

Potential Pathways to Restore β -Cell Mass: Pluripotent Stem Cells, Reprogramming, and Endogenous Regeneration

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Abstract Currently available β -cell replacement therapies for patients with diabetes, including islet and pancreas transplantation, are largely successful in restoring normal glucose metabolism, but the scarcity of organ donors restricts their more widespread use. To solve this supply problem, several different strategies for achieving β -cell mass restoration are being pursued. These include the generation of β cells from stem cells and their subsequent transplantation, or regeneration-type approaches, such as stimulating endogenous regenerative mechanisms or inducing reprogramming of non- β cells into β cells. Because these strategies would ultimately generate allogeneic or syngeneic β cells in humans, the control of alloimmunity and/or autoimmunity in addition to replacing lost β cells will be of utmost importance. We briefly review the recent literature on these three promising strategies toward β -cell replacement or restoration and point out the major issues impacting their translation to treating human diabetes.

Keywords Pluripotent stem cells · Endoderm · Pancreas · Diabetes · Insulin · β cells · Proliferation · Regeneration · Transplantation · Reprogramming · T1DM · Stem cell therapy · Pdx-1 · Endocrine progenitors · β -cell mass · Autoimmunity · Islets of Langerhans · Pancreatic islets

Introduction

Progress in diabetes care over the last decade, such as the routine use of recombinant insulins, continuous subcutaneous insulin infusions, and continuous glucose monitoring, has improved and lengthened the lives of millions of patients. As patients live longer they are more likely to develop impaired awareness of hypoglycemia. Adrenergic responses to hypoglycemia in patients with long-standing diabetes are often severely blunted and nonspecific neuroglycopenic symptoms tend to predominate. As a result, frequent, unanticipated hypoglycemic episodes continue to plague patients. And, although it is well documented that tight glycemic control helps delay or prevent end-organ complications such as retinopathy and nephropathy, it is also clear that the chief barrier to excellent glycemic control is the constant fear of dangerous hypoglycemia.

Ideal control, without glycemic excursions or risk of hypoglycemia, can only be achieved with physiologic β -cell replacement, such as in the form of pancreas transplantation or islet transplantation. One of the major limitations to clinical islet or vascularized pancreas transplantation, however, is the inadequate supply of human cadaver donors. With over 30,000 patients diagnosed with type 1 diabetes each year in the United States and only about 8000 human cadaver donors, the shortfall is obvious (<http://www.jdrf.org/> and <http://optn.transplant.hrsa.gov/>). Therefore, a reliable and clinically

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applicable method to replace lost β cells in patients with diabetes is desperately needed.

Alternative Sources of β Cells

Potential strategies for β -cell replacement can generally be categorized as *ex vivo* strategies involving the generation of β cells *in vitro* and their subsequent transplantation, or *in vivo* regeneration-type approaches. In the former, it is envisioned that cells would be isolated, derived, or grown from other sources and subsequently transplanted either as cells alone or encapsulated cells or cells together with matrices as part of a tissue construct into the diabetic recipient. Examples of this type of strategy might include the differentiation of human pluripotent stem cells through stereotypical developmental stages into pancreatic and endocrine progenitor cells and ultimately into glucose-responsive insulin-secreting β cells followed by transplantation. Alternatively, β cells could be grown from other cell types isolated directly from patients such as blood cells, acinar cells, ductal cells, or liver cells, etc. Advantages of the *ex vivo* strategy is that cells can be characterized, quantified, and safety tested prior to transplantation.

Another strategy involves direct *in vivo* manipulations to promote β -cell regeneration. One approach to achieve this goal that has been explored recently is to reprogram other somatic or parenchymal cells into becoming β cells by overexpressing multiple key pancreatic transcription factors. Although this has been achieved in mice *in vivo*, it may also be worthwhile to explore *ex vivo* conversion of other pancreatic cell types to β cells, such as targeting putative facultative progenitor cells to induce their conversion into β cells.

Regardless of which strategy is pursued, it is generally accepted that in order to be effective in human patients it will be necessary to consider control of alloimmunity and/or autoimmunity in conjunction with replacing lost β cells. The next sections review in more detail three-forementioned promising strategies toward β -cell replacement or restoration that are currently in preclinical studies: derivation of insulin-producing cells from pluripotent stem cells, reprogramming non- β cells into β cells, and methods of promoting endogenous regeneration.

