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Generation of β cells from human pluripotent stem cells: Are we there yet?

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Abstract

In 1998, the landmark paper describing the isolation and culture of human embryonic stem cells (ESCs) was published. Since that time, the main goal of many diabetes researchers has been to derive β cells from ESCs as a renewable cell-based therapy for the treatment of patients with diabetes. In working toward this goal, numerous protocols that attempt to recapitulate normal pancreatic development have been published that result in the formation of pancreatic cell types from human pluripotent cells. This review examines stem cell differentiation methods and places them within the context of pancreatic development. We additionally compare strategies that are currently being used to generate pancreatic cell types and contrast them with approaches that have been used to generate functional cell types in different lineages. In doing this, we aim to identify how new approaches might be used to improve yield and functionality of *in vitro*-derived pancreatic β cells as an eventual cell-based therapy for type 1 diabetes.

Keywords

pluripotent stem cell; β cell; differentiation; endoderm; pancreas; diabetes

Introduction

Isolated from the inner cell mass of the preimplantation embryo, embryonic stem cells (ESCs) are immortal, pluripotent cells that retain the capacity to differentiate into cell types of all three germ layers. The isolation of human embryonic stem cells (hESCs)¹ and their subsequently demonstrated ability to be differentiated toward specific cell fates² raised the possibility that hESC-derived cells could form a basis for future cell-based therapies. In 2007, the generation of human-induced pluripotent stem cells (PSCs)³—an alternate source of PSCs—opened up the possibility of isogenic cell-based therapies. These advances have generated an intense interest in identifying methods to direct PSC differentiation *in vitro*

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Conflicts of interest

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toward therapeutically relevant cell types, such as pancreatic β cells, which could potentially replace the use of cadaveric islets in the treatment of type 1 diabetes. This review examines progress that has been made toward the differentiation of PSCs toward pancreatic β cells. We discuss how this progress was only possible because of our knowledge of pancreas development and how additional knowledge in this area may yield the key for generating fully functional β cells from PSCs *in vitro*.

Developmental pathway of β cells

Successful protocols that promote differentiation of PSCs toward a β cell fate have attempted to recapitulate the signaling processes that function during embryonic pancreas development (Fig. 1). This is achieved by the addition of growth factors and/or inhibitors at specific times during PSC differentiation that are meant to approximate *in vivo* signaling events that guide β cell development. A summation of the critical stages of pancreas development and the growth factors/inhibitors that have been used in attempts to direct differentiation of hPSCs toward β cells is shown in Figure 2.

Formation of definitive endoderm

The first step in differentiating hPSCs toward pancreatic β cells is the formation of definitive endoderm. There is some evidence, both *in vitro*⁴ and *in vivo*,^{5,6} that definitive endoderm derives from a bipotential mesendoderm progenitor that is marked by the expression of the transcription factors Brachyury and Mixl1.^{7–9} Specification of the definitive endoderm lineage is accompanied by the upregulation of a number of transcription factors, most importantly, Goosecoid, Sox17, and FoxA2.^{10–13} This same progression of gene expression occurs during both mouse and human PSC differentiation into definitive endoderm.^{4,14,15}

In the majority of human PSC differentiation protocols, definitive endoderm formation is induced by treating cells with a high concentration of activin A—a transforming growth factor β (TGF- β) family member used to mimic the activity of Nodal. Nodal proteins play an evolutionarily conserved role in initiating gastrulation and in driving endoderm formation in all studied vertebrate species.¹⁶ In fish, frogs, and mammals, Nodal proteins act in a concentration-dependent fashion to promote formation of the mesoderm lineage at low concentrations and endoderm at high concentrations. The levels of Nodal signaling have also been linked to embryonic and germ layer patterning along the anterior–posterior axis such that high levels of Nodal promote anterior mesoderm and endoderm.¹⁷ Consistent with these studies in the embryo, the nodal mimetic activin A has distinct concentration-dependent effects. It was seen by Kubo *et al.* that different concentrations induce different developmental outcomes: high concentrations of activin A favored dorsal mesoderm and endoderm fates, whereas low concentrations of activin A favored more ventral mesoderm fates.¹⁸

