closely associated with the developing pancreas (18, 33). Although the roles of individual signaling pathways have been identified through the analysis of knockout animal models, much remains to be discovered about the interactions and balances between these signaling pathways.

1. Multiple extracellular signaling pathways induce pancreas development in embryogenesis. A number of embryonic signaling pathways, including the Hedgehog, Fgf, Notch, Wnt, as well as TGF-β pathways, control diverse aspects of pancreas and endocrine cell development (reviewed in Ref. 34). During early pancreas development, organ boundaries are specified via inhibitory effects of the Hedgehog signaling pathway present in tissues adjacent to the pancreas anlage (reviewed in Ref. 35). The exclusion of Hedgehog signaling from the dorsal pancreas anlage is achieved through signals provided by the notochord, a transient mesodermal structure that is in contact with the dorsal endoderm at early stages of gut formation. Notochord-derived signals block Hedgehog signaling in the pancreatic endoderm through the secretion of soluble factors such as members of the TGF-β/activin and fibroblast growth factor (Fgf) families (32, 36–38). Hedgehog inhibition allows for the specification of pancreatic endoderm and the expression of critical transcription factors, whereas ectopic expression of Hedgehog ligands perturbs pancreas formation (37, 39–41). Interestingly, Hedgehog activity can be detected in developing pancreas at later stages (E13.5) (41) and appears to regulate the expansion of early endocrine cells (J. Lau and M. Hebrok, unpublished results). In addition, work in zebrafish has revealed a requirement for Hedgehog signaling within the forming endoderm for induction of pancreatic β-cells (42). Thus, temporal and spatial changes in Hedgehog activity regulate different aspects of pancreas and endocrine formation.

Another important multifactorial signaling pathway involved in pancreatic development is the TGF-β superfamily. As mentioned above, TGF-β signals are critical for the specification and early branching morphogenesis of pancreatic epithelium (38, 43). In addition, multiple members of the superfamily, including activin and growth differentiation factors, are involved in the endocrine and exocrine lineage specification (see Refs. 38, and 43–45; for review, see Ref. 46).

Once the notochord has separated from the endoderm, aortic endothelial cells become located in close proximity to the dorsal pancreatic bud. Ligands of Fgf family members, such as Fgf2 and Fgf10, are secreted from notochord (37) and dorsal mesenchyme (47), respectively. These factors play important roles in the specification of the pancreatic primordium through initiation and maintenance of the expression of critical transcription factors (47–49). During later stages, Fgf10 secreted from pancreatic mesenchyme promotes the proliferation and inhibits the differentiation of pancreatic progenitors into endocrine cells via activation of Notch signaling (50–52). Besides Notch and Fgfs, Wnt signaling, another embryonic signaling pathway, has also been implicated in the regulating of pancreatic progenitor proliferation as well as β-cell and acinar cell replication (53–57).

Additional signals guiding islet formation and maturation are provided by signaling molecules derived from blood vessels and capillaries. In return, vascular endodermal growth factor and insulin secreted by endocrine cells regulate aggregation of endodermal cells during islet angiogenesis (28). These coregulatory responses ensure tight contact between endocrine cells and capillaries, connections that are critical for adult islet activity (28). Furthermore, vascular endothelia also regulate insulin gene expression and β-cell proliferation through laminin and laminin receptor interaction (58), thus maintaining and supporting adult islet function (Fig. 2).

2. Transcription factors as markers for distinct pancreatic cell populations. External signaling inputs culminate in changes in transcription factor networks that guide pancreas development. Current evidence supports the notion that the temporal changes in external and internal signals result in the formation of a transcriptional regulatory cascade that promotes step-wise progression from uncommitted progenitors toward specified endocrine precursors and, finally, fully differentiated β-cells. Although the descriptions of these transcriptional cascades are beyond the scope of this review, thorough and in-depth discussion about embryonic signaling pathways and transcription factors can be found elsewhere (reviewed in Refs. 34, 59, and 60). Some transcription factors important for in vivo and in vitro β-cell development are briefly summarized below.