Pluripotent Stem Cells

The quest for alternative sources of β cells has catalyzed an effervescence of studies aimed at translating known molecular pathways of pancreas organogenesis into protocols of stem cell differentiation *in vitro*. The prospect of turning human stem cells, either embryonic (ES) or induced

pluripotent (iPS), into enough β cells to treat many patients first derives merit from the renewal potential of stem cells, whose populations can be amplified to clinically significant levels and can serve as transplantation material. Second, stem cell pluripotency implies that, under the action of the appropriate inductive networks of growth factors, these cells may give rise to any cell type, including fully functional pancreatic endocrine lineages with therapeutic potential in insulin-dependent diabetes. Third, the recent advances in reprogramming human somatic cells to become iPS cells [1–3] potentially allow the *in vitro* derivation of an autologous therapeutic cell population.

Significant progress has been achieved over the last decade in our ability to identify definitive endoderm and pancreatic lineages in stem cell cultures and to directly induce the differentiation of these cell types from human pluripotent stem cells by sequentially recapitulating the major stages in pancreas development. However, the remaining challenge is to match the phenotypic profile of expressed markers and transcription factors with functional qualities such as glucose responsiveness and physiologic insulin secretion *in vitro*, and, more importantly, *in vivo*. Early reports of generating human pancreatic endoderm, capable of differentiating *in vivo* into more mature islet cell types secreting C-peptide, insulin, glucagon, or somatostatin in response to glucose stimulation [4–7] have been followed by recent failed attempts to generate clinically relevant levels of human endocrine hormone secretion [8, 9], casting a shadow of doubt over the reproducibility and efficiency of current protocols of β -like cell differentiation from human pluripotent stem cells.

The past 3 years have brought to light several analogous, yet surprisingly diverse, pancreatic lineage differentiation protocols for human pluripotent stem cells focused on deepening the exploration and applicability of signaling pathways from embryonic pancreas development (summarized in Table 1 and recently reviewed in [10]). Special interest in recent reports has been dedicated to the role of retinoic acid in converting definitive endoderm into pancreatic duodenal homeobox-1 (Pdx1)-positive precursors [11, 12], which were substantially increased in number in the presence of antagonists to bone morphogenetic protein (BMP) and hedgehog [9], epidermal growth factor (EGF) [12], and low-density culture [13]. Additional interest has been focused on fibroblast growth factors (FGFs) in the fine modulation of endoderm differentiation fates. A positive effect of FGF2 in conjunction with BMP4 and Activin A on endoderm differentiation was recently reported by several groups [14, 15–18]. Xu et al. [17, 18] also identified the role of combined FGF2/transforming growth factor- β (TGF- β)/Activin A/BMP4 signaling in endoderm differentiation and FGF2 signaling during later stage patterning of the gut tube, leading to efficient

Table 1 Overview of the most recent studies on pluripotent stem cell differentiation to endoderm and pancreatic lineage cells

Reference	Source	End point	Key factors	Functional	Transplantation in animal models
Borowiak et al. [21]	ES	Definitive Endoderm 57% to 62% Sox17+	IDE1, IDE2, screening	N/A	Cells integrate into developing gut tube
Wang et al. [58]	ES, iPS	73% Sox17+, 39% primitive gut endoderm	CD49e ⁺ , CD141 ⁺ , CD238 ⁺ sorting	N/A	Cells differentiate to endodermal progeny
Chen et al. [22]	ES	Foregut Endoderm 45.8% Pdx1+	ILV, screening	N/A	Cells differentiate to pancreatic progenitors
Johannesson et al. [11]	ES	32% Pdx1+	FGF4, RA	N/A	N/A
Ameri et al. [19]	ES	18% to 20% Pdx1+	FGF2	N/A	N/A
Mfopou et al. [9]	ES	50% to 80% Pdx1+	Noggin, RA; inhibition of FGF10, BMP	N/A	N/A
Maehr et al. [50]	iPS	Endocrine Specification Mature-like C-peptide-producing cells	T1D cell donors	In vitro, glucose-responsive C-peptide release	N/A
Mao et al. [20]	ES	Immature insulin-producing cells	PLGA scaffolds transplantation	In vitro and in vivo low insulin release	In diabetic mice, decreased fasting blood glucose
Zhang et al. [12]	ES, iPS	25% mature-like insulin- and C-peptide-producing cells	EGF, RA, Noggin, FGF7	In vitro, glucose-responsive C-peptide release	N/A
Matveyenko et al. [8•]	ES	0.8% insulin- and C-peptide-producing cells	Novocell protocol reproducibility	In vivo low insulin and C-peptide release	50% of implants develop into islet-like structures
Cai et al. [13]	ES	72% Pdx1+; endo- and exocrine precursors	Low cell density, RA	N/A	N/A
Nostro et al. [14•]	ES, iPS	Up to 25% C-peptide+, immature endocrine	TGF- β inhibition; Wnt	N/A	N/A
Rezania et al. [15]	ES	Immature and mature-like glucagon-producing cells	ALK inhibitors screening	In vitro and in vivo glucagon release	In normoglycemic and diabetic mice, decreased glucagon secretion
Thatava et al. [23]	iPS	Immature and mature-like endocrine cells	ILV, GLP-1,	In vitro C-peptide release	N/A
Xu et al. [17]	ES, iPS	Up to 10% C-peptide-producing cells	Activin, BMP, FGF2	In vitro C-peptide release	N/A