Subsequent studies utilizing hESCs have demonstrated that PI3K signaling must be suppressed for cells to optimally respond to activin/Nodal.¹⁹ Compounds such as wortmannin, which inhibits PI3K signaling, have been found to promote definitive endoderm formation²⁰ and have been used in combination with activin A to induce definitive endoderm from hESCs. To increase the robustness of differentiation and reduce

costs, researchers have sought to discover small molecule alternatives that have the ability to direct hPSC differentiation into definitive endoderm. Two such molecules, IDE1 and IDE2, have been identified and have been shown to be able to induce definitive endoderm formation (in the presence of serum) with similar efficiencies to activin A.²¹ The specific target molecule for these compounds has not been identified, though experiments indicate that activation of TGF- β signaling may be involved.²¹

Other signaling pathways appear to modify the activity of activin A during the definitive endoderm induction step. In the embryo, these signaling pathways function during gastrulation and act downstream of Nodal. One example is the TGF- β superfamily molecule, BMP4, which is expressed in the posterior primitive streak.²² Mouse embryos lacking BMP4 fail to express genes associated with mesoderm formation, such as *Brachyury*, and die during gastrulation (at approximately embryonic day (E) 6.5). A number of studies have reported that the inclusion of low concentrations of BMP4 along with activin A on the first day of differentiation improves the efficiency of definitive endoderm formation.^{23–26} Other signaling pathways that are essential for gastrulation have been shown to play a role in activin-induced definitive endoderm formation. For example, Wnt signaling is essential for vertebrate gastrulation and mouse embryos lacking Wnt3 fail to gastrulate.²⁷ If canonical Wnt signaling is blocked during ESC differentiation, mesendoderm fails to form,^{28,29} whereas activation of canonical Wnt signaling enhances differentiation of hESCs toward definitive endoderm.³⁰

Formation of posterior foregut and pancreatic endoderm from definitive endoderm

In the vertebrate embryo, a number of molecular and morphogenic changes take place between the initial formation of definitive endoderm and emergence of Pdx1-expressing pancreatic endoderm. The definitive endoderm transitions from a two-dimensional sheet into a primitive gut tube that becomes patterned along the anterior–posterior and dorsal–ventral axes. During gut tube morphogenesis, the presumptive pancreatic endoderm comes in contact with mesodermal cell types including the septum transversum mesenchyme (ventral) and notochord (dorsal).³¹

These mesodermal cell types are the source of several signaling molecules that regulate the formation of Pdx1-expressing endoderm, including fibroblast growth factor (FGF), retinoic acid (RA), and hedgehog. FGF signaling is involved in many stages of pancreas development, including global patterning of the endoderm along the anterior–posterior axis as well as posterior foregut patterning. In chick and mouse, low levels of FGF signaling promote posterior foregut fate and expression of Pdx1, whereas higher levels promote more posterior endoderm fates.^{32–34}

The notochord, which is located adjacent to the dorsal endoderm until around E8.0, is an important source of several of these signaling molecules and has been shown to play an essential role in dorsal pancreatic development. Removal of the notochord in chick embryos before pancreatic bud formation resulted in failure to initiate dorsal pancreas development, though ventral pancreas development was unaffected.³⁵ Further studies found that signaling molecules from notochord, including activin β B and FGF2, were able to repress the expression of sonic hedgehog (Shh) in the adjacent prepancreatic endoderm,³⁶ though this

was inhibited in the presence of exogenous Shh, suggesting that Shh acts as an antipancreatic factor.³⁶

RA signaling plays an essential role in the morphogenesis and organogenesis of numerous foregut organs, including the pancreas.³⁷ The distribution of RA in the embryo is tightly controlled by synthesis from circulating retinol in a two-step reaction involving specific retinol and retinaldehyde dehydrogenases (RALDHs),³⁸ as well as by degradation by specific cytochrome P450s (CYP26s).³⁷ A variety of gain and loss studies indicate that retinoid signaling is required for pancreatic specification in a number of species.^{38–41} For example, in zebrafish, it has been shown that retinoid signaling is essential for both pancreas and liver specification, and that exogenous RA treatment induces ectopic expression of pancreatic and liver markers.³⁹ In both *Xenopus*⁴¹ and quail,⁴⁰ it has been shown that retinoid signaling is essential for the formation of the dorsal pancreas, though it has no effect on ventral pancreas genesis. Further studies in *Xenopus* have demonstrated that while RA was sufficient to induce pancreas-specific genes in the dorsal pancreas, it was unable to induce these same genes in the ventral pancreas.⁴² Furthermore, in mice, loss of RALDH activity results in broad foregut organ abnormalities, including dorsal pancreas agenesis. Moreover, it was shown that RA signaling was sufficient to induce Pdx1 expression in the anterior endoderm.^{38,43}

Following from these developmental studies, nearly every published ESC differentiation protocol requires the addition of exogenous RA and/or FGF to promote the transition of definitive endoderm to Pdx1⁺ endoderm in mouse^{44–46} and human (Fig. 2).^{23,25,47–53} Although most protocols use FGF and/or RA, the interpretation of how these pathways promote expression of Pdx1 expression remains poorly understood.

