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# Stem/Progenitor Cell Sources of Insulin-Producing Cells for the Treatment of Diabetes

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## ABSTRACT

**Patients with diabetes experience decreased insulin secretion that is linked to a significant reduction in the number of islet cells. Reversal of diabetes can be achieved through islet transplantation, but the scarcity of donor islets severely hinders wide application of this therapeutic modality. Toward that end, embryonic stem cells, adult tissue-residing progenitor cells, and regenerating native β-cells may serve as sources of islet cell surrogates. Insulin-producing cells generated from stem or progenitor cells display subsets of native β-cell attributes, indicating the need for further development of methods for differentiation to completely functional β-cells. Pharmacological approaches aiming at stimulating the *in vivo/ex vivo* regeneration of β-cells have also been proposed as a way of augmenting islet cell mass. We review the current state of the generation of insulin-producing cells from different sources with emphasis on embryonic stem cells and adult progenitor cells. Challenges for the clinical use of these sources are also discussed.**

## INTRODUCTION

Defects in insulin production or action or both, linked to a severely reduced islet cell mass, characterizes diabetes. Type 1 (insulin-dependent) diabetes is the result of autoimmune destruction of insulin-producing pancreatic β-cells. In type 2 (non-insulin-dependent) diabetes, insulin production is inadequate mainly because of peripheral insulin resistance and subsequent β-cell apoptosis. Diabetes and its devastating effects, which include retinopathy, nephropathy, stroke, and heart attack,[1] afflict more than 194 million people worldwide. According to the World Health Organization, this number will more than double by 2030.[2] Survival of patients with insulin-dependent diabetes relies on recurring insulin delivery, which does not cure the disease or prevent diabetes-associated maladies. Although type 2 diabetes may be managed through a combination of diet, exercise, and prescription of drugs, almost 30% of those affected also require frequent administration of insulin. Therefore, the development of therapies to replace insulin regimens is highly desirable.

To that end, islet transplantation has afforded promising results, with some patients experiencing insulin independence for more than 5 years[3] after the initial procedure. Ongoing procedural improvements aim at increasing the time span of liberation from insulin and reducing the side effects due to immunosuppression. However, the scarcity of available donor tissues hinders wide application of pancreas/islet transplantation Thus, renewable sources of islet β-cells are necessary.

Pancreatic β-cell lines, embryonic stem cells (ESCs), adult progenitor cells (APCs), and regenerating native islet cells are being explored for their potential to serve as β-cell sources. Large numbers of cells can be generated from β-cell lines, although their unrestricted proliferation is also a serious concern in the context of cellular therapies. Stem cells isolated from the inner cell mass of human blastocysts are pluripotent, and several studies have shown that they can give rise to insulin-producing cells. Nevertheless, only a small fraction of differentiated cells produces insulin, and even these cells do not display a complete β-cell phenotype. Furthermore, perplexing ethical questions will need to

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be addressed before human ESCs (hESCs) are used in therapies. APCs residing in various tissues have been shown to (trans)differentiate to insulin-producing cells. In principle, the risk for immune reaction of transplanted APCs is minimal, but significant technical challenges surround their isolation and expansion. Unlike stem and progenitor cells, islet β-cells have limited ability to multiply under normal conditions, although investigations into factors that stimulate β-cell regeneration have yielded promising results. Therapeutic molecules capable of increasing β-cell mass *in vivo* may eliminate the need for invasive surgical procedures. However, the extent of adult β-cell regenerative capacity is unclear.

In this article, we review recent studies on the potential of these sources of β-cells. Emphasis is given to the progress made recently on the differentiation of ESCs and APCs toward insulin-producing cells. Latest findings on factors augmenting the regeneration of native β-cells are also discussed. Finally, we examine important factors associated with the prospective use of stem and progenitor cells in diabetes therapies.

## PANCREATIC β-CELL LINES

Pancreatic cell lines have been widely used as *in vitro* models for research on β-cell physiology. This is in part because cell propagation to large numbers is relatively fast and straightforward, the cells are characterized as clonal, and they exhibit many of the functions found in native β-cells. Pancreatic β-cell lines are derived from insulinoma cells using sub-lethal irradiation[4–6] or via the introduction and expression of tumor genes[7–9] (e.g. SV40 T-antigen (SV40T) or H-ras$^{val12}$).