BMP bone morphogenetic protein; *EGF* epidermal growth factor; *ES* embryonic stem cells; *FGF* fibroblast growth factor; *GLP-1* glucagon-like peptide-1; *ILV* indolactam V; *iPS* induced pluripotent stem cells; *N/A* study not available; *pdx1* pancreatic duodenal homeobox-1; *PLGA* poly(lactic-co-glycolic acid); *RA* retinoic acid; *TGF- β* transforming growth factor- β ; *T1D* type 1 diabetes

pancreatic precursor specification and a highly enriched PDX1-positive population capable of further differentiating into some C-peptide-positive cells. Other recent reports confirm that during the patterning stage optimal levels of FGF2 [19] and FGF4 [11] correlate with improved foregut specification to pancreatic lineage while inhibiting hepatocyte differentiation, however with more modest outcomes. Whereas many protocols use FGF10 during pancreatic precursor differentiation, a recent report validated on multiple human embryonic stem (ES) cell lines associated FGF10 and BMP signaling with hepatocyte differentiation, and showed that inhibiting these factors strongly increases pancreatic Pdx1-positive precursor differentiation achieving 50% to 80% enrichment [9]. The alternative use of FGF7 in foregut endoderm differentiation from definitive endoderm [12, 17] could thus prove to be a solution to circumvent FGF10-driven hepatic lineage specification. Although several recent human stem cell differentiation protocols demonstrated robust endoderm differentiation and enriched PDX1-positive cell populations, endocrine specification was much more restricted and only a fraction of cells in

these studies express insulin or C-peptide. Very few studies were able to report physiologic levels of glucose-responsive endocrine hormone secretion, and even fewer were able to show efficient control of hyperglycemia after transplantation into diabetic mice [6, 8•, 20].

A second approach to protocol optimization is based on high-throughput screening for active small molecules and agonists that mimic protein-based signaling pathways during embryonic development. Treatment with histone deacetylase inhibitors such as IDE1 and IDE2 resulted in 57% to 62% Sox17-positive definitive endoderm differentiation [21], which was reportedly almost as efficient as Activin A. High-content chemical screening identified indolactam V's ability to efficiently induce up to 46% Pdx1-positive pancreatic progenitors from definitive endoderm cultures [22], which prompted inclusion of this compound in other differentiation protocols [23]. Screening for compounds that are active in later stages identified two kinase inhibitors ALK-i I and ALK-i II, which guided the differentiation of pancreatic progenitors to functionally mature endocrine glucose-responsive α cells [15]. Notably,

some of these compound screening reports have been validated *in vivo* by following the differentiated cell progeny after transplantation.