Pancreatic endoderm to endocrine precursor cells

The commitment of pancreatic endoderm to endocrine precursor cells is an obligate step during the formation of β cells, though most differentiation protocols do not incorporate factors specifically designed to promote or enhance this process. This is due in part to a paucity of information about the signaling pathways that initiate endocrine cell formation and guide subsequent differentiation into the β cell lineage. There is a significant amount of information about the transcription factors that regulate endocrine and β cell fate *in vivo*,⁵⁴ and these factors serve as useful markers to identify cell types in ESC-derived pancreatic cultures. For example, the transcription factor neurogenin 3 (Neurog3) has been an important marker because it is expressed early in the specification of the pancreatic endocrine lineage.⁵⁵ However, as it is only expressed transiently and marks all endocrine cells, this makes it problematic to use as a marker to identify cells committing to a β cell fate.

In the mouse, Neurog3 has a bimodal expression pattern, which is thought to mark the first and second waves of endocrine cell formation. The first wave of Neurog3 expression is between E8.5 and E11.5, and the second wave of expression is initiated at E12, with peak expression observed at E15.5.^{56,57} Studies in both chick and mouse have shown that Neurog3 is involved in the epithelial–mesenchymal transition⁵⁸ that is associated with endocrine cell development (Fig. 1), during which delaminating endocrine cells are marked

by the expression of EphB3.⁵⁹ It is thought that only endocrine cells specified during the secondary transition (E12 onwards) form definitive single-hormone cells in the adult pancreas,⁶⁰ while endocrine cells formed during the primary transition (E8.5 through E11.5) do not contribute to the adult pancreas, instead playing a role during embryonic pancreatic function.⁶¹ This paradigm, however, is not supported by all studies of pancreatic cell development, some of which have demonstrated that a small number of endocrine progenitor cells formed before the primary transition are able to form hormone cells during the secondary transition, and hence, can contribute to the adult pancreas.⁵⁶

Perhaps the best-studied developmental signaling pathway involved in controlling endocrine precursor formation is Notch signaling. In a number of reports, it has been found that impaired Notch receptor activation or signaling resulted in upregulation of Neurog3 expression, leading to premature endocrine cell differentiation at the expense of both pancreatic progenitor cell expansion and exocrine cell differentiation.^{62–68} In contrast, active Notch signaling appears to maintain cells as undifferentiated progenitors that are able to contribute to both proliferation and later differentiation. In this sense, the function of Notch signaling during endocrine precursor specification is analogous to its function during early mammalian neurogenesis.^{69,70} Despite a clear role for Notch signaling in repressing endocrine cell fate *in vivo*, relatively few protocols utilize Notch inhibitors such as DAPT during ESC differentiation (Fig. 2). This suggests that the endogenous Notch signaling levels in these cultures is not inhibitory to endocrine differentiation.

FGF10 produced by the surrounding pancreatic mesenchyme is also involved in pancreatic progenitor cell proliferation and acts upstream of Notch signaling. The role of FGF10 was initially identified in mouse knockout studies, where *FGF10*^{−/−} mice exhibit severe pancreatic hypoplasia, owing to a profound reduction in the proliferation of pancreatic epithelial progenitor cells.⁷¹ By contrast, in transgenic mice that overexpress FGF10, pancreatic hyperplasia is seen, which results from the decreased differentiation of endocrine progenitor cells and concurrent expansion of progenitor cell numbers.^{72,73} This proliferative effect of FGF10 has also been observed *in vitro* in both isolated mouse⁷⁴ and rat⁷⁵ pancreatic epithelia. A study using isolated E10.5 mouse pancreatic epithelia found that the exogenous FGF10 increased the proliferation of pancreatic progenitor cells and promoted the expression of Hes1, a downstream target of Notch signaling. Moreover, in FGF10-treated epithelium, inhibition of Notch signaling caused the loss of Hes1 expression and a decrease in pancreatic progenitor cell proliferation, suggesting that Notch is downstream of FGF10.⁷⁴

