Cell Stem Cell
Perspective

Deconstructing Pancreas Development to Reconstruct Human Islets from Pluripotent Stem Cells

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There is considerable excitement about harnessing the potential of human stem cells to replace pancreatic islets that are destroyed in type 1 diabetes mellitus. However, our current understanding of the mechanisms underlying pancreas and islet ontogeny has come largely from the powerful genetic, developmental, and embryological approaches available in nonhuman organisms. Successful islet reconstruction from human pluripotent cells will require greater attention to "deconstructing" human pancreas and islet developmental biology and consistent application of conditional genetics, lineage tracing, and cell purification to stem cell biology.

Introduction

In type 1 diabetes mellitus (T1DM), autoimmune destruction of pancreatic islets of Langerhans leads to a lifelong requirement for insulin replacement to maintain adequate metabolic homeostasis. However, despite nearly a century of progress, current replacement regimes represent approximations of insulin control by native islet β cells, the sole source of insulin. Thus, T1DM is complicated by accumulated damage to tissues and organs like blood vessels, neurons, kidneys, and eyes and by premature mortality.

Advances in our understanding of the mechanisms of pancreas and islet development, the beguiling possibilities of stem cell biology, and improvements in islet function after transplantation have served as landmarks for many research teams and funding programs devoted to developing T1DM therapies. Several routes toward islet replacement (for brevity we use this term to encompass efforts to produce islets in vitro or in vivo, independent of cell source or developmental mechanism) have been suggested by recent research findings, principally in mice. These include regeneration, proliferation, transdifferentiation, and transdetermination to increase β cell numbers, and are reviewed elsewhere (Bouwens, 2006; Zhou et al., 2008; Puri and Hebrok, 2010). For T1DM, concrete advances in immunosuppression are an obligatory therapeutic "partner" for any envisioned cell-based therapy and are also reviewed elsewhere (Cernea and Pozzilli, 2008; Eizirik et al., 2009).

Here we focus on prospects for the use of pluripotent stem cells such as human embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) and knowledge about human pancreas development to create functional cells resembling human islet β cells or their progenitors. Many aspects of applying ESC or iPSC biology toward the goal of pancreas cell replacement have also been reviewed recently (Mayhew and Wells, 2010; Robbins et al., 2010; Van Hoof et al., 2009). Our discussion specifically highlights the need for intensified studies of human pancreas and islet developmental biology and the rigorous application of developmental biology methods to achieve this goal.

There is cautious optimism about the possibility that methods currently under development will produce cells resembling pancreatic or islet progenitors from pluripotent cells like human ESCs or iPSCs that can be used to replace β cells destroyed in T1DM (D'Amour et al., 2006; Kroon et al., 2008; Zhang et al., 2009). These methods attempt to recapitulate the sequence of endogenous signaling pathways that first create progeny cells resembling definitive endoderm, then "primitive" gut tube epithelium, foregut pancreatic progenitors, islet progenitors and, in the final step, hormone$^+$ progeny including insulin$^+$ cells. It is sobering to reflect, however, that these methods are built on developmental biology findings approaching or more than a decade old and mainly reflect studies of nonmammalian or rodent species. Below we review knowledge about human islet development, highlighting areas we feel warrant attention. Prior studies of pancreas and islet development in experimental systems have carefully applied standard, powerful methods to reveal molecular and cellular mechanisms underlying endogenous islet cell differentiation, expansion, maturation, and function. However, such methods have not been systematically applied to stem cell research efforts, and we suggest strategies for doing so.

Prospects for Using Human Pancreas Developmental Biology to Guide Islet Replacement

There has been rapid growth in our understanding of mechanisms underlying pancreas development in the past two decades, making it one of the best delineated among visceral organs. Current strategies to generate replacement β cells from pluripotent cell sources rely on knowledge of pancreas and islet development derived largely from nonhuman experimental models, including rats, chicks, and fish, but primarily mice, and on the premise that cellular and molecular regulation of pancreas development is conserved. In our view, an over reliance on this premise is unwarranted, given apparent unique features in the developmental programs governing human pancreas and islet formation. The mouse pancreas has been