Despite the multitude of differentiation protocols currently described in the literature, current limitations in the ability to generate fully functional β cells from human pluripotent stem cells *in vitro* call for the need to refine the existing protocols, by fine-tuning discrete or reciprocal regulatory mechanisms involved in creating appropriate temporal and spatial differentiation microenvironments in culture. In support of this requirement, elegant studies on mouse development recently showed that the timing and duration of growth factor inductive networks, such as mesodermal BMP and TGF- β signaling, are controlled over very short time frames, in the range of hours, to insure correct cell type specification in the foregut endoderm [24]. Moreover, spatial intra-endodermal cell interactions were shown to be critical for the induction of pancreatic β cells versus non-endocrine pancreas or intestine from adjacent endoderm areas [25]. Furthermore, we know little about the molecular events regulating islet formation and β -cell functional maturation, which typically occurs in the late gestational and early postnatal period. Therefore, we anticipate that future pluripotent stem cell differentiation studies will increase in complexity and that the aim will shift from replicating limited arrays of gene expression profiles of intermediate progeny, to generating fully functional glucose-responsive pancreatic endocrine cells capable of reliably restoring euglycemia in experimental diabetes models.

Endogenous Regenerative Capacity of the Pancreas

The loss of β cells observed in type 1 diabetes mellitus has stimulated great interest in the body's endogenous capacity to regenerate new β cells. Possible regenerative mechanisms include proliferation and/or differentiation from progenitors. β Cells have been shown in numerous studies to replicate after birth in response to pancreatic injury or disease, as well as under certain physiologic conditions such as pregnancy. There is also some evidence to suggest that adult stem cells or other progenitors may give rise to new β cells in the postnatal and adult pancreas (i.e., neogenesis), but this possibility remains controversial, as different laboratories have published conflicting results. Here, we review recent studies, primarily in rodent models, aimed at elucidating the mechanisms and capacity for β -cell regeneration, including evidence for and against adult neogenesis.

Early support for neogenesis arose from immunohistochemical observations, such as the presence of budding insulin-positive cells in the pancreatic duct epithelium

following partial pancreatectomy [26, 27]. However, using a pulse-chase system to label the progeny of pre-existing β cells, Dor et al. [28] provided evidence that all or nearly all new β cells in the adult pancreas—including those generated after pancreatectomy—are derived by replication of pre-existing β cells. Although this finding generated skepticism over the existence of neogenesis, the lineage-tracing approach that was used labeled only β cell-derived β cells, and did not have 100% efficiency, making it impossible to completely rule out the possibility that some β cells arose from other cell types.

In a subsequent study, Nir et al. [29] used a transgenic mouse model to conditionally and selectively induce β -cell death via doxycycline-dependent expression of diphtheria toxin. In addition to conferring cell-type specificity, this strategy also provided exquisite temporal control over both the onset and the duration of β -cell ablation, and eliminates some confounding experimental variables associated with pancreatectomy, such as inflammatory responses. Using this approach in combination with their lineage tracing system, Nir et al. [29] confirmed that most or all regeneration following loss of β cells in mice occurs via replication. Moreover, they found that commonly used immunosuppressants inhibited endogenous regeneration rates by about 80%, suggesting that alternative immunosuppressive strategies may improve outcomes for patients undergoing pancreas or islet transplantation by permitting more active endogenous replenishment mechanisms.

Whereas these studies focused on the pancreatectomy model, other important studies were evaluating the mechanisms responsible for rodent regeneration after pancreatic duct ligation. In 2008, Inada et al. [30] used a cell-lineage tracing approach to determine whether ductal progenitors can give rise to β cells after duct ligation in the adult pancreas. The authors of this study developed a transgenic mouse line in which the progeny of carbonic anhydrase II-positive ductal cells expressed β -galactosidase (β -gal). Following ductal ligation, in which islets distal to the ligation site are destroyed while proximal islets are spared, about 40% of β cells in the distal islets and about 12% in the nonligated islets expressed β -gal, versus about 5% of β cells expressing (endogenous) β -gal in wild-type animals. Together, these results suggest that carbonic anhydrase II-positive ductal cells can give rise to β cells following injury in the adult pancreas.

The potential for neogenesis in the adult pancreas after duct ligation is also supported by results from Xu et al. [31], who mapped the fate of pancreas-specific progenitor cells expressing the transcription factor neurogenin 3 (Ngn3) in adult mice following ductal ligation [31]. The authors demonstrated that Ngn3-positive progenitors can differentiate into β cells *in vitro*, albeit an embryonic pancreas environment.