Two of the transcription factors expressed at the earliest stages of pancreas development are pancreatic and duodenal homeobox 1 (Pdx1) and pancreas transcription factor 1a (Ptf1a). Both proteins are expressed in pancreatic progenitor cells and are important for pancreatic lineage specification (61–64). Pdx1, the homolog of the human insulin-promoter factor, is required for the development of pancreatic progenitor cells in mouse (65, 66) and human (67, 68). Although Ptf1a is somewhat dispensable for the specification of endocrine progenitors (69), the binding of Ptf1a to the Pdx1 promoter indicates a role in the maintenance of Pdx1 expression (70, 71) crucial for pancreatic growth, specification, and maturation of β-cells (72). In the dorsal pancreatic bud, signals from aortic endothelium also induce Ptf1a expression and subsequently sustain the expression of Pdx1 (18, 49). In addition, numerous studies have identified a number of additional transcription factors that are present in the pancreatic progenitor cells, including Nkx6 homeobox 1 and 2 (Nkx6.1 and Nkx6.2), Nkx2 homeobox 2 (Nkx2.2), homeobox b9 (Hbx9), and SRY homeobox 9 (Sox9) (22, 73–77). Thus, a large set of genes can be used to identify pancreatic progenitor cells during embryogenesis.

The subsequent step in pancreatic development requires the specification of endocrine progenitors from the uncommitted pancreatic progenitor cells. Endocrine differentiation is regulated by the opposing effects of Notch signaling that promote expansion of undifferentiated progenitors as well as differentiation of exocrine cells via expression of the hairy/ enhancer-of-split 1 (Hes1) transcription factor and the activity of neurogenin 3 (Ngn3), a basic-helix-loop-helix family transcription factor signaling that is essential for endocrine formation. Notch signaling in nonpermissive cells activates
Hes1, which blocks expression of Ngn3 through direct binding to the Ngn3 promoter (78, 79). In contrast, Ngn3 is transiently activated in the developing pancreas during embryogenesis (61, 80), in cells coexpressing Hnf6 and Sox9 (76, 81, 82), and its function is both necessary and sufficient for the differentiation of all endocrine cell lineages (80, 83, 84). Ngn3 activates NeuroD1, another basic-helix-loop-helix transcription factor initially expressed in cells undergoing endoderm differentiation and later confined to mature islets in which it plays a crucial role in endocrine cell survival as well as insulin gene transcription (85–88).

Expression of Ngn3 is required for the development of all islet cells (80), whereas the specification and maturation of insulin-secreting β-cells requires additional transcription factors, including Pax4, Nkx2.2, Nkx6.1, and MafB (89–91) as well as sustained Pdx1 expression (92, 93). After Pdx1 expression, basic leucine zipper transcription factor MafB, which is expressed in more than 90% of insulin-producing cells in the developing pancreas, is switched off, and another Maf factor, MafA, is turned on in the β-cell lineage (94). Many genes required at these late stages of β-cell differentiation are also indispensable for the function of mature β-cells, including glucose sensing, insulin synthesis and secretion, as well as the replication/survival of the β-cell population after birth (94–100).

In summary, studies from numerous laboratories have identified a hierarchy of secreted signals and transcription factors that guide cell differentiation in endoderm and pancreas. This knowledge provides a blueprint for pancreatic β-cell differentiation that has been exploited in efforts to direct differentiation of hESCs into β-cells.

III. Embryonic Stem Cells as Renewable Source of Functional β-Cells

The success of islet transplantation with the Edmonton protocol has provided a promising new therapeutic option for diabetic patients with significant β-cell loss, especially those with “brittle” type 1 diabetes in which the patients suffer from severe consequences caused by hypoglycemia unawareness (6, 101). However, the current shortage of cadaveric islets significantly impairs the employment of this treatment option. To circumvent this problem, researchers have started to explore new strategies in a search for a renewable source of high-quality β-cells for transplantation. In recent years, significant progress was made in differentiating hESCs toward pancreatic lineages, including endocrine and insulin-producing cells. In this section, we will review the most recent advances in this fast-moving field.