Despite their important attributes, the use of cell lines for cellular therapies is problematic. A major drawback stems from their almost unrestricted proliferative capacity, which creates serious risks for tumorigenesis. Researchers have tried to address this issue in various ways. For example, the βTC[10] cells are derived from transgenic mice expressing the SV40T gene under the control of the rat insulin (II) gene promoter (RIP). Efrat *et al.*[11] introduced a tetracycline-controlled expression cassette to βTC cells to modulate the expression of the SV40T gene. In the presence of tetracycline, SV40T activity ceases, causing the cells to undergo growth arrest. Expression-based systems that can be regulated, however, suffer from leakiness in transgene expression, significantly impeding their clinical use.

Recently, manipulation of proliferation of a human β-cell line was also reported.[12] Primary human islets were propagated in culture after retroviral delivery of the SV40T and the human telomerase reverse transcriptase genes flanked by *loxP* sites. When a sufficiently high cell number was reached, β-cell growth was stopped by infection with an adenovirus carrying the Cre recombinase gene (CRE). These cells express an array of β-cell genes such as Isl-1, Pdx-1, and prohormone convertases 1/3 and 2 and are glucose-responsive, although the levels of insulin content and secretion are lower than in human islet controls. After transplanting the cells to streptozotocin (STZ)-treated severe combined immunodeficient (SCID) mice, normoglycemia was achieved for 30 weeks with no apparent tumorigenesis.

Even if the problem of uncontrolled growth of β-cell lines can be tackled effectively, there are several other problems with the use of β-cell lines for diabetes therapies. Cell lines lose their differentiated phenotype with higher passage numbers. As a result, the cells display aberrant responses to glucose ranging from a left shift in the glucose dose–response curve to a negligible, if any, response to steep changes in glucose concentration. Such aberrations are often associated with faulty regulation in the expression of β-cell genes such as the glucose transporter-2 (GLUT-2),[13] insulin,[14] glucokinase,[15] and other hexokinases.[16] Attempts to engineer function-related genes into β-cell lines have not been effective. For example, glucose responsiveness deteriorates in rat insulinoma RIN cells as GLUT-2 expression is downregulated.[17] Stable transfection with the GLUT-2 gene is sufficient for RIN cells to regain their response to glucose temporarily, but insulin secretion declines again over time, possibly because of a loss of endogenous glucokinase activity. Studies reported to date make it clear that major hurdles should be overcome before β-cell lines can be considered in the context of cellular therapies.

## STEM CELL SOURCES OF β-CELLS

### *Embryonic stem cells*

ESCs are derived from the inner cell mass of a blastocyst (Fig. 1) and differentiate into almost every adult cell type (pluripotency). They can also self-renew extensively—some would claim indefinitely—yielding a large number of cells for downstream applications. In a growing number of studies,[18–20] ESC derivatives are shown to restore the physiological functions of injured organs. In conjunction with the reasonably simple protocols for their genetic manipulation, ESCs represent one of the most promising avenues toward developing cell-based regenerative therapies.

When ESCs are cultivated on non-adherent surfaces, they form embryoid bodies (EBs) in which spontaneous differentiation occurs toward cell types of all three embryonic layers. The process of EB differentiation is poorly defined and relatively non-selective, resulting in a heterogeneous cell population. The presence of agents such as nicotinamide or basic fibroblast growth factor (bFGF) during EB formation appears to enhance the number of cells[21–23] displaying β-cell markers, such as insulin, Pdx-1, Pax-4, Neurogenin-3 (Ngn-3), Nkx2.2, and Nkx6.1. Nevertheless, these cells appear to express additional hormones (e.g. somatostatin and glucagon). The nature and lineage of these cells have not been investigated in detail.