Despite the results of these carefully conducted studies that support the potential for adult neogenesis, the possibility that pancreatic progenitors give rise to β cells in the adult pancreas remains highly contentious. For example, a study by Lee et al. [32] seems to run counter to the result demonstrated by Xu et al. [31] Using *Ngn3-GFP* mice, Lee et al. [32] showed that *Ngn3* is not reactivated after pancreatectomy demonstrating that *Ngn3*, whose expression is transiently required during pancreas development, is not required for regeneration following pancreatectomy even though substantial pancreas regrowth occurred [32]. Other studies by Teta et al. [33] using a sequential pulse of DNA analogues, which incorporate into dividing cells, also seem to support the concept that new murine β cells arise by replication, rather than from tissue-specific progenitor cells, during normal turnover, after pancreatectomy, or other stimuli. Their results in the pancreas were strengthened by the opposite finding in the intestine and hair follicle, tissues in which endogenous progenitors are well characterized [33].

Using *in vivo* lineage tracing strategies, investigators indelibly labeled progenitor cells expressing hepatocyte nuclear factor 1 β (Hnf1 β), *Muc1*, or *Sox9* and asked whether these cells, which give rise to β cells during normal development, can give rise to β cells after pancreatic injury in the adult mouse. Collectively, these studies suggest that regardless of cell type, differing mechanisms exist for the origin of β cells during embryogenesis versus adult life. For example, in a study published in 2009, Solar et al. [34] traced the fate of pancreatic progenitor cells expressing the early pancreatic transcription factor Hnf1 β . The authors found that in the embryonic mouse, Hnf1 β -positive progenitor cells primarily give rise to ductal cells, but can also assume an acinar or endocrine fate (including β cells) if labeled early in development. In the adult, however, Hnf1 β -positive cells assumed a strictly ductal fate, even after ductal ligation or alloxan-induced β -cell ablation, followed by treatment with the neogenesis-promoting factors EGF and gastrin. Two more recent studies have supported the findings of Solar et al. [34]. Kopinke and Murtaugh [35] followed the fate of cells expressing the exocrine pancreatic marker, *Muc1*, and found that these cells can give rise to endocrine cell types during embryogenesis but not after birth. Similarly, Kopp et al. [36] followed the fate of cells expressing *Sox9*, which are found at the interface of small ducts and acini and had previously been shown to be capable of assuming an endocrine fate when injected into fetal pancreatic explants [37]. The authors found that *Sox9*-positive cells were multipotent before birth, giving rise to all types of pancreatic cells. However, in the adult, *Sox9* cells failed to generate endocrine cells under basal conditions or after ductal ligation, although ligation did lead to the presence of *Ngn3* expression in *Sox9*-positive cells [36].

A study from Thorel et al. [38] revitalizes the concept of adult β -cell neogenesis. The authors induced diphtheria toxin-mediated apoptosis selectively in β cells using a transgenic mouse system similar to the one used by Nir et al. [29]. However, in this study expression of the toxin resulted in almost total ablation (> 99%) of β cells. Under these conditions, regeneration of insulin-secreting cells occurred not via β -cell replication but primarily through transdifferentiation of pre-existing α cells. Interestingly, the resulting insulin-positive cells were also glucagon-positive, indicating that they retained at least some fundamental aspects of α -cell identity. Although the physiologic characteristics of these “hybrid” cells remain unexplored, they may prove useful in harnessing the endogenous regenerative capacity of the pancreas to achieve therapeutic outcomes.

As this survey of recent studies illustrates, the cellular origins of new β cells in the adult pancreas remain highly contentious. When comparing the results of these studies, it is important to consider how each study differs from the others. For example, different experimental models of pancreatic injury produce different percentages of β -cell ablation, may trigger apoptotic or necrotic cell death, and may or may not be accompanied by inflammatory responses. Furthermore, each study was specifically designed to test whether β cells can arise from a particular cell type of interest. The key methods and findings of each of these studies are summarized in Table 2.