Table 1. Unestablished Fundamental Properties of Human Pancreas Development

Property	Evidence from Nonhuman Studies
Bipotential endoderm capable of development toward hepatic or pancreatic fates	Deutsch et al., 2001; Wandzioch and Zaret, 2009
Multipotent pancreatic progenitors marked and regulated by Pdx1, Ptf1a, Sox9, and Cpa	Gu et al., 2002; Kawaguchi et al., 2002; Seymour et al., 2008; Zhou et al., 2007
Cells expressing Ngn3 are monoclonal islet progenitors and their development is regulated by the Notch signaling and other pathways	Gradwohl et al., 2000; Jensen et al., 2000; Apelqvist et al., 1999; Desgraz and Herrera, 2009
$Ngn3^+$ cells with limited proliferative capacity and their allocation toward specific islet cell fates is regulated by intrinsic factors, including Pax4 and Arx	Collombat et al., 2003; Collombat and Mansouri, 2009; Sosa-Pineda, 2004
Expansion of pancreatic epithelial mass regulated by multiple extrinsic factors, including Fgf10, Bmp, Wnt, Hedgehog, and Egf	reviewed by Murtaugh, 2007; Oliver-Krasinski and Stoffers, 2008
Expansion of β cells through enhanced proliferation wanes with age and this is regulated by intrinsic factors including CyclinD1, D2, Ezh2, Bmi1, p16/p18, Ir/Irs, p27, and FoxO	reviewed by Heit et al., 2006

Mechanisms thought to underlie pancreatic and islet development, principally in mice, are listed with supporting references.

intensively investigated and provides the best current framework for identifying gaps in our knowledge about human pancreas and islet development.

In mammals, the pancreas and other visceral organs like liver, pharyngeal arch derivatives such as the parathyroids, and the pulmonary organs develop from the definitive endoderm, although lineage-tracing experiments establishing that the pancreas arises from the endoderm have been performed only in mice. Morphologically, the pancreas anlage emerges first from dorsal then ventral mesenchymal condensations that support evagination and branching morphogenesis of underlying endoderm-derived epithelium in the posterior foregut. In both mice and humans, fusion of the initially independent dorsal and ventral pancreatic rudiments culminates in formation of a single organ with mixed endocrine and exocrine functions that nestles in the duodenal loop, which receives exocrine secretions including bicarbonate and zymogens. Exocrine functions of the pancreas originate in ductal and acinar cells, while endocrine function derives from epithelial cell clusters called islets of Langerhans. Distinct islet cells are defined by their principal hormone product, including insulin in β cells and glucagon in α cells (reviewed in Gittes, 2009). Host survival after autoimmune destruction of β cells in T1DM requires insulin replacement.

Genetic studies in mice and humans have revealed conserved regulators of pancreas development and islet cell function. For example, homozygous recessive mutation of *Pdx1* or *Ptf1a*, which encode transcription factors initially expressed by epithelial cells in the dorsal and ventral pancreatic anlagen, causes pancreatic agenesis in mice and humans (Jonsson et al., 1994; Krapp et al., 1998; Sellick et al., 2004; Stoffers et al., 1997b). In humans, heterozygous mutations in *Pdx1* or several other genes (*HNF1a, HNF1β, HNF4α, NeuroD1/Beta2*, and *Glucokinase*) result in β cell dysfunction and a condition known as maturity onset of diabetes in the young (MODY), and in mice, recessive mutations in these genes can also impair β cell function and glucose regulation (reviewed in Fajans et al., 2001; Gittes, 2009). Other features of mouse endoderm and pancreas development revealed via classical embryology, genetic, and developmental biology methods, including organ culture, loss-of-function genetics, lineage tracing, and cell purification, have not yet been demonstrated to apply to human development (see Table 1). Maturation of β cell functions governing energy sensing and coupling of β cell detection of stimuli to insulin secretion occurs in both fetal and postnatal stages in mammals, but regulation of this process is poorly understood in all experimental models. In mature β cells, evidence suggests that conserved factors regulate mammalian insulin processing from a propeptide precursor and modulation of stimulus-secretion coupling during β cell adaptation to physiological stresses like obesity, pregnancy, or normal host growth (Cozar-Castellano et al., 2006; Heit et al., 2006).

Studies of early human organ development are generally hindered by the limited accessibility to early human fetal tissues. Thus, our knowledge of human pancreatic development is largely based on a small number of studies involving retrospective immunohistological analysis of fixed tissues, gene expression profiling of bulk tissue, in vitro organ culture, or xenotransplantation-based assessment of fetal pancreas (Castaing et al., 2005; Lyttle et al., 2008; Piper et al., 2004; Polak et al., 2000; Sarkar et al., 2008). Moreover, these studies are limited to a narrow range of gestational ages (primarily weeks 7 through 21), precluding analysis of early cell-cell interactions and cell fate decisions. Small numbers of tissue samples and different experimental approaches further limit the statistical power of conclusions from such studies. Addressing these deficiencies will require new knowledge, including creation of methods to isolate, profile, and characterize defined cell subsets from human definitive endoderm and fetal pancreas (see below).