Other signaling ligands that are secreted by the pancreatic mesenchyme also play a role in endocrine cell development. For example, mesenchyme-derived TGF- β signals were shown to be involved in regulating cell fate choice between endocrine and exocrine lineages.^{76–78} There was, however, some degree of uncertainty as to which lineages TGF- β signaling was affecting: endocrine, exocrine, or both lineages via a common progenitor cell. Recently, the TGF- β superfamily members, TGF- β 2 and TGF- β 3, have been identified to play key roles in controlling the balance between endocrine precursor proliferation and differentiation in hESC cultures. In their report, Guo *et al.* identified a set of signaling factors, including TGF- β ligands that are expressed by the pancreatic mesenchyme in the embryo and were able to

promote the expansion of hESC-derived pancreatic progenitors through a mechanism that is dependent on the duration of ligand signaling.⁷⁹ It was seen that while a short burst of TGF- β signaling promoted upregulation of both Neurog3 expression and endocrine differentiation, prolonged TGF- β signaling lead to expansion of the progenitor pool and attenuation of endocrine development.⁷⁹

During the formation of the pancreas in human embryogenesis, NEUROG3 expression is observed at week (W) 8 in development, where it is coexpressed with other pancreatic transcription factors such as *PDX1*, as well as hormones such as insulin and glucagon. As in the mouse, NEUROG3 expression levels diminish over time, though it was still observable at W21.⁸⁰ In contrast to Neurog3 expression patterns reported in the mouse, NEUROG3 expression has not been seen to be biphasic during human development.⁸¹ This may indicate that distinct primary and secondary transition events do not occur during human pancreatic development. There is, in fact, some debate about the functional necessity of NEUROG3 in human endocrine pancreas development. Several published reports describe patients with homozygous mutations in *NEUROG3*^{82,83} who were born with varying levels of circulating C-peptide, although one patient described had severely reduced C-peptide levels. Despite all patients' eventually developing diabetes, the fact that these patients often have detectable C-peptide suggests that human *NEUROG3* mutations may not result in complete loss of function, or that NEUROG3 is not required for embryonic specification of all β cells in humans.

Commitment of endocrine progenitor cells to β cells

The signals that control the transition from endocrine precursors into functional β cells are not yet defined. It is, therefore, not surprising that differentiation of PSC-derived pancreatic progenitors into mature, functional β cells *in vitro* has not yet been achieved. Historically, studies have used insulin expression to identify β cells; however, it is now clear from mouse experiments that early insulin-expressing cells do not go on to become definitive β cells. This was initially demonstrated in mice, using a Cre/loxP tagging strategy, in which the early endocrine cells were irreversibly tagged using the activity of Cre recombinase, allowing for cell-lineage tracing. This work showed that the definitive β cells did not arise from early endocrine cells that were specified during the primary transition, but instead arose from a Pdx1⁺ progenitor cell that had not previously expressed insulin.⁶¹ This work suggests that it may be prudent to use other markers in addition to insulin in order to identify PSC-derived β cells.

There have been multiple studies that describe the emergence of hormone-expressing cells during human pancreas development. One report found that insulin-expressing cells first appear at W8 before other hormone-expressing cell types, such as glucagon or somatostatin,⁸⁴ which were detected at W8.5, followed by pancreatic polypeptide (PP)-expressing cells at W10.⁸⁵ At these early stages, insulin-expressing cells coexpress glucagon at frequencies ranging from 10% to 92%^{84–86} and coexpress somatostatin at frequencies ranging from 10% to 97%.^{84,86} The existence of tri-hormonal (insulin/glucagon/somatostatin) cells has also been reported. In contrast, PP-expressing cells have only been

observed as monohormonal cells during human fetal development.⁸⁶ Polyhormonal cells were seen to decrease in frequency after W9⁸⁴ and were not detected in pancreata at W22.⁸⁷

The use of hormone (markers) as a readout of PSC-derived β cells has misdirected many researchers into focusing on primary transition insulin⁺ cells. As is observed during embryonic development, human PSC-derived insulin-expressing cells are generally polyhormonal, expressing glucagon and/or somatostatin. These *in vitro*-derived polyhormonal cells are functionally immature and lack the capacity for glucose-stimulated insulin secretion (GSIS),^{24,47} which is the key hallmark of a functional β cell. As yet, however, there have been no reports suggesting that polyhormonal endocrine cells give rise to functional monohormonal endocrine cells, which is consistent with lineage-tracing experiments previously performed in mice.