Finally, it must be noted that most of what is known about β -cell regeneration, including all of the studies described above, comes from rodent models. Because of the inherent difficulty of studying in human subjects, little is known about turnover of β cells over the human life span. In a recent study, Perl et al. [39] showed that β -cell turnover primarily occurs in the first three decades of human life. The authors analyzed iododeoxyuridine/bromodeoxyuridine expression in pancreas tissue from 10 deceased patients who had previously received thymidine analogues as part of a cancer-related clinical trial. Cells co-labeled with analogues and insulin were only observed in patients younger than 30 years old. The authors further confirmed this finding using a recently developed DNA carbon-dating technique [40] on tissue from three cadaver donors. Thus, although these findings are based on a relatively small sample sizes, it would appear that the capacity for β -cell generation is extremely limited in the adult human pancreas. However, the replicative power of β cells may be revealed during conditions such as pregnancy [41] or autoimmune disease [42]. The relatively low β -cell proliferation rate under basal conditions and significantly higher replication rate under provocative conditions indicates that β -cell mass is tightly regulated, yet there is substantial potential for expansion of existing β cells.

Table 2 Some recent murine models studying mechanisms of endogenous β -cell regeneration in the adult pancreas

Reference	Ablation method	Cell type traced	Supports adult neogenesis?
Dor et al. [28]	Partial pancreatectomy (70%)	Existing β cells	No
Teta et al. [33]	Partial pancreatectomy	Not specific, replicating cells	No
Nir et al. [29]	DTX ablation of β cells (~ 80%)	Existing β cells	No
Inada et al. [30]	Ductal ligation	Carbonic anhydrase II+ differentiated ductal cells	Yes
Xu et al. [31]	Ductal ligation	Ngn3+ progenitor cells	Yes ^a
Solar et al. [34]	Ductal ligation, alloxan-induced diabetes	Hnf1 β + progenitor and ductal cells	No
Thorel et al. [38•]	DTX ablation of β cells (> 99%)	α cells	Yes
Kopinke and Murtaugh [35]	–	Muc1+ progenitor and adult ductal and acinar cells	No
Kopp et al. [36]	Ductal ligation	Sox9+ progenitor and ductal cells including duct/centroacinar cells	No

^aNgn3+ cells became β cells in an embryonic pancreas environment

DTX diphtheria toxin; Hnf1 β hepatocyte nuclear factor 1 β ; Ngn3 neurogenin 3

Reprogramming Cells into β Cells

Methods for de- and transdifferentiation of mature cell types, or now more commonly called cellular reprogramming, are likely to contribute to the future of regenerative medicine because they could facilitate restoration of cellular functions lost in disease states. In the case of diabetes, a primary focus has been on reprogramming different cell types to become glucose-responsive, insulin-secreting β -like cells.

There has been long-standing interest in the possibility of transforming hepatocytes into β cells. Liver and pancreas tissues share a common endodermal origin and hepatocytes, like β cells, express glucose transporter 2 (GLUT-2) and glucokinase, two key proteins involved in stimulus secretion coupling in the β cell. However, it is well known that hepatocytes lack several important components of insulin secretion, such as enzymes that convert proinsulin to insulin. In one study, adenovirus-mediated gene transfer of the transcription factor Pdx1 into mice was used to reprogram 60% of hepatocytes [43]. The authors detected increased quantities of insulin and proinsulin in the liver and were able to show decreased blood-glucose levels in animals with diabetes induced by streptozotocin. Subsequently, another study from Wang et al. [44] tried to use adeno-associated virus (AAV) instead of adenovirus to reprogram liver cells. Interestingly, the authors of this study found that AAV-mediated transfer of β -cell markers Pdx1 or Ngn3 alone failed to reduce symptoms of diabetes, but when it was coadministered with an irrelevant adenoviral vector the treatment corrected hyperglycemia and weight loss, increased blood insulin levels, and improved glucose tolerance in diabetic mice. The authors suggested that the

immune response against adenovirus is required for reprogramming of hepatocytes into a β -like cell fate.

Several other transcription factors important for β -cell differentiation have also been introduced into the liver. For example, Kaneto et al. [45] showed that adenovirus transfection of *MafA*, *Pdx1*, and *NeuroD* induced production of insulin from hepatocytes and helped control blood-glucose levels in diabetic animals. Sapir et al. [46] reprogrammed cells from human liver biopsies using adenovirus-mediated transfer of *Pdx1*. The reprogrammed cells secreted C-peptide and expressed GLUT-2 as well as several important transcription factors present in mature β cells, such as Nkx2.2, Nkx6.1, and Pax4. The cells also expressed the neuroendocrine vesicular markers SCG-2 and SGNE1, as well as prohormone convertase 2. After these cells were transplanted into streptozotocin-induced diabetic mice, the animals exhibited decreased blood-glucose levels compared with animals that received non-transformed human liver cells. Although these results are promising, it remains unclear whether the cells maintain the β -like phenotype for a long period of time, especially if transplanted into the liver where they could conceivably revert to a hepatocyte identity.