A. In vitro differentiation of human embryonic stem cells

ESCs are cells derived from the inner cell mass of preimplantation blastocysts (9, 10, 102). The most important characteristics of ESCs include the capacity to self-renew and the potential to differentiate into all embryonic cell types, a potential termed pluripotency, under in vivo and in vitro conditions (103). Given this capacity for pluripotency, great interest exists in exploring guided ESC differentiation into any desired cell types in vitro for cell replacement therapies to treat degenerative diseases, including diabetes mellitus.

Another advantage of ESCs is their ability to maintain their stem cell properties upon proliferation under certain cell culture conditions, thus allowing almost unlimited expansion without compromise of their differentiation capacity. Given the need to generate large cell numbers for therapeutic purposes, this property favors ESCs over cells in a more differentiated state that in general have a quite limited proliferation capacity. However, directed differentiation of ESCs toward specific cell lineages has proven to be a challenging task. This is in part because directed induction of a desired cell type has to be balanced with the inhibition of the formation of unwanted cell fates. Thus, to generate a specific cell type successfully, such as insulin-producing β-cells, protocols have been established that simulate the differentiation steps that occur during normal embryonic development. These protocols have been based on the findings obtained from decades of developmental biology research (summarized in Section II) that has outlined a cascade of signaling molecules and transcription factors that guide undifferentiated progenitor cells toward fully mature, functional cells. Here, we will review strategies that have been applied to the derivation of insulin-producing cells from ESCs.

1. Early attempts for in vitro β-cell differentiation. After the isolation of mammalian ESCs and establishment of in vitro culture conditions that allow them to maintain an undifferentiated state, numerous approaches have been used to generate insulin-producing β-cells from hESCs. Although many reported the generation of cells with some degree of insulin production from mouse (104, 105), monkey (106), and hESCs (107, 108), none of the studies has affirmed the in vitro production of fully functional β-cells that can secrete physiologically sufficient amounts of insulin in response to glucose. One of the reasons for this failure is the fact that although pancreatic β-cells are the main source of insulin production in mammals, they are not the only cell type that can synthesize and release insulin. Other insulin-producing cells can be found in yolk sac, fetal liver, and certain neuronal cell types (109–111). In addition to insulin, these extrapancreatic cell types express several other genes in common with true β-cells, and a β-cell type specific profile has therefore proven difficult to define. This is of consequence as ESC-derived, β-like cells usually produce insulin at much lower levels compared with true β-cells, and the machinery for regulated, high-level insulin secretion based on physiological conditions is not present.

Many early attempts to generate β-cells from ESCs were focused on the selection of cells positive for nestin (104, 112, 113), an intermediate filament protein that served as a marker for stem/progenitor cell populations in other tissues (114, 115). Later differentiation protocols have shown that the selection of nestin-positive cells likely leads to the generation of neuronal cell types, which agrees with the hypothesis that nestin is a marker for neural progenitors and pancreatic exocrine progenitors but does not mark endocrine progenitor cells (114, 116, 117).

In addition, early studies were hampered by the fact that insulin immunohistochemistry was used as a standard...
marker to identify β-cells, a strategy that turned out to give misleading results because differentiating ESCs and apoptotic cells can take up insulin from the culture medium (118, 119). More strict criteria were instituted to identify the mature β-cell phenotype. Lineage-specific gene markers are now routinely used to confirm the differentiation of ESCs first into definitive endoderm (DE) and subsequently pancreatic cell types (120, 121). Terminally differentiated cells are examined for insulin synthesis and release through the measurement of C-peptide, a cleavage product that is generated during processing of proinsulin into mature insulin (119).

Lessons from the unsuccessful attempts to generate β-cells have underscored the importance of understanding biological problems in their physiological context. Therefore, strategies were developed that more closely followed the differentiation steps found during development of β-cells during embryogenesis.