Although much of our knowledge of human pancreatic development is based on prior studies in mice, it is clear that there are fundamental differences between these two species that may reflect differences in developmental mechanisms. For instance, the morphology of the human and mouse pancreas differs at both the organ and cellular level (Piper et al., 2004; Polak et al., 2000). Unlike the mouse pancreas, which has an abundance of mesenchymal cells, especially at the beginning of development, the mesenchyme in fetal human pancreas is sparse and loosely associated with the epithelial component. Thus, the quality and nature of epithelial-mesenchymal interactions, which have crucial, established roles in mouse pancreas specification

(Bhushan et al., 2001; Deutsch et al., 2001; Golosow and Grobstein, 1962; Wandzioch and Zaret, 2009), may be distinct in human fetal pancreas. Islet β cells and non-β cells in rodents like mice appear to segregate, but this segregation is less clear in human (and nonhuman primate) islets. These morphological differences may reflect both species-specific expression of islet cell adhesion molecules like NCAM and Ep-CAM (Cirulli et al., 1994, 1995, 1998, 2000) and the comparatively high proportion of α cells in human versus mouse islets. These differences between human and rodent islet architecture may have functional consequences. For example, β cell oscillatory activity (based on membrane depolarization) is not coordinated within individual human islets as robustly as in mouse islets, and human islets have been demonstrated to secrete insulin in response to lower concentrations of glucose than rodent islets (Cabrera et al., 2006).

The mechanisms regulating the speed and duration of islet progenitor cell formation, progenitor maturation toward β cell fates, and β cell replication may also be distinct in humans and mice. In mice, fetal pancreas development occurs in 10 days, and a period of accelerated β cell development (called the "secondary transition") and proliferation occurs in roughly the latter half of gestation (reviewed in Gittes, 2009). There is a lack of evidence for such punctuated β cell development in humans (Sarkar et al., 2008). On the contrary, descriptive immunohistology studies of human pancreatic morphogenesis (Jeon et al., 2009; Lyttle et al., 2008; Piper et al., 2004; Polak et al., 2000; Sarkar et al., 2008) suggest that the development process, particularly for islets, spans several months. This difference is further highlighted by the finding that "rescue" of experimental diabetes by transplanted human fetal pancreatic tissue requires a "maturation" period of up to 10 weeks (Hayek and Beattie, 1997). Do these distinctions reflect underlying differences in the mechanisms governing islet development? Recent human fetal pancreas studies of NGN3, a marker and essential regulator of islet progenitor cell development in mice, support that possibility. *Ngn3* mRNA expression in the developing mouse pancreas peaks around E15.5, roughly equivalent to week 7–8 (Carnegie Stages 21–22) in human development. Consistent with this timing and the hypothesis that human islet cells derive from $NGN3^+$ progenitors, human pancreatic cells expressing hormones like insulin and glucagon emerge by 7–8 weeks, and islet morphogenesis occurs continuously thereafter. However, expression of *NGN3* in the developing human pancreas is prolonged and *NGN3* mRNA peaks between weeks 11 and 19, possibly reflecting the prolonged period of islet morphogenesis and accumulation observed in a recent study by Jeon et al. (2009). Unlike in islet cells in mice, NGN3 expression is readily detectable by immunostaining in $hormone^+$ cells (Wang et al., 2009), suggesting that the regulation of *NGN3* may differ in humans and mice.

Mice harboring homozygous null *Ngn3* mutations develop an exocrine pancreas, including ducts and acini, but lack islets (Gradwohl et al., 2000) as well as enteroendocrine cell subsets (Lee et al., 2002). Recent studies pioneered by Martín and colleagues (Wang et al., 2006) identified patients harboring homozygous recessive alleles of *NGN3* with aplasia of enteroendocrine cells (dubbed anendocrinosis) accompanied by congenital diarrhea. These subjects had detectable serum insulin C-peptide perinatally and did not manifest neonatal diabetes, providing evidence for development of islet β cells in these subjects. Although some of these patients subsequently developed overt diabetes, treated by insulin replacement, these collective findings raised the possibility that, unlike in mice, islet development occurred in human subjects lacking NGN3. Subsequent studies suggested that the human *NGN3* alleles linked to congenital intestinal anendocrinosis were hypomorphic, not null, alleles, but this remains controversial (Jensen et al., 2007). Thus, definitive studies are required to establish the role of NGN3 in human islet development.