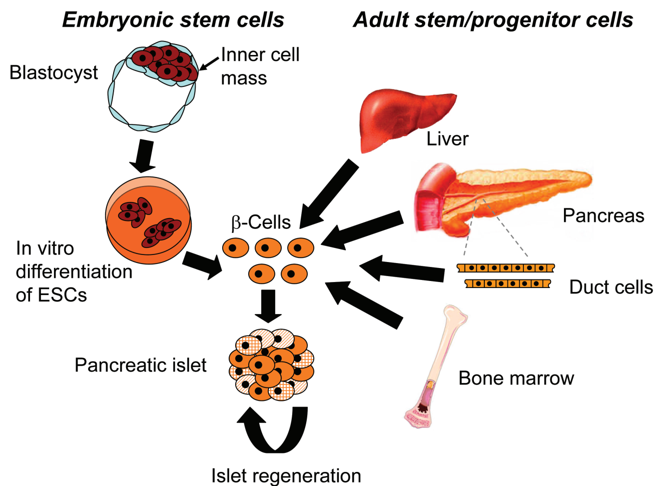


**FIG. 1.** Potential sources of β-cells. Embryonic stem cells (ESCs) derived from the inner cell mass of a blastocyst can be manipulated *ex vivo* to differentiate toward β-cells. Adult stem and progenitor cells residing in various organs such as the liver, pancreas, and bone marrow may also serve as sources of insulin-producing cells. Regeneration of native β-cells can contribute to increased islet mass. Color images available online at www.liebertpub.com/ten.

Treatment of dispersed ESCs or EBs with different compounds has also been attempted (Table 1) in combination with selection of cells positive for the neurofilament protein nestin. Nestin is detected in islets and neuronal tissues. Similarly, insulin is present in neuroectodermal and β-cells, although its amount, intracellular processing, and physiological role in the two cell populations are different. Lumelsky *et al.*[24] reported the generation of insulin-producing cells from ESCs by implementing a protocol originally intended for neuronal differentiation. The procedure entailed EB formation, selection of nestin$^{+}$ cells and incubation with supplements including bFGF, nicotinamide, and insulin-transferrin-selenium-fibronectin (ITSFn). The fraction of differentiated cells that stained positive for insulin was low. More importantly, transplantation of these cells in diabetic mice failed to reverse hyperglycemia. Hori *et al.*[25] also adopted a multi-step protocol, selecting nestin$^{+}$ cells that were treated with a phosphatidylinositol-3 kinase inhibitor (LY294002). The obtained cells exhibited glucose-stimulated secretion of hormone and improved glycemic control in diabetic mice upon transplantation. Later studies,[26,27] however, suggested that the cells' insulin immunopositivity was due to insulin uptake from the medium instead

**TABLE 1. EXAMPLES OF REPORTED EMBRYONIC STEM CELL (ESC) DIFFERENTIATION *IN VITRO* TO INSULIN-PRODUCING CELLS**

| *Differentiation agents (cell type)* | *Markers* | *Glucose-stimulated insulin secretion* | *Reversal of diabetes (rodents)* | *Reference* |
|---|---|---|---|---|
| EB formation, high-density adherent cultures (hESCs) | Pdx1, Glucokinase (GK), Insulin, GLUT-1,-2 | Yes | N/A | 21 |
| EB formation, ITSFn, bFGF, B27, Nicotinamide, KGF, EGF, progesterone, (Ins2-βgeo$^{+}$ mESCs) | Insulin 1 & 2, Pax4, Kir6.2, Glut-2, Ngn3, Nkx2.2, Nkx6.1, Isl-1, Pax4, Pax6, GK | Yes | N/A | 22 |
| EB formation, nestin$^{+}$ cell selection, bFGF, N2, B27, ITSFn, nicotinamide (mESCs) | Insulin, GLUT-2, Pdx1, IAPP, HNF3β, glucagon | Yes | No | 24 |
| EB formation, ITSFn, nestin$^{+}$ cells, B27, N2, bFGF, nicotinamide, LY294002 (mESCs) | Insulin, Pdx1, GK, Isl-1, Ngn3, GLUT-2, carboxypeptidase A | Yes | Yes | 25 |
| EB formation, nicotinamide (Pax4$^{+}$ or Pdx1$^{+}$ mESCs) | Pdx1, Isl-1, Ngn3, Insulin, GLUT-2 | Yes* | Yes | 29 |
| EB formation (Nkx2.2$^{+}$ mESCs) | Pdx1, Nkx2.2, Insulin 1 & 2 | No | N/A | 30 |
| EB formation, Nkx6.1 gene selection, co-culture with pancreatic rudiments, nicotinamide, Shh anibodies (Nkx6.1-neo$^{+}$ mESCs) | Pdx1, Nkx6.1, Insulin, GLUT-2, SUR1, GK, | Yes | Yes | 31 |
| EB formation, nicotinamide, ITSFn, B27, N2, bFGF (hESCs) | Ngn3, Insulin, Pdx1, GLUT2, Nkx6.1, Isl-1, IAPP, GK, SUR1 | Yes** | N/A | 135 |
| Activin, Wnt3a, FGF10, cyclopamine, retinoic acid, exendin-4, IGF1, HGF, DAPT (hESCs) | Insulin, Sox17, Foxa2, HNF1β, Pdx1, Nkx2.2, Nkx6.1, Pax6, C-peptide. | Yes | N/A | 41 |