2. Directed differentiation of hESCs: lessons from in vivo pancreatic development. In 2001, Assady et al. (107) reported that insulin-secreting cells can be generated from spontaneous differentiation using hESCs. Although the number of insulin-producing cells and the insulin content in these cells was low, it was the first proof-of-principle experiment showing that hESCs were a potential source for generating β-like cells. However, the real breakthrough in our efforts to generate fully differentiated β-cells came from subsequent studies that used the signals that regulate embryonic endoderm and pancreas formation. As discussed in Section II, embryonic pancreas development is a complex process that requires the sequential activation of several signaling pathways. Because these signals had been identified in prior efforts, it became possible to mimic the in vivo orchestra of events through a multistep protocol that promotes differentiation of ESCs through the normal developmental stages. In the past few years, many groups have reported the generation of β-like cells using numerous protocols. The recent reports from D’Amour et al. (122) and Kroon et al. (123) represent the most successful attempts for the differentiation of pancreatic lineage in vitro and will be discussed in detail below in this section (Fig. 1, lower panel).

The first crucial step in the differentiation of hESCs toward the pancreatic lineage is the specification of DE. Because hESC differentiation is nonreversible under normal culture conditions, efficient DE formation is important for the subsequent induction toward the pancreatic cell fate. hESCs that do not assume the endodermal fate are unlikely to generate pancreatic progenitors and subsequently hormone-producing β-cells. In addition, nonendodermal cells may secrete signals that can interfere with the progression of DE cells toward pancreas fate. Previous studies had implicated TGF-β and Wnt signals as critical signals that induce DE formation in vitro (124–128). Thus, activin A, a member of the TGF-β family, was used at high concentration in combination with Wnt5a to effectively induce DE in vitro (122, 123, 129, 130). Further studies showed that serum components essential for maintenance of ESC survival and self-renewal contain activators of the phosphatidylinositol 3-kinase pathway that inhibit the induction of DE (131). As a consequence, recent protocols require the removal of serum during the initial differentiation step (122, 123, 129).

It is important to note that the efficiency of DE formation can be assessed by comparing quantitative PCR and immunohistochemical staining patterns of transcription factors to those found in a developing embryo. For example, hESCs respond to the initial activin treatment by activating markers of mesendoderm, a transient tissue that gives rise to both the mesodermal and endodermal germ layers. Expression of the gene BRACHYURY/T, a marker of this cell lineage, spikes rapidly upon activin treatment and then quickly decreases when cells assume endodermal properties. The formation of DE can be confirmed by testing for the up-regulation of endodermal markers, including SOX17, FOXA2, and GATA4 (122, 123, 129, 132). Importantly, it is necessary to control for the absence of genes like SOX7 expressed in primitive endoderm, parietal and visceral endoderm, tissues that are quite similar to DE but do not give rise to the organs of the gastrointestinal tract during embryogenesis (129). Fluorescence-activated cell sorting provides additional tools for purification of DE cells due to the expression of cytokine receptor CXCR4 in these cells (129, 132). Thus, controlled differentiation of ESCs into DE can be achieved via combined treatment with activin and Wnt5a and monitored via the expression profile of an array of cell lineage-specific markers.

During embryogenesis, DE is partitioned in subsequent steps along the anterior-posterior axis into defined regions that eventually will give rise to epithelial-derived organs, including liver, lung, intestine, and pancreas (reviewed in Ref. 17). Fgf signaling has been shown to regulate the specification of the gut-derived organs and Fgf proteins were added to the differentiation media upon the removal of activin A for the induction of foregut endoderm from DE (122, 123). Fgf treatment results in the continuous expression of FOXA2, a marker for DE, and the induction of transcription factors HNF4α and HNF1β, genes generally expressed in the developing gut tube (133–136).