To better identify cell types that can give rise to mature β cells, the field has focused on transcription factors, including Nkx6.1, MafB, and MafA, which mark progenitors and maturing β cells *in vivo*. For example, Nkx6.1 is initially broadly expressed in the mouse pancreas at E10.5⁸⁸ but becomes restricted to the insulin-expressing cells and scattered ductal cells between E13 and E15.5. In the adult mouse, Nkx6.1 is exclusively seen in the β cells.⁸⁹ A similar pattern of NKX6.1 expression has been observed during human development, where NKX6.1 is broadly expressed in the pancreatic epithelium from W9, with subsequent downregulation of NKX6.1 expression in noninsulin-expressing cells by W13. Similar to the mouse, NKX6.1 expression is restricted to β cells in the adult human pancreas.⁹⁰ Loss-of-function studies in the mouse showed that Nkx6.1 is required for β cell specification⁸⁹ and *in vitro* overexpression studies suggest that it may improve β cell function,⁹¹ although this was not true *in vivo*.⁹²

There are now several reports examining the expression of NKX6.1 in differentiating hPSC cultures. Two early studies identified expression of NKX6.1 RNA⁴⁸ and protein⁴⁷ following the expression of earlier transcription factors such as PDX1; however, neither report demonstrated coexpression of NKX6.1 with other markers of β cell differentiation, such as insulin. More recently, NKX6.1 was observed to be coexpressed with C-peptide,²⁰ PDX1,^{20,52,93,94} or insulin.²⁵ However, these cells either demonstrated limited functionality²⁰ or functionality was not examined.^{25,93,94} It is therefore unclear if coexpression of NKX6.1 with either PDX1 or insulin will be a useful identifier of the functional β cell lineage. A recent study sorted differentiated hESCs on the basis of high (~80%) or low (~25%) NKX6.1 expression, then transplanted these different populations into diabetic mice. It was seen that while diabetes was reversed within 3 months in NKX6.1-high recipient mice, NKX6.1-low recipient mice remained hyperglycemic.⁹⁵ This would suggest that high NKX6.1 expression may be an indicator of the definitive β cell lineage.

MafA and MafB are transcription factors that are expressed in both the mouse and human pancreas in a temporospatially regulated fashion. In the mouse, MafB is first observed at E10.5 during the primary transition⁹⁶ in Neurog3⁺ progenitors and in insulin⁺ and glucagon⁺ cells. MafB expression then becomes restricted to glucagon⁺ cells (β cells) in the adult.^{96,97} In contrast, MafA expression is not observed until later in development—at E13.5 in the insulin⁺ cells specified during the secondary transition.⁹⁸ In addition to their different

temporal and spatial distributions, MafB and MafA play different functional roles during β cell differentiation. Surprisingly, only MafB is required for β cell development in the mouse — β cell numbers are not affected in mice lacking pancreatic *Mafa*. However, it is possible that MafB is able to compensate for the loss of MafA in this context.⁹⁷ In contrast, MafA has been shown to control the glucose-responsive transcription of insulin and other associated genes in the definitive β cells.^{99,100} *Mafa*^{-/-} mice have impaired glucose tolerance and defects in insulin secretion, emphasizing the importance of MafA in the maintenance of an appropriate GSIS response.¹⁰¹

Interestingly, the expression pattern of MAFA and MAFB differ in the human, both spatially and temporally, as compared to the mouse. MAFA has been seen to be expressed throughout the developing pancreatic epithelium, including in the nascent endocrine cells from W9.⁹⁰ In insulin⁺ cells, a strong nuclear expression of MAFA was seen, in contrast to weaker expression throughout the remaining epithelium. From W13 onwards, MAFA expression was downregulated, with a low level of expression restricted to the insulin⁺ cells, which was followed by complete loss of expression by W21. However in the adult, nuclear-localized MAFA expression was observed in β cells, similar to that seen in mice,⁹⁰ suggesting possible biphasic expression of MAFA in humans. A second study found that *MAFA* transcripts increased from W9 through to W23, albeit at levels lower than those observed in the adult.¹⁰² The expression of MAFB initially resembled that of MAFA, in that at W9, expression is seen in both the pancreatic epithelium and in insulin⁺ cells. However, by W14, expression of MAFB is seen to be restricted to both insulin⁺ and glucagon⁺ cells. This spatial patterning is maintained throughout development, with expression of MAFB in both the β and β cells of the adult.⁹⁰ This differs from the mouse, where MafB expression has not been observed in the β cells of the adult islet.⁹⁶