Loss-of-function genetic studies were instrumental in establishing the roles of key regulators like Ngn3 in mouse pancreas development. What are the prospects for such studies in human pancreas developmental biology? Although genetic studies of human pancreatic development remain highly constrained, important opportunities for investigating developmental genetics of human pancreas have been created by newer methods like iPSC derivations from specific patients. Thus, we foresee value in systematic analysis of cell lines derived from patients harboring the heterozygous or homozygous mutations previously described (Table 2), coupled with studies of native fetal pancreatic cells in vitro (see below). Such investigations could prove useful for discovering the mechanisms that regulate human pancreatic cell development, thereby defining the cellular targets we hope to reconstruct from pluripotent cell sources. Collectively, these considerations argue that intensified molecular and cellular studies of pancreas development in humans and in species with embryological homologies, including simians, are warranted.

Methods for Deriving and Assessing Replacement Islet Cells

The expansion of our knowledge about pancreas and islet development, growth, and function stems in large part from incisive use of standard tools in the developmental biology toolkit. These include cell lineage tracing, cell purification, cell ablation, cell mixing, mutant cell analysis, conditional genetics for gain- or loss-of-function, organ reconstitution by cell transplantation, and use of small molecule libraries. In the past several years, many groups have adapted knowledge about the pancreas and islets to attempt development of functional islet β cells or their progenitors from renewable sources like ESCs or iPSCs (D'Amour et al., 2006; Kroon et al., 2008; Zhang et al., 2009; Borowiak et al., 2009) and from cell subsets from organs like adult pancreas (Zhou et al., 2008). But, with very few exceptions, these studies did not use the most powerful and precise developmental biology tools. Most frequently, cell cultures are exposed to a sequence of factors or conditions culminating in development of the desired cell type as a minor subset in a mixed population of cells. Typically, the frequency of factor or condition changes is based somewhat arbitrarily on 8, 12, or 24 hr periodicity. No prior study has achieved resolution of the heterogeneity at the final "stages" of in vitro differentiation by cell sorting or other methods. To our knowledge, lineage tracing studies have also not been reported. It therefore remains unknown whether, for example, cells thought to resemble endoderm from the expression of SOX17 protein engender progeny in the next stages that resemble pancreatic progenitors expressing PDX1,

Table 2. Genetic Regulation of Human Pancreatic Development and Islet Function

Gene	Phenotype	References
PDX1	homozygous: congenital pancreatic agenesis; heterozygous: monogenic diabetes (MODY4) and type II diabetes	Stoffers et al., 1997a, 1997b
PTF1A	homozygous: pancreatic and cerebellar agenesis	Sellick et al., 2004
SOX9	heterozygous: campomelic dysplasia, pancreatic malformation	Foster et al., 1994
NEUROD1	heterozygous: monogenic diabetes (MODY6)	Malecki et al., 1999
NGN3	homozygous: congenital malapsorptive diarrhea, intestinal anendocrinosis, diabetes	Wang et al., 2006
GCK	homozygous: persistent neonatal diabetes; heterozygous: monogenic diabetes (MODY2)	Njølstad et al., 2001; Vionnet et al., 1992
HNF1α	heterozygous: monogenic diabetes (MODY3)	Yamagata et al., 1996
HNF1β	heterozygous: monogenic diabetes (MODY5); cystic kidney disease	Horikawa et al., 1997
HNF4α	heterozygous: monogenic diabetes (MODY1)	Fajans, 1989
MEN1	heterozygous: multiple endocrine neoplasia	Chandrasekharappa et al., 1997
CDKN1B	heterozygous: multiple endocrine neoplasia	Pellegata et al., 2006
CDKN2C	heterozygous: multiple endocrine neoplasia	Agarwal et al., 2009
ARX	hemizygous males: lissencephaly, epileptic encephalopathy, abnormal genitalia	Kitamura et al., 2002
PAX4	heterozygous: monogenic diabetes (MODY9)	Plengvidhya et al., 2007
GLIS3	homozygous: neonatal diabetes and congenital hypothyroidism	Senée et al., 2006
KCNJ11	homozygous: hyperinsulinemic hypoglycemia; heterozygous: persistent neonatal diabetes	Thomas et al., 1996; Gloyn et al., 2004
RFX6	homozygous: neonatal diabetes, pancreatic hypoplasia, intestinal atresia, and gall bladder aplasia or hypoplasia	Smith et al., 2010

