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REVIEW

In Vitro Differentiation and Expansion of Human Pluripotent Stem Cell-Derived Pancreatic Progenitors

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■ Abstract

Recent progress in understanding stem cell biology has been remarkable, especially in deciphering signals that support differentiation towards tissue-specific lineages. This achievement positions us firmly at the beginning of an era of patient-specific regenerative medicine and human disease modeling. It will be necessary to equip the progress in this era with a reliable source of self-renewing progenitor cells that differentiate into functional target cells. The generation of pancreatic progenitors that mature *in vivo* into functional beta-cells has raised the hope for new therapeutic options in diabetes, but key challenges still remain including the production of sufficient numbers of cells for research and transplantation. Recent approaches to this problem have shown that the presence of organ- and stage-specific mesenchyme improves the generation of progenitors, from endoderm to endocrine cells. Alternatively, utilization of three-dimensional culture may improve the efficiency and yield of directed differentiation. Here, we review the current knowledge of pancreatic directed differentiation and *ex vivo* expansion of pancreatic progenitors, including recent advances in differentiation strategies for the generation of pancreatic progenitors, and we discuss persistent challenges which will need to be overcome before personalized cell-based therapy becomes a practical strategy.

Keywords: diabetes · pluripotent stem cell · definitive endoderm · pancreas · insulin-producing cells · differentiation · self-renewal · expansion

1. Introduction

The idea of tissue regeneration, especially of human organs, is not new, appearing first in ancient mythology, in the story of Prometheus, whose liver was ripped out by a vulture every night only to regenerate the next morning, which is one of the best known examples of regeneration phenomenon. More scientifically related examples of long-term interest in regeneration come from seventieth century observations of lizards which can regenerate their own tails after amputation. The desire to live longer, replace injured tissues or simple renew the body continues to the present day. However, the most practical application of regenerative biology is the cell replacement seen in different types of disease. One example is diabetes mellitus type 1, an autoimmune disease that manifests itself by the destruction of insulin-secreting beta-cells in the pancreas. Diabetes mellitus type 2 is characterized by failure to maintain proper glucose balance and results from inadequate beta-cell mass and function that can no longer compensate for insulin resistance.

Diabetes is a metabolic disease resulting in the failure of glucose homeostasis. In consequence, it leads to hyperglycemia, causes damage of different tissues and organs, and finally shortens the life span. Type 1 diabetes (T1D) is essentially an ideal candidate for cell therapy as one cell type, the beta-cell, is missing. According to the International Diabetes Federation, diabetes currently af-

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fects approximately 360 million people worldwide, a number which is likely to increase to 550 million by 2030. Approximately 10% of the patients will be diagnosed with T1D.

Several clinical studies have shown that the replacement of endogenous beta-cells with cadaveric islets results in the restoration of insulin independence. However, large-scale application of islet tissue transplantation in T1D patients is hampered by the limited availability of cadaveric donor tissue. Therefore, there is significant interest in finding new, robust sources of beta-cells. Currently, there are several promising strategies to achieve this end, including directed differentiation of pluripotent stem cells, reprograming and trans-differentiation of other mature cell types to a beta-cell. Pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), are characterized by the unique ability to self-renew almost indefinitely and to give rise to every mature cell type. PSCs hold exceptional promise as a source of functional differentiated cells for use in regenerative medicine and as an *in vitro* model system to study human development and diseases. Successful examples of PSC differentiation to neural, epidermal, cardiac, and other cell types are typically defined by progressive, stepwise lineage specification and involve progression through an intermediate progenitor state prior to terminal differentiation. Discussed below are recent advances in the application of PSCs to generate pancreatic cells *in vitro* together with their advantages and disadvantages.

2. Generation of pancreatic progenitors from pluripotent stem cells: successes and obstacles

2.1 Pancreatic progenitor specification and expansion during embryonic development

Cells destined to form the pancreas arise from the primitive gut tube which develops from definitive endoderm. The pancreatic domain first appears around embryonic day 9.5 (e9.5) in mice with the thickening of dorsal gut tube epithelium. Shortly afterwards, two ventral buds simultaneously grow from the endodermal epithelium in close proximity to the hepatic bud. After the dorsal and ventral gut tube endoderm commits to a pancreatic fate, self-renewing multipotent pancreatic progenitors are formed around e11.5 and express *Pdx1*, *Ptf1a*, *Sox9*, *Nkx6.1* and *Hnf6*. Deficiency of either gene causes partial or complete pancreas agenesis or malfunction, in mice and humans [1-5]. Despite these and other insights, the genetic blueprint of pancreatic progenitors is not yet fully understood.

Abbreviations:
Alk5 - activin receptor-like kinase 5
BMP - bone morphogenetic protein
Cdx2 - caudal-type homeobox transcription factor 2
c-Myc - v-Myc avian myelocytomatosis viral oncogene homolog
CPA - carboxypeptidase A
CXCR4 - chemokine (C-X-C motif) receptor 4
DAPT - N-(N-(3,5-difluorophenacetyl)-L-alanyl)-S-phenylglycine t-butyl ester
DE - definitive endoderm
ECD - E-cadherin
EP - endocrine progenitor
ESC - embryonic stem cell
Ex4 - exendin 4
FGF - fibroblast growth factor
FoxA2 - forkhead-box protein A2
Gata4 - GATA-binding protein 4
GFP - green fluorescence protein
GLP1 - glucagon-like peptide 1
Gsc - goosecoid
Hes1 - hairy and enhancer of split 1
hESC - human embryonic stem cell
Hex - hematopoietically expressed homeobox protein
HGF - hepatocyte growth factor
Hnf6 - hepatocyte nuclear factor 6
hPSC - human pluripotent stem cell
IDE1/2 - inducer of definite endoderm 1/2
IGF - insulin growth factor
iPSC - induced pluripotent stem cell
Irx1 - iroquois homeobox protein 1
KGF - keratinocyte growth factor
Lgr5 - leucine-rich repeat-containing G-protein-coupled receptor 5
MafA - v-maf musculoaponeurotic fibrosarcoma oncogene homolog A
Mes2 - maternal effect sterile protein 2
mRNA - messenger ribonucleic acid
NeuroD - neurogenic differentiation
Ngn3 - neurogenin 3
Nkx6.1 - Nk6 homeobox protein 1
Oct4 - octamer binding transcription factor 4
Pax4 - paired box homeodomain transcription factor 4
PDGFR - platelet-derived growth factor receptor
Pdx1 - pancreatic and duodenal homeobox 1
PE - pancreatic endoderm
PI3K - phosphatidylinositide 3 kinase
PSC - pluripotent stem cell
Ptf1a - pancreas transcription factor 1 subunit alpha
RA - retinoic acid
RBJ - Ras-associated protein Rap1
Shh - sonic hedgehog
Sox9 - sex-determining region Y box 9
T1D - type 1 diabetes
TGF-beta - transforming growth factor beta
Ucn3 - urocortin 3
VEGF - vascular endothelial growth factor
WNT - wingless-type MMTV integration site family