Other tissues important for β cell development

Many tissues are known to play a role in controlling β cell development (Fig. 3). Earlier, we have discussed the role of both notochord-and mesenchyme-derived signals. The signaling molecules from these tissues have been at least partially identified. In contrast, it has been observed that both neural crest cells and endothelial cells play important roles in β cell development, though the signaling molecules derived from these tissues have not been well characterized.

Neural crest

Neural crest cells derive from the dorsal neural tube, where they migrate ventrally and infiltrate the foregut mesenchyme at ~E10.0. Neural crest cells play an important role in control of progenitor cell replication and β cell maturation.¹⁰³ A study utilizing mice where *FoxD3* was specifically deleted in the neural crest cells resulted in a loss of neural crest cells and, interestingly, increased insulin⁺ cell proliferation but decreased β cell maturity.¹⁰⁴ This correlated with *in vitro* studies that identified a role for neural crest cells in β cell replication. A study in which isolated mouse islets were cocultured with neural crest stem cells found up to a 10-fold increase in β cell replication, as compared to β cells found in isolated islets.¹⁰⁵ To date, no study has reported the coculture of neural crest cells with human PSC-derived pancreatic endoderm or endocrine cells. Given the role that neural crest plays in β cell

maturation, this may be an approach that is able to influence maturity of human PSC-derived pancreatic cells *in vitro*.

Endothelium

The endothelium or vasculature plays multiple roles in controlling pancreatic and β cell development. Early in development, at around E8, fusion of the dorsal aortae displaces the notochord and the dorsal foregut. These aortic endothelial cells have been shown to induce pancreatic-bud structures as well as Pdx1 expression in the adjacent endoderm.¹⁰⁶ In *Xenopus* that were engineered to lack a dorsal aorta, there was a loss of pancreatic marker expression.¹⁰⁶ Interestingly, it was seen that recombination of prepancreatic endoderm with other tissues (such as mesenchyme or umbilical artery) that contained endothelial cells also allowed for the induction of pancreatic differentiation (as marked by the expression of insulin).¹⁰⁶ Further studies found that while the endothelial cells of the dorsal aorta were not required for the initial induction of Pdx1, they were, however, required for the maintenance of Pdx1 expression, as well as inducing expression of the pancreatic transcription factor Ptf1a in the dorsal pancreas. In contrast, ventrally located endothelial tissues are unnecessary for Pdx1 and Ptf1a expression in the ventral pancreas.¹⁰⁷

Work in the chicken has also suggested a role for endothelial signals early in pancreatic development. Before formation of the blood vessels, it was seen that CXCR4-expressing angioblasts migrated to the prepancreatic endodermal region because of recruitment by the CXCL12-expressing endoderm. Following migration, the angioblasts were able to induce Pdx1 expression in the prepancreatic domain.^{108,109} Studies have demonstrated that over-expression of the CXCL12 ligand resulted in ectopic recruitment of angioblasts and a concurrent expansion of the pancreatic domain, while blockade of the CXCR4 receptor resulted in the absence of dorsal aorta formation, and consequently a decrease in Pdx1 and insulin expression.¹⁰⁸

The endothelium is also known to play a role later in pancreatic development, in addition to its earlier role in dorsal bud induction. At ~E13.5, endocrine cells express the angiogenic factors angiopoietin and vascular endothelial growth factor A (VEGF-A) and are located in close proximity to the newly forming capillaries.¹¹⁰ As the developing endocrine cells are unable to synthesize their own basement membrane, they rely on the adjacent endothelial cells for the formation of their basement membrane.¹¹¹ In addition, VEGF-A expression by β cells is required for establishing the capillary network that is necessary for islet function in the control of glucose homeostasis. In mice that have a β cell-specific inactivation of VEGF-A, impaired GSIS was observed, despite normal β cell mass and insulin contents in the islet. It is thought that this defect in GSIS is due to a decreased intra-islet capillary network leading to a reduction in insulin transport via the endothelial fenestrae.¹¹⁰