and so on (Figure 1A). Thus, the lineage of progeny cells produced in culture experiments is not established. Thanks to the widespread use of homologous recombination to produce "reporter" cells, sequential lineage tracing with lineage-marking methods based on, for instance, Cre-recombinase, is well within reach for mouse ESC and iPSC studies. However, enhancements of methods for gene targeting by homologous recombination (Porteus and Baltimore, 2003; Zou et al., 2009; Zwaka and Thomson, 2003) are needed to foster this approach in human ESC and iPSC lines. Direct isolation of enriched cell subsets followed by "replating" and continuation of development may act as a surrogate for lineage-tracing experiments. Yasunaga et al. (2005) described replating mouse ESC progeny isolated by FACS for the Cxcr4 antigen to differentiate endoderm-like cells, but to our knowledge, this general approach has not been used in attempts to produce islet or pancreas-like cells from ESC lines (Figure 1B). By contrast, similar studies aiming to produce functional cardiomyocytes or cardioblasts have fruitfully used such approaches (Bu et al., 2009; Yang et al., 2008). It is therefore not surprising that maturation of functional glucose-sensing cells resembling islet β cells from prior in vitro approximations of pancreatic islet development has not been achieved (Kroon et al., 2008). Below, we discuss cell purification, lineage tracing, and genetic strategies for enhancing attempts to control the complex programs underlying development of islet-like cells from renewable or expandable human cell sources.

What Is the Lineage and Quality of Pluripotent Stem Cell Progeny?

Studies of gene expression in FACS-purified native cardiomyocyte and hematopoietic progenitor/stem cell lineages has accelerated experimental attempts to generate these cells from cultured hESCs and iPSCs (Bu et al., 2009; Yang et al., 2008). Even without such precedents, it seems self-evident that an understanding of fundamental phenotypes of native pancreatic cells, like gene expression profiles and cell surface markers for FACS, would enhance cell culture-based efforts to produce cells resembling them; just what are we trying to make in our experiments? There is a slowly accumulating body of data on gene expression in isolated cell subsets from the developing and postnatal mouse pancreas, but these data focus largely on mature β cells (Gu et al., 2004; White et al., 2008). Moreover, some gene expression profiles may unfortunately be "contaminated" because of imprecisely defined experimental strategies to label and sort relevant cells. For example, perdurance of the eGFP signal derived from a *Ngn3-eGFP* transgene led to labeling of both $Ngn3^+$ $hormone^-$ islet progenitors and their $hormone^+$ progeny, including $Insulin^+$ β cells (Sugiyama et al., 2007). Thus, gene expression profiles of *Ngn3-eGFP*$^+$ cells (Gu et al., 2004) may reflect this mixture of cells.

Analysis of purified native cell subsets from definitive endoderm, primitive gut tube, foregut, the developing pancreas, and associated organs like the liver may help to define pathways that regulate cell differentiation, growth, survival, and other phenotypes. Such studies may also reveal novel gene interactions or developmental or islet cell maturation regulators, and may identify cell surface markers for FACS purification of cell subsets, independent of genetic background (Gadue et al., 2009; Sugiyama et al., 2007; Xu et al., 2008). Although genomic-scale studies for isolated rat α and β cells have been reported (Kutlu et al., 2009), the majority of studies using methods like DNA microarrays have focused on whole pancreas, islets, or immortalized islet cell lines (White and Kaestner, 2009) in model organisms such as rodents. The lone genomic-scale study of human fetal pancreatic gene expression described results from analysis of whole pancreatic tissue (Sarkar et al., 2008). Thus, development of methods for isolating native pancreatic and islet progenitor cells will be crucial for advancing