Although *Pdx1* expression is a dominant feature of multipotent progenitors, not all *Pdx1*$^+$ cells have the ability to generate multiple lineages. Furthermore, fate regulation has a temporal basis; cells are multipotent as early as e10.5, but not later than e12.5 *Pdx1*$^+$. The e12.5 *Pdx1*$^+$ trunk progenitors become developmentally restricted with loss of their ability to generate ducts [6]. However, at this stage, distal tip cells of pancreatic epithelium that co-express *Ptf1a*, c-*Myc*, and *CPA* together with *Pdx1* can differentiate to endocrine, exocrine and ductal cells [7]. The tip cells are located in close proximity to the surrounding mesenchyme, and signaling by FGF and/or other yet unidentified molecules in the mesenchyme is thought to participate in maintaining a *Pdx1*$^+$ progenitor pool. Consequently, *Pdx1*$^+$ cells located in the distal part of the trunk viewed from the mesenchyme lose their multipotent character earlier [8]. Loss of FGF signaling leads to a block in progenitor proliferation [9]. Accordingly, ectopic expression of FGF10 stimulates the proliferation of pancreatic progenitors and simultaneously revokes pancreatic differentiation.

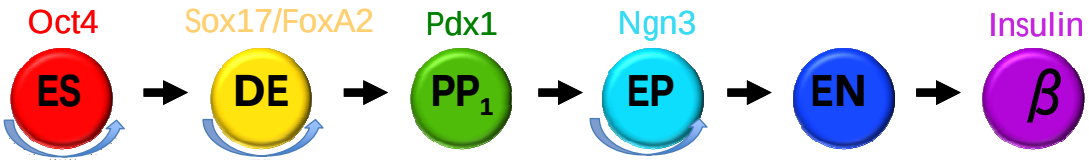
Once the pancreatic progenitors emerge, they have two choices: they can either self-renew or differentiate into ductal, endocrine or exocrine progenitors. The two independent effects are coordinated by Notch signaling. Previous studies have shown that Notch represses Ngn3 expression and maintains the progenitor state. Genetic studies on loss of function in various Notch pathway genes, such as delta-like ligand and intracellular mediators such as RBJ and Hes1, show that progenitor depletion results from upregulation of the transcription factor neurogenin 3 (*Ngn3)* and premature endocrine differentiation. Conversely, the expression of Notch intracellular domain in pancreatic progenitors leads to their proliferation and prevent endocrine differentiation [10-14]. A recent study by Maike Sander showed that the function of Notch during pancreatic specification is more dynamic and that Notch does not operate in simple on/off switch mode. High levels of Notch block endocrine differentiation, while intermediate Notch activity is not sufficient to maintain the expression of *Hes1* repressor, and thus enables Ngn3 expression and endocrine differentiation. Therefore, these findings indicate that a gradient of Notch activity leads to distinct cell fate decision during pancreas differentiation [15].

Although Notch signaling seems to play an important role in directing pancreatic progenitors to either proliferate or differentiate, the mechanism by which responding cells interpret Notch signal is unclear. A possible candidate mechanism includes the Sox9-positive progenitor population since Notch signaling promotes the *Sox9* expression that in turn cell-autonomously activates *Ngn3* expression. Whatever the explanation, the expansion of multipotent progenitors must be precisely regulated, as different mature pancreatic populations are generated at different time points during development. Indeed, any disturbance in normal pancreatic progenitor proliferation and in cell cycle exit has an undesirable impact on the organ size and cellular composition of the pancreas [16].

The endocrine program in the pancreas is initiated by a single key transcription factor: *Ngn3*. Mice with ablated *Ngn3* lack islets and die shortly after birth from hyperglycemia [17]. Moreover, lineage-tracing experiments in mice confirmed that *Ngn3* marks all endocrine cells within islets [18]. Its expression is tightly regulated by Notch signaling, which represses *Ngn3* and maintains the progenitor state within pancreatic epithelium [10], with a peak at around e15.5 in the developing pancreas corresponding to the time window for endocrine specification. Enforced ectopic expression of *Ngn3* at a different time window can stimulate differentiation to different endocrine cells, depending on the cellular context. The results of several studies indicate that random *Ngn3* overexpression leads to over-representation of somatostatin- and glucagon- but not insulin-positive cells [19, 20]. Thus, endocrine specification requires precisely regulated expression of *Ngn3* at a time when pancreatic epithelial cells are competent to respond to this signal. Ngn3 also marks endocrine cells in stomach and intestine [21, 22]. Interestingly, the null mutation of human Ngn3 gene leads to the loss of intestinal but not pancreatic endocrine cells, suggesting that pancreatic endocrine specification is divergent in human and mice [23].

Downstream of *Ngn3* there are several transcription factors that regulate the formation of specific endocrine cells within islets. Loss of *Nkx6.1*, for example, results in severe beta-cell depletion in mice [24], while loss of another family member, *Nkx2.2*, leads to complete absence of beta-cells with a reduction in the number of glucagon- and pancreatic polypeptide- producing cells [25]. Other transcription factors that regulate endocrine cell development include *Isl1*, *Pax4* and *6*, *NeuroD*, and *Irx1/2*. Many of these factors are also expressed at postnatal stages and modulate beta-cell function and maintenance [26].