Focusing on an abundant, nonendocrine pancreas cell type, Zhou et al. [47•] converted exocrine cells of immune-deficient mice into insulin-secreting cells through viral-mediated gene transfer of the transcription factors *Ngn3*, *MafA*, and *Pdx1*. This transformation partly reduced the elevated blood-glucose concentration observed following ablation of pre-existing β cells with streptozotocin, but failed to fully normalize glycemia. The lack of full rescue may have been due to insufficient numbers of new insulin-secreting cells, or to a lack of organized islet structures that could respond properly to glucose stimuli. Nonetheless, the transformation of cells

already present in the adult pancreas to assume a β -like function is an important and promising advance.

In another important study illustrating cellular reprogramming to β cells, Collombat et al. [48] used a Cre/lox transgenic mouse system to selectively drive ectopic expression of *Pax4* in cells that expressed either Pdx1 (pancreatic progenitor cells), Pax6 (endocrine progenitor cells), or glucagon (α cells) in postnatal adult mice. Expression of *Pax4* in each of these cell types increased the number of insulin-positive cells present in the pancreas at the expense of other endocrine cell types. Pax4 expression also restored normoglycemia in younger (≤ 4 weeks old) mice following ablation of β cells with streptozotocin, and 41% of these animals lived for 2 months. However, older (> 4 weeks old) animals died spontaneously and showed signs of hyperglycemia, in spite of also having increased insulin-positive cell counts. Furthermore, all older animals expressing ectopic Pax4 died following treatment with streptozotocin, indicating an age-restricted effect. It is unclear whether insulin-positive cells generated by ectopic Pax4 expression are nonfunctional in the older animals, or whether these animals have become insensitive to insulin. Nonetheless, this study provides important evidence that α cells may be a useful progenitor population from which insulin-producing cells could be derived. Future investigations may be directed toward achieving direct cellular reprogramming without introduction of exogenous genes and/or by specifically altering chromatin states.

Although considerable progress has been made in reprogramming cells toward a β -like state, there remains no proven method for generating a continual source of glucose-responsive, insulin-secreting β cells with long-term stability suitable for the treatment of diabetes. Future challenges will include not only establishing such a source, but also making it safe for the treatment of human patients. Nevertheless, the possibility of acquiring cells from a patient's own body, transforming them into a stable line of glucose-responsive, insulin-secreting β -like cells, and surgically reintroducing them would likely revolutionize the treatment of diabetes.

Translational Considerations

To fulfill their therapeutic potential, alternative sources of β cells must meet a challenging set of requirements, such as functional maturation to clinically relevant numbers of endocrine cells, and persistent engraftment and survival, but also must satisfy safety concerns related to tumorigenicity, genetic stability, adventitious pathogens, containment, host tissue toxicity, and dosage.

To begin with, solid evidence that human pancreatic precursors can mature functionally in vitro or in vivo and

cure experimental diabetes in animal models is absolutely necessary, yet is currently still missing. In addition to differentiating into functional endocrine cells for the duration of experimental assays, ideally pancreatic precursors must also be capable of engrafting and surviving in vivo for extended periods of time. To this end, innovative solutions, such as controlling the size of precursor clusters by covalent microcontact printing of laminin on cell culture substrates [49], show promise to allow the efficient production of human uniform-size islet-like structures with optimized nutrient and oxygen access after engraftment in vivo.