The next desired step after the formation of primitive gut endoderm is the induction of pancreatic epithelium. Suppression of the activity of Shh, a member of the Hedgehog signaling pathway, is required for the initial specification of pancreatic buds in vivo (39). Hedgehog inhibition can be mimicked in cell culture via treatment with cyclopamine, a cholesterol analog known to block the function of Smoothen (137, 138), an essential element of the Hedgehog signaling cascade. Addition of cyclopamine to the differentiation media at this stage promotes the formation of pancreatic endoderm marked by the expression of PDX1, the earliest marker of pancreatic epithelium, among a collection of other transcription factors important for pancreas organogenesis (122, 123). Retinoic acid is added to promote the commitment of the PDX1-positive pancreatic progenitors toward the endocrine lineage over pancreatic exocrine cells (122, 123, 139). At the end of this stage, expression of NGN3, a transcription factor essential for endocrine cell differentiation, is significantly up-regulated.

During embryogenesis, NGN3-positive endocrine progenitors give rise to five different endocrine cell types located within the islets of Langerhans. Although cells expressing endocrine hormones have been generated under cell culture
conditions, current analysis indicates that these cells have not reached the full maturation stage found in adult human islets. Immunostaining with antibodies against multiple hormones showed that insulin-producing cells coexpress other hormones, such as glucagon and somatostatin. The percentages of insulin-positive cells were also relatively low (around 7% of total cell population), possibly due to the lack of the second wave of PDX1 expression during the final stage of differentiation that is present in embryonic endocrine cells. PDX1 is not only important for β-cell differentiation but is also required for optimal β-cell function, and its lack might explain in part the marginal response to glucose stimulation. In addition, expression of MAFA, a transcription factor normally up-regulated during the final stages of β-cell formation, is not observed. All these data indicate that insulin-producing cells differentiated in vitro have not progressed to fully matured, functional β-cells (122).

Interestingly, ESC-derived endocrine precursors that give rise to multihormone positive cells observed under culture conditions do carry the ability to form functional endocrine cells, including β-cells, when given the appropriate stimuli. Final differentiation of β-cells and other hormone-secreting cells was achieved when endocrine precursors were transplanted into immunocompromised severe combined immunodeficiency mice, suggesting that as of yet unidentified signals or cell-cell interactions are required to promote final endocrine differentiation (123). Importantly, implantation of hESC-derived endocrine precursors resulted in insulin-positive cells capable of rescuing hyperglycemia induced by streptozotocin, a compound that elicits apoptosis in murine but not human β-cells. Removal of the transplanted cells caused recurrence of the diabetic phenotype, further supporting the hypothesis that restoration of normoglycemia was dependent on hESC-derived cells (123). These studies underscore the notion that hESCs carry the ability to develop into fully differentiated cells, including endocrine cells of the pancreas.

3. Generation of insulin-secreting cells through nuclear reprogramming. As mentioned in the previous section, β-cell specification and differentiation depends on a battery of transcription factors that function in an exquisitely coordinated temporal and spatial manner in the developing pancreas. In the differentiating ESCs, this process can be mimicked by adding growth factors that induce expression of the right combination of transcription factors. A more direct genetic engineering approach, nuclear reprogramming, has also been examined by constitutively expressing critical transcription factors in ESCs (112, 140–142). Early reports have shown that more robust pancreatic differentiation could be achieved when ESCs express high levels of exogenous Pdx1, Ngn3, and Pax4, although no functional β-cells were generated from these protocols (112, 140–142). Although direct expression of transcription factors can guide cell differentiation, it is important to point out that both the level and duration of these transcriptional signals is precisely controlled during in vivo pancreatic differentiation. Forced expression of these factors at an inappropriate time or at non-physiological levels leads to alterations of normal ESC properties or changes in cell fate choice during differentiation. For example, prolonged overexpression of Pdx1 failed to induce the specification of insulin-secreting cells despite enhanced differentiation toward pancreatic lineages in general (140). Similarly, precocious expression of Ngn3 during pancreas development results in the predominant formation of α-cells at the expense of other endocrine cell types (84, 143).