The key regulators of beta-cell maturation have not yet been fully discovered. However, mouse knockout studies on the Maf transcription factor



A: Inducers		WNT Activin A/Nodal LY294002 (inhibitor PI3K) FGF2 BMP4 IDE1, IDE2	FGF7, 10, 2 or 4 Noggin or LDN-193198 (BMP antagonists) KAAD-cyclopamine (Hh inhibitor) RA EGF Indolactam V (PKC agonist)	DAPT (gamma-secratase inhibitor) Noggin or LDN-193198 (BMP antagonists) Alk5 inhibitor II (TGF-beta antagonist) SANT or KAAD-cyclopamine (Hh inhibitors) PdBU (PKC inhibiotor) Forskolin (PKA inhibitor) Dexamethasone Activin A EGF	BMP, HGF, IGF, GLI or *in vivo* maturation
B: Expansion *in vitro*	Yes, LIF for mES FGF2 for human PSCs	Yes, FGF2, BMP4, VEGF Ectopically expressed Sox17 Co-culture with mesenchyme (MES1, MES2) Matrigel+mefs+BMP4+ FGF2+EGF+VEGF		Yes, Co-culture with mesenchyme (MES3, MES4)	
Freezing down	Possible	Possible	Possible		
C: Transplantation *in vivo*			Possible	limited success	
D: Challenges	Limited markers available to purify specific stages. Undifferentiated PSCs must be removed before transplantation. In case of iPSC-based therapy, usage of viral factors. Genomic instability. Beta-cell phenotype must be sustained in culture. Progressively decreasing differentiation efficiency.				

Figure 1. Stepwise differentiation of pancreatic stem cells (PSC) into pancreatic beta-cells. Pancreatic directed differentiation *in vitro* consists of four major steps which can be distinguished by the expression of a panel of specific markers such as Oct4 for ES cells, Sox17 and FoxA2 for definitive endoderm (DE), Pdx1 for pancreatic endoderm (PE), Ngn3 for endocrine progenitors (EP), and insulin for beta-cells. **A**: Signals utilized for *in vitro* progressive specification of PSCs into beta-cells. **B**: Options and conditions for expansion and long-term storage of PSC-derived pancreatic populations. **C**: Options for transplantation. **D**: Major challenges for clinical application of PSC-derived beta-cells.

family indicate that a switch from *MafB* to *MafA* members is pivotal for unraveling the beta-cell phenotype. Mature beta-cells maintain the expression of *Pdx1*, *Nkx6.1*, *Pax6*, and *MafA* throughout adulthood [27-29].

2.2 In vitro differentiation of pancreatic progenitors from PSCs

Formation of endoderm. Several *in vitro* protocols have been devised to direct the differentiation of PSCs into the pancreatic lineage. The most successful approaches promote the transition of cells through a series of intermediate stages designed to mimic development *in vitro*. In the pancreas, this entails progressing from PSCs, marked by expression of octamer-binding protein 4 (Oct4; also known as Pou5f1), to definitive endoderm, marked by expression of the transcription factor SRY-box containing gene 17 (Sox17) and the gene encoding forkhead box protein A2 (FoxA2). This developmental population is followed by pancreatic progenitors, marked by expression of the transcription factor, pancreatic and duodenal homeobox1 (Pdx1) gene, endocrine progenitors, marked by expression of Ngn3, and finally mature beta-cells that express insulin.

Under optimal conditions, the transition through these developmental stages should proceed at the expense of other germ layers and of other endoderm lineages. D'Amour and colleagues achieved a major breakthrough in driving pancre-

atic differentiation by manipulation of signaling pathways in a stepwise manner to recapitulate pancreatic development *in vitro* (**Figure 1**) [30, 31]. Briefly, they used a combination of WNT and TGF-beta signaling in absence of serum to coax cells in the culture to develop into definitive endoderm. This was followed by a short incubation with FGF for gut tube patterning. Multipotent *Pdx1*$^+$ pancreatic progenitors were induced by simultaneous application of retinoic acid (RA), FGF family members, and BMP inhibitors. Endocrine progenitors were induced by Notch inhibition via gamma-secretase antagonist (DAPT) along with exendin 4 (Ex4), glucagon-like peptide analog. After 18-21 days, this protocol generated insulin-producing cells from hESCs, a high proportion of which were poly-hormonal and expressed another endocrine hormone, glucagon. Moreover, these *in vitro*-generated cells did not respond properly to the glucose in the medium, so that the final products of this protocol were beta-like cells rather than functional beta-cells. Thus, whether *in vitro* conditions can be suitably modified to produce functional beta-cells remains to be demonstrated.

Other laboratories have reproduced the initial protocol with several hESC and iPSC lines, albeit with different efficiency. Furthermore, a number of modifications to this original protocol have been reported that the aim of which was to improve efficiency and shorten the time of differentiation as well as to define media conditions in chemical terms. The increasing body of pancreatic differentiation protocols published makes evaluation of all of them into a daunting challenge. Especially since it has been reported that cell lines vary in their ability to generate pancreatic and other lineages [32, 33]. In general, the most reproducible strategies involve the manipulation of signaling pathways to recapitulate key steps in pancreatic development in the early embryo.

Developmental biology studies in mice identified nodal, a member of the TGF-beta pathway as a key factor for definitive endoderm induction and patterning. Most protocols utilize nodal-related activin A, a highly bioactive protein that mimics nodal activity. However, functional evaluation of nodal or activin A-induced definitive endoderm revealed different behaviors *in vivo* when injected into the gut tube of developing mouse embryos. Only nodal, but not activin A-treated endoderm incorporates into the gut tube and forms ectopic gut tube after transplantation [34]. Besides the TGF-beta family, it was also shown that LY294002- or wortmannin-mediated inhibition of PI3K pathways could positively modulate definitive endoderm induction in the presence of activin A [35]. Furthermore, a combination of activin A and FGF2 or BMP4 can be used to optimize the efficiency of PSC transition into definitive endoderm. Lastly, small molecules such as IDE1 and IDE2 were also shown to induce endoderm from mouse and human ESCs without the use of activin A (**Figure 1**) [36].

One important aspect of this early endoderm specification and patterning is the concentration of activin A and the timing of its application. For instance, lower concentrations of activin A induce mesoderm, while prolonged exposure to this growth factor promotes the hepatic lineage, with restricted activin A exposure followed by WNT and FGF4 favoring intestine development [37]. Together, these studies highlight the dynamic and precise regulation of TGF-beta signaling that is required for proper recapitulation of pancreatic development *in vitro*. The TGF signaling requirement might also vary depending on the global signaling make-up of different PSC lines and results in a different efficiency of endoderm induction among PSC lines.

Once definitive endoderm is formed, the cells need to be coaxed into becoming pancreatic epithelium at the expense of other endodermal derived organs. This step has been achieved by temporally manipulating FGF, BMP, sonic hedgehog (Shh), and RA signaling (**Figure 1**). FGF signaling induces posterior and represses anterior gene expression and foregut tube development. FGF7 (also known as KGF) and FGF10 are expressed in human pancreatic mesenchyme and induce proliferation of human pancreatic explants [38]. The importance of endoderm patterning was shown by studies in which FGF7 was excluded from protocols or FGF pathway was blocked by small molecule inhibitors (LY294002 and U1026); in both cases pancreatic progenitor induction was repressed [39]. Other FGF family members, such as FGF2/4 and EGF, have been incorporated into some differentiation protocols to improve pancreatic progenitor derivation [40].