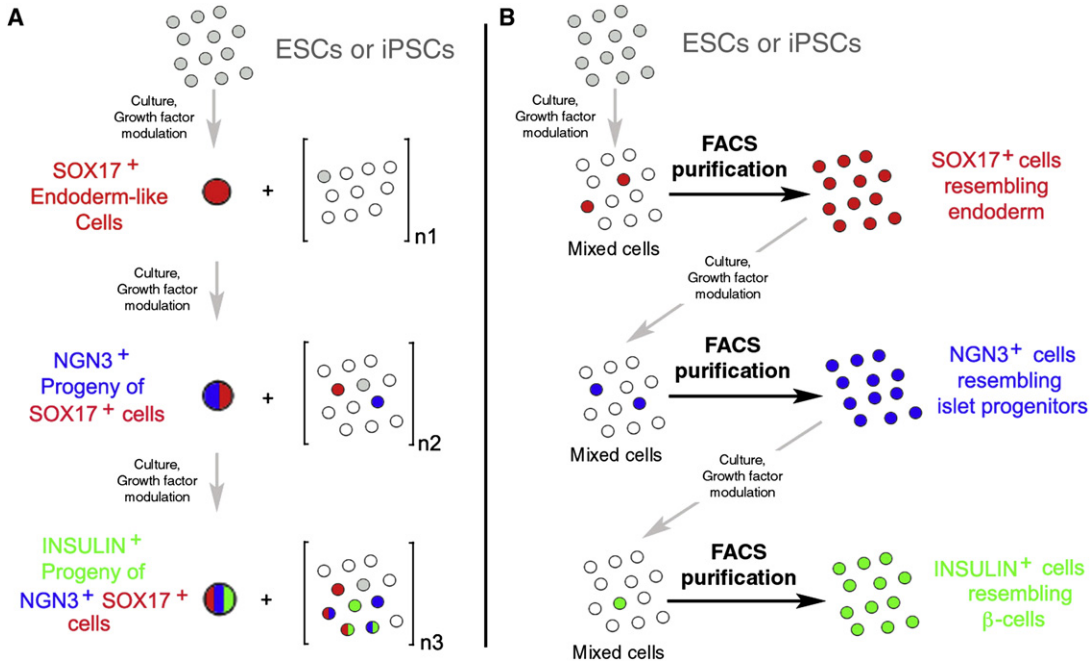

Figure 1. Schematic Summarizing Two General Strategies to Assess and Improve the Quality of Progeny Produced from Pluripotent Stem Cell Cultures

(A) Marking of embryonic stem cell (ESC) or induced pluripotent stem cell (iPSC) progeny via Cre recombinase-based methods to induce expression of probes signaling sequential passage through a SOX17$^+$, then NGN3$^+$ and INSULIN$^+$ fates. n1, n2, and n3 represent numbers of indicated, undifferentiated, or partially differentiated cells in cultures, where n1 < n2 < < n3.

(B) Sequential flow cytometry-based enrichment to reduce heterogeneity of ESC or iPSC progeny at discrete stages of differentiation toward SOX17$^+$, then NGN3$^+$ and INSULIN$^+$ fates.

our understanding of pancreas developmental biology in humans and experimental animals (Figure 1B). Recently, Grompe and colleagues used a "decoy" strategy to identify monoclonal antibodies for fractionating and isolating human islet and exocrine cell subsets from adult human pancreas (Dorrell et al., 2008), but additional studies are needed to test whether these reagents will also permit sorting of human fetal pancreatic cell subsets.

Flow cytometry-based purification methods for developing cell subsets in the fetal human pancreas could significantly enhance outcomes of tissue differentiation studies from ESC or iPSC sources. For example, flow cytometry strategies could be used to isolate specific cell subsets for analysis and resolve undesirable cell heterogeneity; this in turn could prevent hazards like formation of teratomas or other tumors known to arise from ESC and iPSC types (Takahashi et al., 2007). Isolation of cells expressing native surface markers would also impose a type of "quality control" on in vitro culture methods; for example, cells produced from ESC cultures could be enriched for cell progeny with greater similarity to endogenous cells. Such an approach was recently used to isolate cardioblast-like cells from ESC cultures by Keller and colleagues (Yang et al., 2008). Undifferentiated ESCs may express cell surface markers that would otherwise serve to fractionate progeny, thereby limiting use of some antigens for these approaches. For example, CD133 (also known as prominin) is well established as a marker of endogenous stem or progenitor cell populations in neural, hematopoietic, skin, and pancreatic lineages. However, undifferentiated mouse and human ESCs express high levels of CD133 (Kania et al., 2005), precluding use of this marker for studies in the early stages of in vitro ESC developmental studies. Yet, if CD133 is downregulated during initial stages of differentiation and subsequently reexpressed, it could be combined with other cell surface antigens to isolate in vitro-derived pancreatic progenitor cells. Also, although dissociation of epithelial cells is possible, flow cytometric analysis of epithelial cells is often complicated by eventual cell death from anoikis and other mechanisms (Alexander et al., 2009). Thus, although isolation and replating of mesodermal or mesenchymal cells has been described (Yang et al., 2008), purification and replating schemes like that outlined in Figure 1 have been difficult to achieve with epithelial lineages derived from ESC cultures. However, an armory of other fruitful approaches based on cell separation by magnetic beads or by immunopanning, which has been used to overcome the challenge of isolating neural cell subsets like astrocytes or projection neurons (Dugas et al., 2008), could be applied more broadly to epithelial differentiation schemes and combined with methods that increase cell-cell interactions, like sphere culture (Seaberg et al., 2004). Alternately, depletion of cells via selection with drugs like blasticidin, G418, or hygromycin combined with lineage-specific activation of drug-resistance markers is a strategy that can be used to avoid the need for dispersion-based cell isolation (Kawaguchi et al., 2010). Such drug selection-based cell enrichment schemes may also help to address an unanswered fundamental question in derivation of specific cells from ESC and iPSC cultures: is the cell heterogeneity inherent to these cell cultures *required* for derivation of

specific lineages? And if so, when? Experimental approaches to address this question may require systematic removal of specific cell subsets from heterogeneous cultures, which might be facilitated by drug selection schemes.