An additional challenge to the in vivo survival of pancreatic precursors in human patients is the susceptibility of β cells to inflammation-induced cell death and the presence of autoimmune and alloimmune responses that may compromise an otherwise functional transplant. In this respect, the breakthrough of generating iPS cells from human tissue [1, 2] and differentiating them to pancreatic cell types [12, 14, 18, 23, 50, 51] that potentially may be transplanted back to the patient from whom the iPS cells were derived for β -cell replacement holds a significant therapeutic promise for circumventing allogeneic immune responses that ES-derived precursors might otherwise elicit. A proof of concept of using iPS cell progeny in curing diabetes has been recently reported in a mouse system. Alipio et al. [52] derived iPS cells from murine skin fibroblasts and differentiated them into insulin-secreting β -like cells in vitro that responded to glucose stimulation under physiologic or pathologic conditions. These insulin-secreting β -like cells were transplanted via intraportal vein injection into the livers of two different mice lines modeling type 1 and type 2 diabetes. Although cells engrafted and subsequently corrected hyperglycemia in both models, the insulin-deficient model that was used was not an autoimmune model. In fact, studies testing the efficacy of syngeneic iPS cells in autoimmune diabetes models in vivo or alternatively testing the susceptibility of type 1 diabetic human iPS cells to autoimmune destruction in vitro have yet to be reported. Nonetheless, whether iPS cell-derived β cells would be destroyed upon transplantation into subjects with type 1 diabetes is an important clinical issue as some past human experiments might predict [53]. In these cases, pancreatic grafts from a nondiabetic twin were transplanted into the diabetic twin without immunosuppression. Pathologic evaluation of these pancreas transplants, which failed early post-transplant, exhibited classic insulinitis and patients reverted to hyperglycemia, whereas nonpancreatic grafts would normally be accepted between these twin siblings. A similar pancreas transplant that was subsequently performed with low-dose immunosuppression succeeded long term [54]. Based on these data, the possibility exists that destruction of any autologous β -cell population, be it derived from iPS cells in vitro, induced by reprogramming, or by endogenous

regeneration, when placed in an autoimmune environment, may occur and will thus need to be formally tested.

With the rapid advance of preclinical research on new sources of β cells for cell-replacement therapies, a number of safety and good manufacturing practice issues begin to arise. For instance, one of the principal unique aspects of using ES or iPS cells as alternative sources of β cells is the need to thwart the teratogenic potential that transplantation of non-purified stem cell-derived populations might hold. Moreover, recent studies on numerous human iPS cell lines reprogrammed using a variety of methods showed that iPS cells may harbor protein-coding point mutations, which could have arisen in the original donor tissue or resulted from reprogramming [55, 56] as well as an assortment of epigenetic mark abnormalities [57], all adding to a possible increase in their tumorigenic potential. One prospective solution to these challenges is the sorting of target precursors from heterogeneous populations based on surface molecules associated with differentiation. Newly described CD141 and CD238 surface markers allowed for higher efficiency of human definitive endoderm generation by cell enrichment [58] and similarly, selection of CD326- (i.e., epithelial cell adhesion molecule) [59] or CD24 [60]-expressing cells resulted in higher yields of pancreatic endoderm cells, while significantly diminishing the teratogenic/tumorigenic cell numbers allowing for safer transplantation [58, 59]. As an alternative, bioengineering innovations such as encapsulation devices that permit molecular diffusion while preventing precursor cell escape or immune cell penetration are currently being considered [61].

A new level of translational complexity is introduced by reprogramming protocols. In addition to future efficacy and scalability tests that would need to be done in humans, safety concerns arise related to various gene delivery systems. Primary among these concerns is the issue of random integration and potential for mutagenesis. Bystander effects and toxicities of vectors and viral delivery systems are likewise critical issues. Even though a cellular reprogramming clinical trial would likely be approached with caution, such an approach holds significant potential to materialize into relevant therapies while nongenetic means of reprogramming are actively being investigated. Altogether, there is significant evidence in recent literature surveys that innovative resources and sophisticated new methodologies are actively being applied to address many of the challenges that translational integration of alternative β -cells sources currently face.

Conclusions

Society's ability to ultimately confront the dramatic increase in diabetes prevalence worldwide and the

limitations of existing therapeutic options will largely depend on the aggressiveness to which research pursues developing alternative sources of β cells. Whether new β cells are derived from human pluripotent stem cells in vitro and transplanted, or generated in vivo by either reprogramming adult cells or regenerating precursors, these therapies offer tremendous advantage over islet or pancreas transplantation by virtue of their renewal potential, ample supply, syngeneic source, ability to be quality control tested, and capacity for cell number control through homeostatic regulation. With these beneficial attributes comes the need to control the outcome of their differentiation to achieve the desired functional endocrine maturity in addition to finding ways to overcome autoimmunity. Collaborative multidisciplinary research bringing together the fields of autoimmunity, tolerance, encapsulation, biomaterials, stem cells, and developmental biology will undoubtedly furnish future therapeutic opportunities.

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