The role of BMP signaling in pancreatic differentiation is more complex. There are strong experimental data from several model organisms suggesting that suppression of BMP signaling is required for induction of the ventral pancreatic bud at the expense of hepatic fate. In accord with these data, the BMP inhibitors applied during *in vitro* differentiation in combination with other factors improved the efficiency of *Pdx1*$^+$ cell induction and promoted endocrine fate [30]. Without BMP inhibitors at this step, endoderm will proceed into hepatic lineages. However, endogenous BMP sig-

naling levels have been shown to vary among cell lines, so that inhibition of BMP signaling is necessary for some, but not all, PSC lines to induce pancreatic progenitors. Furthermore, the hESC lines with high BMP expression levels differentiate to hepatoblasts more readily [37]. Two other modulators commonly used for pancreatic specification, RA and Shh, are required for dorsal pancreas specification in several model organisms.

Generation of endocrine progenitors. All of these strategies induce *Pdx1* expression, but they do not simultaneously ensure that all *Pdx1*$^+$ cells have the same capacity to produce endocrine progenitors. Besides pancreas, *Pdx1* is also expressed in the dorsal foregut, together with Sox2 in the stomach, and with Cdx2 in the intestinal endoderm and extrahepatobiliary system [41-43]. As specific markers that distinguish between ventral and dorsal pancreas are limited, it is often unclear whether *Pdx1*$^+$ cells induced *in vitro* correspond to the dorsal or ventral region, which might explain some differences in efficiency among published protocols. Thus, better characterization of induced *Pdx1*$^+$ cells could shed some light on how to manipulate these progenitors to the desired mature cell type. In this regard, there is a number of other genes besides *Pdx1*, including *Sox9, Ptf1a* and *Nkx6.1,* that are necessary for endocrine specification.

Therefore, the endocrine specification is less well studied. There is no clear consensus between laboratories on which signaling pathways should be manipulated for proper and efficient specification into *Ngn3*$^+$ endocrine progenitors. One signaling pathway modulated in various protocols is Notch, and Notch inhibitors such as DAPT are put into culture following pancreatic epithelium specification, with variable success depending on the cell line. Based on studies of pancreatic development in *Smad4*-deficient mice, Nostro and colleagues included the inhibition of Bmp4 and TGF-beta into an endocrine progenitor induction regimen [37]. Detailed analysis revealed that Bmp signaling inhibition was primarily responsible for an increase in insulin expression, while TGF-beta inhibition promoted cell survival, which led to the increase in endocrine cells. Accordingly, Rezania *et al*. identified Alk5 inhibitor II as a small molecule that promoted endocrine fate in the presence of noggin [44]. However, the promotion took only place when BMP4 was inhibited in addition, which means that both BMP and TGF-beta pathways need to be inhibited. Similarly, studies by Nostro *et al*. identified Alk5 inhibitor as inducer of endocrine fate in hESC-derived pancreatic progenitors [45]. Additional small molecules identified in the independent screen for endocrine inducers were forskolin (an adenylate cylase activator) and dexamethasone. Although, these studies provide knowledge of how to improve the efficiency of endocrine specification, little is known about maintaining endocrine progenitors *in vitro* and the development of reproducible methods to generate mature beta-cells requires further studies.

Terminal differentiation to beta-cells. The final stage of pancreatic differentiation involves the maturation of endocrine progenitors to single hormonal beta-cells that are insulin-secreting in a glucose-dependent manner. Engraftment of hESC-derived pancreatic endocrine cells into mice supports their maturation into beta-cells that release C-peptide in a glucose-dependent manner [46]. However, currently there are no signaling modulators known to allow the efficient induction of physiologically competent mature beta-cells *in vitro.* Proposed signals included HGF, IGF, and glucagon-like peptide 1 (GLP1) or its analog, Ex4, but the incorporation of these signals into a current differentiation regimen did not lead to the generation of mature beta-cells [30, 47].

One of the challenges here, which prevents full understanding of the maturation mechanism, is the lack of markers that allow separation of immature from mature beta-cells. Two such candidates include, but are not limited to, MafB and Ucn3 [48, 49]. Further studies are necessary to evaluate their potential for use as markers in large-scale screens for mature beta-cells. Furthermore, the signals that govern maturation *in vivo* are not sufficiently well understood. Together, this highlights how further studies of beta-cell maturation *in vivo* may help to guide beta-cell generation *in vitro.*

The beta-cell by definition is capable of synthesizing, storing, and secreting adequate insulin levels in response to spikes in blood glucose concentration. These are highly specialized cells, as 20% of mRNA mass in beta-cells represents insulin mRNA and total human pancreatic insulin content is approximately 10 mg (1% of beta-cell weight), which corresponds to secretion for 10 days by a healthy person. Freshly isolated primary beta-cells contain 10 ug of insulin per million of cells. Fasting serum C-peptide levels in healthy individuals range from 343-1803 pmol/l [50]. The level of released C-peptide should be dependent of glucose dose and time of glucose administration. In the current differentiation protocols, the C-peptide level of *in vitro* generated beta-cell-like cells is

barely detectable, less than 100 pM, and does not correlate properly with fluctuations of glucose concentration in media. C-peptide levels, either fasting or after glucose stimulation, remain very low during the first few weeks after transplantation into mice. Fasting and glucose-stimulated serum levels of human C-peptide become similar to the levels measured in mice with 3000-5000 human adult pancreatic islets, but only approximately 3 months after implantation of human stem cell-derived pancreatic populations into mice [51]. However, this maturation process is variable and not well understood.

As optimal PSC-derived beta-cells cannot currently be efficiently generated *in vitro*, the minimum acceptable requirements for cell transplantation into T1D patients should contain a high percentage of endocrine progenitors that express at least some of the pancreatic transcription factors such as Pdx1 and Nkx6.1. The remaining genetic beta-cell makeup should become manifest after *in vivo* maturation along with secretory granules and glucose-response components such as Sur1, Glut2, and Kir6.1. The implants after *in vivo* maturation should secrete insulin in a glucose-dependent manner and reverse diabetic hyperglycemia, though with minimum requirements, some abnormal glucose tolerance may be acceptable. The implants should be physiologically functional over longer time-periods like 2-3 years. Finally, for safety reasons, the presence of undifferentiated pluripotent stem cell and/or foreign genes cannot be tolerated [52].