In addition, many transcription factors important for pancreatic development are also involved in neuronal differentiation. Thus, overexpression of these factors, such as Ngn3 and Pax4, at inappropriate time points may lead to the generation of nonpancreatic, neuronal cell types that display some but not all characteristics of pancreatic cells. In this regard, it is unclear whether the insulin-secreting cells produced by this method are of endodermal origin or whether they represent cell types from the ectoderm. Recently, Bernardo et al. (144) used a more sophisticated system to temporally regulate the expression of Pdx1 in differentiating mouse and hESCs to mimic the biphasic expression of Pdx1 in normal pancreas development. With the induction of the second wave of Pdx1 expression in late differentiation stages, an increase in β-cell numbers was achieved. However, unlike β-cells in mature islets, these insulin-secreting cells express MafB instead of Mafa. Furthermore, the cells also did not respond to high glucose stimulation, suggesting that full activation of the β-cell state has not been achieved and might require the exogenous expression of other factors (144).

B. Remaining challenges

The promising outcome of recent islet transplantation efforts in human patients has opened the door for novel treatment options for patients suffering from diabetes mellitus. However, cell-based therapeutics are still in the experimental phase, and many hurdles remain before they can be applied as standard treatments. Some of the risks and side effects, including ischemic and enzymatic damage caused by the islet isolation and purification protocol as well as the concerns of thrombosis and portal hypertension induced by transplanting islets into the liver portal vein, are associated with the islet transplantation procedure itself (reviewed in Refs. 145 and 146). Herein, we will discuss the obstacles that challenge the application of ESC-derived cells for transplantation purposes.

1. Risk of cancer formation upon transplantation of hESC-derived cells. Besides the controversial ethical issues that hamper the derivation of hESCs, stem cell-based therapies need to be cautiously tested in animal models before they can be employed in human patients. One of the major problems concerns the persistence of undifferentiated ESCs competent to cause tumor formation after cell transplantation (147, 148). Teratomas, the most likely tumor type expected to form upon hESC-derived cell transplantation, have been observed when a population of cells enriched in pancreatic progenitors was grafted into mice (123). Teratomas are benign tumors that are likely to arise from pluripotent stem cells that have remained in the mixed population and they contain cells that have differentiated into tissues from multiple germ layers (123). One obvious way to avoid the risk of tumor formation is to transplant a purified, homogenous population of partially or
fully differentiated cells generated from hESCs. Such an approach would require the development of cell type-specific cell surface markers present on the endocrine progenitor or fully differentiated β-cells. Fluorescence-activated cell sorting and magnetic-activated cell sorting could then be used to purify transplantation-quality cell populations with high efficiency.

2. Full maturation of β-cells in vitro. As of today, many groups have reported the generation of insulin-secreting cells from hESCs in vitro; however, these cells likely correspond to immature β-cells because they present with low insulin content, express multiple hormones in the same cell, or show very little response to glucose stimulation (122, 123, 149). A possible explanation as to why fully differentiated β-cells have so far not been generated under cell culture conditions might come from the absence of cell-cell interactions between mesenchymal and epithelial cells that occur during embryonic pancreas development in vivo. Pancreas organogenesis is an interactive process in which tissues from different germ layers secrete and respond to inductive signals (see Refs. 18, 28, 31, and 32; for review, see Ref. 150). In contrast, in the most recent in vitro differentiation protocol, cells are grown in two-dimensional cultures as monolayers. Thus, three-dimensional interactions between epithelial cells as they occur during islet formation in vivo are lacking. In addition, hESCs differentiate into DE and posterior gut cells with high efficiency (122, 123), and this relatively pure cell population may lack signals secreted from mesodermal or ectodermal cells necessary for successful differentiation of pancreatic lineages during the final stages of endocrine cell differentiation. Thus, a coculture system with pancreatic mesenchyme or endothelial cells may improve the efficiency of β-cell specification and maturation. Screens for genes differentially expressed in pancreatic mesenchyme might also provide cues for additional factors to be included in the differentiation media.