Functionality of β cells generated from PSCs

Although there have been no reports describing the generation of a fully functional β cell *in vitro*, ESC cultures routinely give rise to polyhormonal insulin-expressing cells that might be useful as insulin delivery vehicles. However, transplantation of these cells has not ameliorated diabetes in mice under nonfasting conditions^{50,51,112} and in some cases

transplanted cells were unable to maintain an insulin⁺ phenotype, instead giving rise to grafts that consisted solely of glucagon-expressing cells.^{24,113} In lieu of attempting to generate functional β cells *in vitro*, Kroon *et al.* used a different approach by engrafting pancreatic progenitors (PDX1⁺ endoderm) into mice and allowing them to mature *in vivo*. Cells matured *in vivo* became insulin-expressing, glucose-responsive cells capable of regulating blood sugar levels in these animals. It was necessary to allow these grafts to develop for substantial periods of time (90–140 day posttransplant) before secretion of human insulin was seen. Following selective ablation of mouse β cells with streptozotocin (STZ), the engrafted cells were able to maintain normoglycemia until surgical graft removal (up to 88 days post-STZ treatment).¹¹⁴

This same approach has now been used by a number of groups^{49,52,53,115,116} and was most recently used to demonstrate that hESC-derived pancreatic cells were able ameliorate diabetes in both mice and rats.⁵³ This is an important proof of principle in efforts to translate this approach into a clinical setting, as it is the first time that PSC-derived pancreatic cells have been shown to function in a nonmouse model. A study performed by Kelly *et al.* highlighted the difference in transplanting progenitor cells as compared to polyhormonal cells. Following transplantation, progenitor cells consistently gave rise to insulin⁺ cells that ameliorated diabetes, whereas polyhormonal cells gave rise to grafts containing glucagon⁺ and somatostatin⁺ cells that were therapeutically ineffective.⁵²

Form to function: role of morphogenesis in PSC differentiation

Perhaps one reason why, as yet, there has been failure to create wholly *in vitro* PSC-derived β cells is a lack of supporting tissues being present—such as the mesenchyme and endothelium, whose essential roles in β cell development were earlier discussed. Another deficiency is the two-dimensional nature of many culture systems, which do not allow for the morphogenetic processes that occur during organogenesis *in vivo*. Several recent reports have incorporated morphogenetic processes into differentiation protocols to generate three-dimensional intestinal, retinal, and pituitary gland tissues.

One key morphogenetic event in endoderm organogenesis is the transition of definitive endoderm into a primitive gut tube, from which all endoderm organs are derived. The primitive gut tube is a simple cuboidal epithelium surrounded by a primitive mesenchyme. It was recently shown that manipulation of Wnt and FGF signaling was sufficient to induce human PSC-derived definitive endoderm to undergo gut tube–like morphogenesis. The resulting three-dimensional structures were a simple cuboidal epithelium surrounded by a primitive mesenchyme. These gut tube–like structures were competent to form complex intestinal organoids *in vitro*. Developing intestinal organoids underwent similar developmental processes as the embryonic intestine and gave rise to a complex array of functional intestinal cell types¹¹⁷ (Fig. 4A).

The importance of morphogenesis was also demonstrated in approaches to generate retinal cell types. Using aggregates of mESCs, Ikeda *et al.* were able to induce the formation of Pax6-expressing retinal cells; however, no clear formation of retinal epithelial structures was seen.¹¹⁸ Subsequent modifications to their protocol, most importantly, the addition of either

matrigel or the combination of purified laminin, enactin, and nodal, stimulated optic cup morphogenesis and resulted in the formation of three-dimensional optic cup-like structures. Optic cup structures matured into tissues that contained all of the major cell types of the retina¹¹⁹ (Fig. 4B). A similar approach was used to generate three-dimensional neural tissues resembling the anterior pituitary gland structure Rathke's pouch. In this case, mESC aggregates that were cultured in the presence of a Smoothened agonist (thus activating Hedgehog signaling) and BMP4, followed by the inhibition of Notch signaling, were able to form a structure *in vitro* that morphologically resembles Rathke's pouch and contained multiple endocrine cell types that were able to secrete adrenocorticotrophic hormone (ACTH)¹²⁰ (Fig. 4C).