3. Opportunities and challenges for pluripotent stem cell-based approaches in regenerative medicine

The most reproducible and efficient strategies for ESCs to become terminally differentiated beta-cells involve stage-specific activation and inhibition of different signaling pathways in defined culture conditions. Although the concept and basis for beta-cell differentiation are well developed, there are a number of challenges still preventing the wide application of cell-based therapy for diabetes. One of them is the generation of sufficient numbers of beta-cells for high throughput studies and transplantation. The efficiency of conversion between stages decreases with the progression of pancreatic differentiation. Therefore, the induction of definitive endoderm is achievable at 90% efficiency, but that of the endocrine progenitors at 20-30% only. The decrease is inherent to pancreatic differentiation *in vivo*, as only a small proportion of pancreatic progenitors during embryogenesis will become an endocrine component of the pancreas, as the islets constitute 1-2% of the mass of an adult pancreas and beta-cells themselves make up 60-80% of islets. One possible solution to increase the number of *in vitro*-generated pancreatic cells is to incorporate the conditions that allow expansion of populations at different stages.

To date, most widely applied therapy based on stem cells is bone marrow transplantation. It has been used for a spectrum of patients with hematologic malignancies, solid cancer, rare hereditary deficiency diseases, and autoimmune syndromes. However, despite many efforts, it is still not possible to expand bone marrow stem cells *ex vivo*. More successful, but still in its infancy, is the approach involving the expansion of hematopoietic stem cells and multipotent progenitors [53]. The same applies to the pancreas: even though considerable progress has been made in understanding pancreatic development and growth factors that support the maturation of beta-cells, the factors that govern self-renewal of pancreatic progenitors continue to be elusive.

The idea of expanding progenitors or adult stem cells *in vitro* has been pursued for different organs with varying degrees of success. To date, it has been most efficiently utilized for adult stem cells. Although progenitor cells have been isolated, expanded, and re-transplanted from skin and cornea only to date, self-renewing cells are proposed to exist in every specific tissue and organ such as intestine, skin, eye, and blood. In both cases, a single differentiated cell type was produced. The presence of adult stem or progenitor cells in the pancreas is controversial. Pancreatic stem cells were proposed relatively recently [54, 55], but efforts to substantiate their existence and origin have not yielded conclusive results. Even if such stem cells are identified, it will be a major challenge to induce them to contribute to the beta-cell pool.

Given the uncertainty over the relevance of adult pancreatic stem/progenitor cells to beta-cell generation, there is high interest in understanding the mechanism that governs self-renewal and later maturation of embryonic progenitors as a promising strategy to establish a new robust source of beta-cells.

4. Self-renewal of pluripotent stem cell-derived endoderm and pancreatic populations *in vitro*

Recently, several studies have identified endodermal populations that possess proliferative ca-

pacity, while retaining their ability to give rise to differentiated progeny.

One of the early successful protocols to generate endoderm from ESCs robustly was developed by Nishikawa and colleagues [56, 57], who applied one of two TGF-beta family members, activin A or nodal, to mouse ESCs expressing GFP gene in goosecoid (GSC) locus and selectively induced mesendoderm marked by $Gsc^{-}GFP^{+}$E-cadherin(ECD)$^{+}$PDGFRα(αR)$^{+}$ expression. Subsequently, the mesendoderm differentiated *in vitro* into definitive endoderm and mesoderm lineages. In this elegant study, the authors purified the endodermal populations based on a GFP reporter and cell surface markers and propagated the definitive endoderm over 6 months in serum-containing media. However, long-term propagation of endoderm resulted in the loss of potential to give rise to *Pdx1*$^{+}$ or *Albumin*$^{+}$ cells either *in vitro* or when transplanted under the kidney capsule. Nonetheless, this was the first demonstration that *in vitro*-generated endoderm possesses the capacity to undergo sustained proliferation *in vitro.*

Early in this decade, Morrison and colleagues devised conditions to generate and expand anterior endoderm that subsequently forms the ventral gut tube *in vivo* and contains bipotent pancreas and liver precursors [58, 59]. For this purpose, the authors employed a reporter mouse ESC line that carries red fluorescent protein under control of the *Hex* locus. Hex is a homeobox transcriptional repressor which is one of the earliest markers of anterior definitive endoderm. The Hex/CXCR4 double-positive population was expanded approximately 2000-fold *in vitro* in media supplemented with FGF2, BMP4, and VEGF. Expression of anterior definitive endoderm markers was maintained during passaging *in vitro.* After several passages, endodermal cells differentiated further towards pancreatic and hepatic progenitors. Although the Hex^{+} cells were generated with 20% efficiency only, this study provides an elegant example illustrating that the desired endodermal population can be purified, specifically expanded, and thus enriched for anterior definitive endoderm. Consequently, this approach offers an optimal platform for further research, including queries related to liver and pancreatic progenitor generation.

Transient or stable expression of master transcription factors has been successfully applied to induce numbers of different cell types. In the case of endoderm, Rossant and colleagues constitutively expressed two lineage-determining transcription factors, *Sox17* and *Sox7,* to drive differentiation of hESCs into definitive and extra-embryonic endoderm, respectively [60]. None of the commonly used exogenous growth factors, such as activin A, was necessary to induce definitive endoderm when *Sox17* was ectopically expressed. Gene expression profiling was used to verify the identity of endodermal cell types generated by this approach. Remarkably, these populations exhibited a stable extra-embryonic and definitive endoderm phenotype for 30 passages, even in the absence of exogenous growth factors. Importantly, Sox17-induced definitive endoderm had the capacity to generate hepatocyte and insulin-producing pancreatic beta-cells. Hence, a single transcription factor, *Sox17*, might be sufficient to induce a stable phenotype of proliferating endoderm.