A major difference between β-cells in the pancreas and the β-cells generated in vitro lies in the different environment they reside in. Pancreatic islets are complex structures that consist of multiple cell types, including different hormone-secreting cells, neuronal cells, and vascular endothelial cells. This three-dimensional structure that provides interactions between β-cells and endothelial cells, as well as the interactions among β-cells themselves and cells of the other endocrine lineages, appear to be critical for insulin sensing and secretion (151, 152). Provision of in vitro-generated β-cells with a three-dimensional scaffold loaded with extracellular matrix promoting β-cell aggregation and angiogenesis may facilitate β-cell maturation and improve β-cell function upon transplantation into patients.

A novel strategy for β-cell generation is inspired by the success of nuclear reprogramming. Introducing critical transcription factors into acinar cells can convert exocrine cells into insulin-secreting cells (153). Thus, it should be possible to reinforce the normal differentiation from hESCs to endocrine cells through expression of “master” genes that drive β-cell differentiation. Transient transfection of different transcription factor combinations can be used to avoid permanent genetic modification for future clinical use. Pancreatic progenitor and endocrine precursor cells, both of which can be generated via directed in vitro differentiation of hESCs, should also be tested because they might be more amenable to gene delivery than hESCs and may also provide a more suitable genetic “context” for β-cell maturation.

Alternatively, small chemical compounds have been shown to direct cell differentiation in the absence of permanent genetic changes (154). Considering the increasing number of libraries containing diverse chemical compounds and the technical advances that allow rapid screening of these libraries, identification of molecules that guide specific steps during hESC to β-cell differentiation has become feasible (155, 156).

3. Transplantation-related immune rejection. A final concern that needs to be addressed before widespread transplantation of hESC-derived endocrine cells can become a reality is how to circumvent the recipient’s immune response. There are two types of immune responses that need to be resolved with regard to islet transplantation. In type 1 diabetes patients, β-cells are destroyed by an autoimmune immune response, and the suppression of autoimmunity is crucial for the success of islet transplantation-based therapy. Improved immunosuppression regimens are tested and continuously optimized in animal models and clinical trials (6, 158), recent breakthroughs in cellular reprogramming might present alternatives that would alleviate or completely abrogate the allograft reaction. Studies have demonstrated that a cocktail of only four different transcription factors is sufficient to reprogram somatic cells into induced pluripotent stem (iPS) cells. The iPS cells are similar to hESCs and can differentiate into cells of all three germ layers, suggesting that patient-specific stem cell populations may become a reality (159–162). In support of this notion, iPS cells have recently been generated from cohorts of human patients suffering from a variety of diseases, including type 1 diabetes (163). Although the first-generation iPS cells were generated via transfection with lentiviruses or retroviruses and some of the transcription factors used for reprogramming are known oncogenes, recent studies indicate that some of the factors can be substituted for by small chemicals or histone deacetylase inhibitors (164, 165), suggesting the possibility of eventually generating iPS lines safe for transplantation into human patients. Most recently, a virus-free reprogramming strategy has been developed, which marks another milestone toward the full exploration of iPS cell-based therapies in clinics (166, 167). Ongoing studies in several laboratories will address the question of whether iPS cells can respond to the current hESC differentiation protocol and whether iPS-derived β-cells will be fully functional.

IV. Additional Approaches to Expand β-Cell Mass in Adult Pancreas

Unlike skin, intestine, hair, and other adult organs that show great dynamic responses in tissue homeostasis (with fast turnover of old cells and rapid replacement with new cells), the pancreas has a relatively quiescent cell population.
Although changes in signaling pathways such as Wnt regulate its growth to some extent (54), pancreatic organ mass is mainly determined by the size of the progenitor cell pool early during embryogenesis (24). β-Cells in particular are known to have a low proliferation rate due to tight cell cycle control (168). However, recent studies have shown that β-cell mass is indeed dynamically regulated in adult vertebrates. Several physiological conditions are known to significantly increase β-cell mass, including obesity and pregnancy (169–173). Currently, an ongoing debate concerns the extent of neogenesis from non-β-cells vs. proliferation of existing β-cells. Although evidence for the reactivation of the endocrine differentiation program from cells located in pancreatic ducts exists (174–176), cell-lineage tracing experiments have confirmed that new β-cells are mainly generated from proliferation of preexisting β-cells (177, 178). However, during pancreas regeneration after injury, cells lining the ducts can turn on Ngn3, a transiently expressed pancreatic progenitor marker in embryonic pancreas, and differentiate into hormone-secreting cells including β-cells (179, 180). These data point to the plasticity of adult pancreatic cells with regard to their differentiation potential, a capacity that eventually could be used to generate functional β-cells. The most recent reports concerning β-cell neogenesis from pancreatic progenitors, acinar and ductal cell transdifferentiation, and hepatocyte transdifferentiation and transdifferentiation are summarized below.