Interrogating the Quality of Stem Cell Progeny with Genetics

Emerging data on gene expression and epigenetic profiles should prove useful for evaluating cell products of ESC or iPSC cultures. To date, the Food and Drug Administration, which oversees cell replacement therapy safety in the US, has not required expression profiles of key genes or analysis of the epigenome in these cellular products. However, once this sort of evaluation has been performed and validated, it may be extremely helpful in characterizing or improving cell populations. For example, Mutskov and Felsenfeld (2009) recently reported unique features of the epigenetic "landscape" of the *INSULIN* gene locus in human cells. We envisage that fine-scale molecular mapping of epigenetic regulation at native loci encoding hallmark β cell factors like *INSULIN* could help validate candidate cell populations produced from ESC or other stem cell cultures.

To supplement corroborative, descriptive studies of gene expression and epigenetic status, loss-of-function genetics can provide powerful ways to interrogate the quality of these cell products. For example, genes like *Sox17, Pdx1*, other MODY genes, and established key regulators like *Ptf1a, Sox9*, and *Ngn3* have been modified to create conditional null alleles in mice and mouse ESC lines (Kawaguchi et al., 2002; Gu et al., 2002; Seymour et al., 2007). Prior in vivo studies after inactivation with Cre recombinase have permitted identification of products encoded by these genes as key developmental regulators of definitive endoderm, foregut epithelium, pancreatic progenitors, and insulin-secreting β cells. Likewise, studies of regulators like *Men1, $p27^{Kip1}$, $p18^{INK4c}$, Ezh2*, and *Bmi* have permitted identification of key functions in mature β cells regulating chromatin dynamics, proliferation, and fate control (Chen et al., 2009; Crabtree et al., 2001; Dhawan et al., 2009). Thus, loss-of-function analysis of these well-studied loci would provide important genetic benchmarks for comparing progeny from in vitro cultures to native cellular counterparts formed during normal organogenesis. For example, islet development is blocked in mice lacking *Ngn3*, and this phenotype is recapitulated in some culture methods (Sugiyama et al., 2007); however, inactivation of Ngn3 has not been used to validate current methods for generating hormone$^+$ cells from any ESCs or iPSCs (Borowiak et al., 2009; D'Amour et al., 2006; Kroon et al., 2008; Zhang et al., 2009). Moreover, genetic resources in human ESC and iPSC lines are still in an early stage of development. Systematic use of genomic-scale RNAi resources might provide another means for analysis, but current methods based on this approach remain limited by incomplete or inconsistent loss of function and technical challenges in transplantation-based settings. Thus, although we emphasize the importance of human pancreatic studies here, there are clear opportunities for exploiting existing (but underused) genetic resources from organisms like mice. Development of similar resources in human pluripotent cells, including iPSC lines, could be facilitated by prior identification of patients with pancreatic or diabetic phenotypes linked to mutant alleles, including null alleles, in *IPF1/PDX1*, *HNF1α, HNF1β, HNF4α, GLUCOKINASE, NEUROD1/BETA2*, *SOX9*, *NGN3, PTF1A, MEN1*, and other genes (Table 2).

Assays of Developmental Potential

Developmental and stem cell biologists attempting to reconstitute organogenesis in vitro are challenged by the knowledge that many features of endogenous developmental signaling may not be recapitulated in their culture systems (Huppert and Magnuson, 2009; Wandzioch and Zaret, 2009). Reconstitution of cell-cell interactions by cell mixing may improve attempts to recapitulate some aspects of cell-cell signaling. For example, coculture of pancreatic epithelium with vascular endothelial cells may recapitulate aspects of early signaling coordinating in vivo pancreas development (Lammert et al., 2001; Yoshitomi and Zaret, 2004). However, developmental cell fates in the pancreas probably result from a series of transient, complex cell-cell interactions and other signaling events in a three-dimensional space, whose elements have not yet been reconstituted. Thus, inefficiencies are probably *inherent* to in vitro approaches that, ultimately, are mere molecular approximations of the actual development programs. Ultimately, preclinical assessment of developmental potential and physiological function will depend on imaginative use of in vitro systems that approximate native stem/progenitor cell niches (Lutolf et al., 2009), and for human ESC or iPSC progeny, xenotransplantation-based assays. For subsets of cells in the pancreatic or islet lineage, however, current methods for assaying developmental potential are limited, with the majority of in vivo studies employing heterotopic graft sites like the renal subcapsular space or omentum of *SCID* or *NOD scid* mouse hosts (D'Amour et al., 2006; Kroon et al., 2008).