Cheng and colleagues generated a self-renewing endodermal progenitor cell line from human ESCs and iPSCs [61]. In optimized growth conditions, these definitive endoderm cells have vast proliferation capacity and can be maintained for more than 20 passages with an expansion of $>10^{16}$ fold. Endodermal cells were expanded on matrigel and mouse fibroblast feeders in serum-free media supplemented with BMP4, bFGF, EGF and VEGF. Despite massive proliferation, no karyotypic abnormalities were detected. Microarray gene expression analysis revealed that the endoderm progenitors express a mixture of pluripotency markers (*Nanog*, *Sox2*, and *Oct4*) together with an endoderm signature (*Sox17*, *FoxA2*, *FoxA3,* and *Gata4*). Importantly, the endodermal cells differentiate *in vitro* and *in vivo* into a number of endoderm derivatives including pancreatic beta-cells, hepatocytes, and intestine cells. In contrast, when endoderm progenitors were subjected to conditions permissive to the neuronal or mesoderm lineage, upregulation of neuroectoderm or mesoderm markers was not detected, suggesting that these progenitor cells are exclusively committed to the endoderm germ layer.

Another promising approach is the co-culture system. We have derived 17 mesenchyme cell lines from mouse and human pancreas and other endodermal organs at various stages, from e12.5 to adulthood. In a screen performed to detect the ability of each mesenchyme line to induce proliferation of mouse and human ESC-derived pancreatic population in co-culture systems, we identified two mesenchyme cell lines, called Mes1 and Mes2, both of pancreatic origin, that induce the self-renewal of Sox17- and FoxA2-positive definitive endoderm [62]. The definitive endoderm expansion was 9.7- to 12- fold after 6 days, and 3 million- and

6 million-fold after 7 passages for Mes1 and Mes2 respectively and 65 million-fold for human endoderm expanded for 9 passages on Mes2. The gene expression profile of long-term expanded endoderm correlated closely (R2 = 0.92 for Mes1 and 0.96 for Mes2) with that of non-passaged endoderm. Furthermore, two independent mesenchyme cell lines, termed Mes3 and Mes4, were identified as inducers of mouse *Ngn3*$^+$ endocrine progenitor renewal. This effect appears specific to the responding cell, as *Ngn3*$^+$ cells did not substantially expand when cultured on Mes1 or Mes2 cells. Similarly *Sox17*$^+$ endoderm did not massively proliferate in the presence of Mes3 and Mes4. Importantly, the pancreatic differentiation potential of expanded endoderm was not diminished. On the contrary, progenitors serially expanded on mesenchyme gave rise to glucose-sensing, insulin-secreting cells when transplanted *in vivo* under the kidney capsule.

Expansion of pancreatic progenitors offers the possibility of assessing the pancreatic progenitors functionally either *in vitro* or *in vivo* during animal studies and testing for safety, as well as cell banking, prior to the clinical applications. It should be stressed, however, that the expansion of endoderm or pancreatic progenitors in the presence of other cells, such as pancreatic mesenchyme and embryonic fibroblasts, has some limitations. These include the presence of other cell types of animal origin and these supportive cell lines may vary between passages, leading to changes in the expanded cell and variable differentiation outcomes. Hence, it will be important to decipher the molecular mechanism of pancreatic progenitor expansion. Clearly, it is unlikely that only one growth factor will be found to govern such expansion; rather an orchestrated interaction between soluble growth factors and cell-cell contact molecules will be responsible for pancreatic progenitor self-renewal.

Expanding intestinal endoderm involves 3D culture in the presence of growth factors or coculture with supportive cell lines, such as stromal cells. The first system has been elegantly developed as an organoid culture established from single *Lgr5*$^+$ cells sorted from murine intestine or derived from human ESCs. Lineage tracing experiments showed that *Lgr5* marks an adult stem cell population in intestine. The organoids derived from *Lgr5*$^+$ can be expanded approximately 100,000-fold over 140 days, without loss of tissue identity and genomic integrity. These organoids can then be differentiated into various intestinal cell types, such as enteroendocrine cells, enterocytes, goblet, and Paneth cells [63]. Recently, protocols have been developed to expand human small intestine and colon organoid cultures from sorted stem cells or small biopsies based on *EphB2* expression [64].

Lgr5 is a stem cell marker expressed not only in the intestine, but also in the stomach, hair follicle, and mammary gland. Furthermore, recent studies showed that following an injury to hepatocytes and oval cells, *Lgr5*$^+$ cells appear also in the liver [65]. It is still unclear whether *Lgr5* labels a true stem cell or progenitor population in the liver. Nonetheless, these *Lgr5*$^+$ cells actively contribute to liver regeneration via *de novo* formation of hepatocytes and duct cells. When *Lgr5*$^+$ cells were isolated from liver and cultured *in vitro*, they formed organoids without exposure to mesenchyme. These organoids can be maintained in defined medium for longer than a year, allowing prolonged *in vitro* expansion. *Lgr5*$^+$ cells expanded over time retain their differentiation potential and upon exposure to a differentiation regimen, 30-50% of the cells will acquire hepatocyte markers. Innate liver regeneration capacity is severely affected by chronically damaged or diseased liver. For example, in the case of the cirrhosis, hepatocytes are replaced by fibrotic tissue. This damaged liver tissue thus loses its regenerative potential leading to liver failure. It remains to be determined whether *Lgr5*$^+$ or generally committed hepatic progenitors/stem cells can be isolated from diseased liver, and whether they can be expanded and differentiated *in vitro* to generate a large hepatic population to undertake transplantation for the prevention of liver failure.

Tissue-specific stem cell populations that can be expanded have also been identified in other endodermal tissue. Adult lung contains multipotent bronchioalveolar stem cells and basal cells that can regenerate airway epithelium [66].

5. Number games: a bioengineering approach to the scaling up of pluripotent stem cell differentiation to pancreatic progenitors

Most reports on pancreatic differentiation describe the efficiency of conversion of one cell type into another. For example, several published protocols allow 90% of cells in the dish to be coaxed into definitive endoderm, although data on the cell survival and growth rates are missing for the most part. Overall, the commonly described ESC culture and definitive endoderm differentiation protocols yield approximately 0.4 endoderm cell per input

hESC [67]. These approaches are indeed promising and useful for basic research requiring millions of desired cells per experiment or time point. However, for transplantation needs, the Edmonton protocol recommends a patient dose of 10,000 islet equivalents per kg of body weight to achieve insulin independence [68]. Thus, for transplantation of PSC-derived progenitors into patients, one would need in the order of 10^8 to 10^9 or even more cells for one patient. Therefore, the efficiency of conversion is not the only factor to be considered when evaluating the differentiation protocol. To ensure that adequate numbers of PSCs and their derivatives are available for transplantation, it will be necessary to develop bioengineering approaches and cell banking.