Another approach to generate three-dimensional tissues from PSCs is through the incorporation of other supporting cell types such as stromal and vascular cells. For example, addition of vascular endothelial cells and mesenchymal stem cells into PSC-derived liver cultures resulted in the formation of liver bud-like structures.¹²¹ These liver bud structures formed highly differentiated hepatocytes and contained well-formed capillaries that were capable of forming a patent, functional vascular plexus when engrafted into mice. Given the importance of vascular cells for endocrine pancreas development, incorporation of vascularity into human PSC-derived pancreatic cultures is a promising approach to promote endocrine development.

Summary

Since the first publication reporting the generation of PSC-derived pancreatic cells in 2006, a vast number of protocols have been reported. Many of these protocols utilize the same ontogenic approach to PSC differentiation, and the most success in generating functional β cells has come from transplanting PDX1⁺ progenitor cells, to allow for *in vivo* cell maturation into glucose-responsive β cells. The use of progenitor cells as a treatment for type 1 diabetes has a number of advantages as compared to the use of more differentiated insulin⁺ cells. Importantly, progenitors give rise to therapeutically effective insulin-expressing cells *in vivo* and are ready for use in clinical trials. There is no need to understand or identify the signals required for β cell maturation in order to achieve a therapeutic end point. However, pancreatic progenitor cell therapy is somewhat of a black box approach since they also give rise to the other pancreatic endocrine cell types that could be therapeutically counterproductive. When engrafting progenitors, one can only hope that they give rise to the desired number of β cells following *in vivo* maturation. For this reason, it is still critical to develop methods to generate fully functional β cells *in vitro*. These cells could be highly characterized and quality controlled, and transplanted into patients with a more predictable therapeutic outcome.

Given the recent advances in generating more functional, complex three-dimensional tissues, it should be possible to generate more functional pancreatic tissues for transplantation. This approach would allow for the incorporation of other important tissues, such as mesenchyme and endothelium, and may provide the correct signaling molecules and three-dimensional architecture to allow for the formation of mature, functional β cells.

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Figure 1. Schematic depicting key developmental stages and corresponding morphogenetic processes occurring in the embryo during pancreas formation. The lower rows show the relative mouse and human developmental timelines, as well as some of the pivotal genes used to identify these stages.

Figure 2.

(A) Timelines of published multistep procedures that have been used to induce differentiation of hESCs toward insulin-secreting cells before cell transplantation. (B) Characteristics of hESC-derived pancreatic cells that have been differentiated toward insulin-secreting cells. ActA, activin A; ALK5i, ALK5 inhibitor; BSA, bovine serum albumin; CDM, chemically defined media; Cyc, cyclopamine; D/F12-B, DMEM/F12 with BSA; EGF, epidermal growth factor; FBS, fetal bovine serum; FCS, fetal calf serum; Hep, heparin; HrgB, heregulin 1b; Nic, nicotinamide; Nog, Noggin; RA, retinoic acid; RPMI-B, RPMI1640 with BSA; SFM, serum-free medium; TBI, TGF- β R1 kinase inhibitor; TT, TTNPB.

Figure 3.

Schematic depicting the influence of surrounding tissues on β cell development. (A) At E8.0, the notochord plays an important role in the suppression of Shh in the pancreatic endoderm. (B) At E10.5, the mesenchyme surrounding the pancreas produces FGF10 that signals to the pancreatic epithelium and plays an important role in endocrine progenitor cell proliferation, replication, and differentiation. (C) At E13.5, endocrine cells produce proangiogenic factors, such as angiopoietin and VEGF-A, in order to attract the developing endothelium. Ang, angiopoietin; DS, dorsal somite; Ec, ectoderm; En, endoderm; IM, intermediate mesoderm; LPM, lateral plate mesoderm; No, notochord; NT, neural tube; Shh, sonic hedgehog; VS, ventral somite.

Figure 4.

Schematic depicting self-organization of PSCs in order to form differentiated tissues. (A) hPSCs can be differentiated into intestinal cell types. As differentiation occurs, spheroids bud from a posterior endoderm monolayer and go on to form lumen-containing organoids with both epithelial and mesenchymal tissue that contain the major cell types of the intestine. (B) mESC cell aggregates self-organize to form spherical vesicles on the edge of the aggregates, which then evaginate to form optic cup-like structures. (C) mESC cell aggregates form ectoderm on the surface that can self-organize to form anterior pituitary-like structures.