Over the last few years increasing evidence has been obtained to support the existence of a long-debated pancreatic progenitor population located in the adult ductal epithelium that can be cultured and differentiated to insulin-expression cells in vitro (174, 179, 181–185). Overexpression of Ngn3 in human duct cells can further increase the percentage of insulin-producing cells (186, 187). On the other hand, fully differentiated nonendocrine cell types can convert to insulin-secreting endocrine cells. Several groups have shown that acinar and ductal cells can transdifferentiate into endocrine cells in vivo (188–191). The most recent breakthrough in this area is the direct conversion of exocrine cells into pancreatic β-cells by introducing Pdx1, Ngn3, and MafA, three transcription factors known to drive β-cell differentiation (153). This direct reprogramming does not seem to involve dedifferentiation of acini, nor does it require cell proliferation, although substantial evidence suggests that β-cells can also be generated from acinar cells through transdifferentiation after dedifferentiation (reviewed in Ref. 192).

Transdifferentiation between hepatocytes and endocrine cells also offers an attractive strategy for generating more insulin-secreting cells, considering that liver and pancreas share a common embryonic origin and both organs have a similar glucose sensing system (reviewed in Ref. 193). Ectopic expression of Pdx1 and Pdx4 led to transdifferentiation of mouse hepatic cells into insulin-positive cells that can revert hyperglycemia in streptozotocin-induced diabetic mice (194–199). Overexpression of Ngn3 or NeuroD have also been reported to induce liver to pancreas transdifferentiation (200, 201). However, the most recent report indicates that establishment of the full pancreatic phenotype is due to the transdifferentiation of hepatic stem cell rather than the transdifferentiation of mature hepatocytes (201).

It is important to point out that although β-cell replication and transdifferentiation/transdetermination could be exploited for β-cell expansion, there are several important issues that could limit the application of these methods. Ex vivo expansion of β-cells appears to be difficult because cells lose insulin expression over time, and culture conditions that permit replication of fully functional β-cells have not been identified. Promotion of endogenous β-cell replication with small molecules or biological signals has not been achieved, and target molecules would have to be tested with regard to their effect on other cell types within the body to exclude unwanted neoplastic effects. Transdifferentiation/transdetermination of pancreatic or hepatic nonendocrine cells into insulin-producing cells would need to result in fully differentiated cells that possess all characteristics of pancreatic β-cells, including the ability to adjust insulin secretion to physiological demand. Thorough studies need to be performed to “phenotype” the newly generated β-cells and compare their activities to endogenous, pancreatic β-cells. Thus, whereas certainly exciting, significant research efforts are needed to validate the promise of these novel strategies.

V. Conclusion and Future Perspective

The past 15 yr have seen great progress in our understanding of normal and pathogenic pancreas development. Today, cell replacement therapy for both type I and type II diabetic patients has become a promising scenario that could be achieved in the near future. The continuing advances in our understanding of pancreas development and regeneration, insulin gene regulation, stem cell differentiation and manipulation, immunosuppression regime, transplantation, and other related techniques will be instrumental in devising novel strategies for diabetes treatment. Finally, breakthroughs in the field of nuclear reprogramming and transdifferentiation carry great promise to provide “customized” diabetes cell therapy by generating patient-specific β-cells.

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