Schultz *et al*. described a strategy to scale-up the production of pancreatic progenitors that is conceptually different from approaches used for definitive endoderm expansion [69]. Briefly, they expanded the starting population of hESCs as uniform aggregates, which were subsequently differentiated in suspension *en masse* to form pancreatic endoderm clusters. The pancreatic progenitors generated during four steps in a 12-day protocol were then transplanted *in vivo*, where mature endocrine cells were formed. The transplants were verified functionally in mice based on glucose-induced insulin secretion and the ability to maintain blood glucose homeostasis in a streptozotocin-induced beta-cell ablation model. The authors also developed conditions that allow cryopreservation of end-stage pancreatic progenitor aggregates without loss of their *in vivo* functional capacity. Hence, this strategy offers not only an impressive scale-up potential, but also provides the consistency needed for follow-up studies. The generated pancreatic endoderm cells can first be evaluated before releasing them for the further studies or clinical applications.

Ungrin *et al*. employed the predictive bioprocess design strategy for hESC expansion and DE generation in feeder- and matrix-free defined medium suspension culture [67]. The authors noted a 10-fold improvement in the total number of endoderm cells produced by this set-up, as compared to a directed differentiation approach in adherent culture. Taking into account four rounds of expansion of hESCs, more than 10^9 C-kit and CXCR4 double-positive cells were generated from 1.5×10^4 undifferentiated hESCs in 22 days. Definitive endoderm produced in suspension was subsequently used to generate hepatic and pancreatic progenitors confirming endoderm developmental competence.

6. Open questions remaining before transition to clinical application

The safety of PSC-derived populations and their expanded *in vitro* cell products is critical for the future development of any cellular therapies. Undifferentiated PSCs are tumorigenic and therefore must be removed from their derivative populations before transplantation into patients. With judicious use of cell surface markers and flow cytometry, it is now possible to enrich for adult human endocrine cells, including beta-cells as well as acinar and ductal populations. Similar strategies have been extended to pancreatic progenitors. Recent studies show that CD142 marks pancreatic endoderm, while CD200 together with CD318 labels endocrine cells. These two markers can be efficiently used to enrich for pancreatic populations in differentiated hESC cultures [39]. Another study described a combinatorial approach to the use of cell surface markers to purify ESC-derived endodermal populations ($CD49^+/CD141^+/CD238^+$) [70]. The resultant enriched pancreatic population did not form teratomas for more than 160 days when transplanted into mice. However, additional studies are required to investigate the reliability of this method, as only a few hundred undifferentiated cells are sufficient for tumor growth. Furthermore, common teratoma assays involve transplantation into immuno-compromised mice that might not fully recapitulate all biological aspects or the different transplantation sites of the recipient. Moreover, flow cytometry sorting associated with cell loss must be also taken into consideration for larger clinical studies. Thus, the development of reliable techniques allowing the detection of harmful or undesirable populations from PSC-derived transplants might well be critical for the future clinical applications of expanded PSCs.

Other important safety issues are related to the application of patient-specific iPSCs in regenerative medicine. Initially, iPS cell lines were derived by virally mediated ectopic expression of four transcription factors, including a potent oncogene such as c-Myc or Klf4 along with pluripotency factors such as Oct4 or Sox2. Novel approaches to delivery of reprogramming factors include the application of synthetic modified RNA, synthetic proteins or nonintegrating episomal vectors. Furthermore, recent studies showed that the oncogene c-Myc is dispensable for reprogramming or can be efficiently replaced by a GLI-like transcription factor, *Glis1*, [71]. With a view to introducing large-scale iPSC-based therapy into the clinic, the replace-

ment of reprogramming transcription factors with small molecules is highly desirable. Even though a combination of small molecules that would eliminate the need for transcription factors during human cells reprogramming remains to be identified, a number of different small molecules have greatly improved the efficiency and decreased the time of reprogramming [72]. Recently, a novel combination of 7 small molecules was published to reprogram mouse somatic cells into iPSCs [73]. Despite enormous progress in pluripotency research, reprogramming is still a lengthy, arduous, and complex process that generates embryonic stem-like cells with variable developmental potential. The molecular mechanism of the reprogramming must be better understood and characterized to provide robust and reliable methodology for therapeutic application on a large scale.

Furthermore, reprogramming and long-term *in vitro* expansion of undifferentiated as well as multipotent progenitors might be a cause for genomic instability. Novel techniques for genome and epigenome assessment are necessary to evaluate whether the genome and chromatin landscape of expanded progenitors accurately reflects that of the cells' non-manipulated and *in vivo* counterparts, and to clarify how data may be helpful in predicting cell behavior and differentiation capacity. Some studies suggest that iPSCs might retain an epigenetic memory of their tissue of origin [74, 75]. Lastly, it would be beneficial if an approach to generate pancreatic populations was amenable to automation. Cost considerations may ultimately affect the large-scale application of the pluripotent stem cell-derived populations.

7. Maintaining the beta-cell phenotype *in vitro*

Despite the enormous efforts to establish a new source of beta-cells *in vitro*, it is unknown how to sustain beta-cell phenotype or promote replication over time. After 2-3 days in culture, half of all isolated beta-cells are lost, with the remaining cells showing reduced glucose-stimulated insulin secretion. Furthermore, islet cells cultured for a few days have decreased ability to restore proper glucose homeostasis *in vivo* when transplanted into diabetic mice [76]. Therefore, current protocols exclude the use of beta-cells cultured *in vitro* for longer than a few days for the purposes of cell therapy applications. It is recognized that preserving the 3-dimensional beta-cell arrangement may contribute to the long-term survival and function of transplanted and PSC-derived cells [77, 78].

Suitable differentiation conditions might be even more challenging to set up in the case of hESC-derived beta-cells. The design of pancreatic differentiation aims to produce preferably only beta-cells with highest efficiency. Currently, these cultures lack any other cell types present in the pancreatic niche during embryonic development and adulthood, in contrast to the highly organized nature of pancreatic islets *in vivo* characterized by distinctive endocrine arrangements and other cell types. In the native pancreatic niche, which consists of mesenchyme, endothelial cells, and nerves, there is evidence of cross-talk among niche cells. Hence, it might be beneficial to recapitulate this elaborated multicomponent environment in the culture dish. An organ-isolated islet does not consist of beta-cells only and might already autonomously provide part of the pancreatic niche necessary to *in vitro* culture. Pancreatic development depends on instructive signals derived from adjacent tissues, including the mesenchyme and blood vessels [79, 80]. Indeed, co-culture of mouse islets together with human endothelial cells and skin fibroblasts significantly promoted islet survival *in vitro* and post-transplantation *in vivo* [81].