These assays constitute a kind of "graft and hope" approach that limits interpretation, especially in the analysis and quantification of developmental phenotypes. To assess the developmental potential of native mouse pancreatic Ngn3$^+$ cells, we surveyed multiple culture conditions and found that insulin$^+$ and glucagon$^+$ cells developed when Ngn3$^+$ islet progenitors at low density were cocultured either with mitomycin C-treated mouse embryonic fibroblasts (MEFs) or with PA6 mouse stromal cells, a feeder cell layer shown to promote neural differentiation by embryonic stem cells (Kawasaki et al., 2000). Although the coculture assays helped confirm the developmental potential of isolated Ngn3$^+$ cells to form β cells and other islet cells (Sugiyama et al., 2007), they also had limitations. Specifically, there was poor survival of input pancreas cells, measured to be about 0.01%–0.05% after 3 days' culture, and no evidence of cell proliferation. Heimberg and colleagues recently reported use of *Ngn3* mutant fetal mouse pancreas as a platform for assessing developmental properties of injected mouse Ngn3$^+$ cells (Xu et al., 2008). Use of specific feeder cells or surrogate niches like fetal pancreas is an approach that could be extended to in vivo assays of developmental potential. The lack of a "reconstitution" assay analogous to bone marrow transplantation is impeding attempts to isolate and assess candidate progenitor cells derived from ESC and iPSC sources for visceral organs like the pancreas and islet progenitor cells. This is a major limitation in many fields, not just pancreas biology, and restricts efforts to use, for example, genetic screens to discover regulators of islet maturation or proliferation. We speculate that sites in organs

with native endocrine and exocrine tissues that support islet engraftment, like adult pancreas (Hayek and Beattie, 1992) or mammary stroma (Outzen and Leiter, 1981), as well as experimentally modified sites (Dufour et al., 2005) could be explored as potential surrogate niches for developmental studies of pancreatic and islet progenitor cells.

Summary

Bone marrow transplantation (BMT) remains a bewitching paradigm of cell-based therapy for biomedical researchers. BMT was originally applied as an experimental therapy several decades ago for patients with diseases like chronic myelogenous leukemia in blast crisis, who could otherwise expect to live for an average of only 6 weeks. By contrast, the stakes for cell-based therapies in T1DM have been heightened by the transformation of this once rapidly fatal disease, through insulin replacement and other treatment advances, into a chronic illness with an average patient lifespan of more than six decades. As a result, any cell-based therapy for diabetes would need to have a high benefit to risk ratio. Rigorous application of standard molecular methods to studies of pluripotent cells will probably both accelerate production of functionally superior islet replacement cells and enhance the safety of such cells. For example, the risk of tumor development from pluripotent cell sources in immunosuppressed patients must be eliminated. However, current claims of reduced or eliminated potential of ESC- or iPSC-derived progeny to form tumors like teratomas made in prior studies are problematic for at least two reasons. First, there is a manifest need to transplant large numbers of cells (whether "progenitors" or more differentiated insulin-secreting cells) to replace islet function in patients with T1DM; however, such large numbers have not be generated or transplanted in any prior study, to our knowledge. Second, the establishment and prolonged maintenance of appropriate genetic and epigenetic regulation in cells produced from ESCs or iPSCs has not been demonstrated. In our view, this is an important practical milestone, because of the established role of reduced gene dosage or expression of tumor suppressor loci encoding *Men1*, $p27^{Kip1}$, and $p18^{Ink4c}$ in pathogenesis of human endocrine tumor syndromes (reviewed in Agarwal et al., 2009). However, no study has yet determined whether "appropriate" expression of these known regulators of growth and cell fate, and their molecular targets, exists in the progeny of stem cell cultures.

"Regenerative medicine" has become a common phrase that expresses more hope than experience. Prior studies and evidence argue that considerable further progress is required before such a title can be earned and applied to stem cell approaches for the treatment of diabetes. However, armed with powerful experimental approaches, we and others remain undaunted by this challenge.

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