Moreover, in recent studies by Borden *et al.*, genetic or pharmacologic ablation of sympathetic innervation during development caused altered islet architecture, reduced insulin secretion, and impaired glucose tolerance in mice [82], while in a neuron-islet co-culture system, the presence of neurons promoted beta-cell migration. The transplantation outcome of rat beta-cell-enriched aggregates is similar to that of rat islets into immunocompromised mice [83]. However, other studies reported that mixing beta-cells with non-beta-cell islet components is beneficial for longer transplant functionality [84]. Overall, this indicates that endocrine non-beta-cells and donor endothelium may be beneficial to the function of transplanted islet grafts, but other than beta-cells, do not seem to be essential for successful transplantation.

Finally, bona fide beta-cell development and later function occur in a three-dimensional environment, whereas a significant amount of experimental work on beta-cells is carried out in two-dimensional *in vitro* culture settings. Moreover, to a large extent, a lot of these conditions are based on a methodology normally used for pancreatic cancer cell lines.

Taken together, modifications to current culture conditions might improve beta-cell survival and function during *in vitro* culture and vitality after transplantation. One approach may consist in the co-culture of beta-cells with other cell types

such as endothelial, mesenchymal cells, and nerves. Alternatively, three dimensional suspension cultures might also improve beta-cell identity survival.

8. Beta-cell expansion *in vivo* and *in vitro*

Shortly after birth, the beta-cell mass significantly expands in mice and humans. At this time, approximately 3% of fetal beta-cells replicate, which decreases to 0.5% by 6 months in humans. Rapid expansion in the neonatal phase comes from proliferation of existing beta-cells rather than a stem/progenitor pool. During adulthood, beta-cells are long-lived and the majority of adult murine beta-cells do not proliferate; only 1 in 400 cells is believed to divide every 24 hrs in 1-year-old mice. Genetic tracing experiments showed that, during adulthood under normal physiological conditions, only beta-cells themselves are a source of new beta-cells [85, 86]. Similar to other cells, like hepatocytes, the proliferative capacity of beta-cells diminishes with age. In studies of beta-cells ablated by diphtheria toxin, the regenerative capacity of beta-cells is higher in young mice than in old mice; it reaches 7.5% in young mice, whereas in old mice it is at most 1% [87].

Under special circumstances, beta-cell replication is modified in response to augmented metabolic demands during pregnancy and in obesity, but the mechanism of the increase is not fully understood. In the case of pregnancy, hormones such as prolactin and lactogen are suggested to increase beta-cell mass [88, 89]. According to some hypotheses, the primary physiological cue for replication is glucose. Human islets transplanted into mice can be induced to proliferate along with rodent host islets following glucose infusion. Furthermore, humans with glucokinase mutation that increases affinity for glucose show enlarged islets caused by increased beta-cell proliferation [90]. In T2D, however, the adaptive mechanism to align proper insulin secretion with increasing physiological demands clearly fails. Hence, efforts have focused on small molecule screens for specific inducers of beta-cell replication. Identification of the beta-cell replication molecule might also be very useful for T1D, even for patients diagnosed a long time ago, as they may still have some intact beta-cells. Furthermore, the beta-cell proliferative molecule might be used for expansion of the beta-cells generated by other approaches, including from PSCs.

9. Engraftment of PSC-derived pancreatic population: when and where?

Another challenge is to determine the stage at which differentiated pancreatic cells are the most suitable for transplantation. If the PSC-derived pancreatic progenitors are transplanted too early during development, they might not become beta-cells and there could be a higher risk of tumor formation. Conversely, if designated pancreatic populations are transplanted too late, the cells may be unable to adapt to the *in vivo* environment and function correctly. Initial transplantation studies in mice suggest that after an *in vivo* incubation period of a few months, pancreatic progenitors expressing *Pdx1* and *Nkx6.1* rather than $Ngn3^{+}$ endocrine progenitors or immature endocrine cells generate more beta-cells that release C-peptide in a glucose-dependent manner [39]. The origin of these glucose-responsive beta-cells remains unclear as the transplanted cells are not a homogenous population, but are enriched for pancreatic progenitors, and lineage-tracing data are missing. Finally, it remains uncertain whether other cell types, either pancreatic, endothelial, neuronal, or mesenchymal, transplanted along with pancreatic progenitors might facilitate *in vivo* maturation, physiologic function, and survival of implants.

The ideal beta-cell or its precursor transplantation would be one that promotes maturation, long-term survival, and function. Furthermore, it should be easily accessible for the purposes of engraftment procedures and later for monitoring the health status of transplants. Currently, cadaveric islets are transplanted into the portal vein of the recipient patient, which is chosen because of its proximity to the liver and the advantage of minimal invasion. However, about half of the islets introduced into the liver die during or shortly after transplantation [91-93]. Transplantation studies with mice showed that the kidney capsule, peritoneum or omental pouch provide a permissive environment for pancreatic population maturation and function. In particular, subcutaneous transplantation might be an attractive prospective site for transplantation into patients due to the easy access, but might require additional strategies to provide a sufficient nutrient and insulin transport via vasculature.

10. Conclusions and future directions

The treatment of T1D and other diseases by organ transplantation has been impeded by the lim-

ited availability of donor tissue. Stem cell therapy offers the promise of a new, robust, and reproducible source of beta-cells. In recent years, the understanding of human PSC differentiation and lineage commitment has grown rapidly leading to the development of several robust and reliable methods to generate pancreatic progenitors that mature *in vivo* into glucose-sensitive, insulin-secreting beta-cells.

Although efforts to induce stepwise pancreatic differentiation have been partially successful, a number of major problems remain, including insufficient rate of conversion of one cell type into another and the generation of insufficient quantities of target cell population. During embryogenesis, specification of progenitors is followed by amplification and further differentiation, and the balance between the two is probably responsible for determining final organ size. These two steps, renewal and differentiation, can be effectively mimicked *in vitro*, enabling the separate control and manipulation of each step. This approach permits the expansion of progenitors to an extent that may exceed that occurring during normal *in vivo* development. Importantly, the wider use of a combination of directed differentiation and self-renewal might bring us closer to the projected era of regenerative medicine and closer to fulfilling the promise of stem cell therapy for patients with diabetes.

Finally, despite the exciting progress to date, daunting challenges remain, not least of which is the need to shield cell-based therapies from host immune response without resorting to immunosuppressive drugs. Lastly, the use of patient-specific beta-cells would be important for our understanding of the disease.

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