*Diabetes was ameliorated also in mice receiving wild-type ESCs.
**Cells were challenged with 3.3 mM glucose.

of *de novo* insulin production. Further analysis[28] showed that mESC-derived insulin-releasing cell clusters incubated in medium supplemented with $^{35}$S-cysteine, did not produce detectable amounts of radioisotope-labeled insulin. Secreted C-peptide, a byproduct of insulin synthesis, was less than 1% of the stoichiometric equivalent to insulin. Surprisingly though, approximately 50% of the cell clusters subjected to immunofluorescence staining and immunogold labeling were positive for intracellular C-peptide. These findings, in conjunction with mass balance calculations, supported the notion that the insulin secreted by differentiated cells comes both from hormone sequestration and *de novo* synthesis. From these studies, it became evident that determination of insulin secretion alone is not a conclusive criterion for claiming differentiation of stem or progenitor cells to β-cells. Active formation of insulin in differentiated cells should also be analyzed via several methods, such as C-peptide staining, metabolic labeling, demonstration of biphasic insulin release upon glucose challenge, and transplantation assays.

Heterogeneous populations of differentiated ESCs can be enriched with a desired cell type through the implementation of genetic methods. A popular approach is the introduction of transcription factor genes to ESCs to direct the differentiation to a β-cell fate or expedite the selection of cells resembling β-cells. For example, constitutive expression of the *Pdx-1*[29], *Pax4,*[29] or *Nkx2.2*[30] transcription factor leads to the differentiation of ESCs toward cells expressing pancreatic endocrine markers. Pax4 overexpression[29] and selection of nestin$^{+}$ cells gives rise to clusters with cells displaying mRNA for *Isl-1*, *insulin*, *islet amyloid polypeptide*, *GLUT-2,* and *Ngn3.* Injection of these clusters in STZ-treated mice resulted in normalization of blood glucose. Unexpectedly, however, normoglycemia was also observed in diabetic controls receiving wild-type ESCs.

Cell trapping is another approach using gene insertion for selection of insulin-producing cells. ESCs are stably transfected with a construct of a drug-resistance gene downstream of a promoter that is active in β-cells. Examples of such promoters include those of the *Nkx6.1*[31] (active among other cell types in maturing β-cells) and the human insulin[32] genes.

The cells undergo differentiation by forming EBs while treated with nicotinamide or mouse pancreatic explant-conditioned medium. Subsequent treatment with an appropriate drug ablates all cells but those with a β-cell-like phenotype, leading to an increase in the percentage of insulin-producing cells. Leon-Quinto *et al.*[31] reported the differentiation of mESCs stably transfected with a cassette of the Nkx6.1 promoter upstream of the hygromycin resistance gene. After culturing mESCs as EBs with factors such as nicotinamide, sonic hedgehog antibodies, and mouse embryonic pancreatic explants, differentiated cells were selected by incubation with hygromycin. Approximately 20% of the cells became positive for *insulin* and *Pdx-1* after differentiation. This fraction increased to almost 100% of insulin-positive cells upon selection with hygromycin. The cells expressed Pdx-1, Nkx6.1, glucokinase, and sulfonylurea receptor (SUR1) and secreted insulin upon glucose stimulation. Diabetic mice receiving these cells[33] became normoglycemic for more than 42 weeks.

Nevertheless, stimulating ESC differentiation by engineering the expression of transcription factor genes or their respective promoters is problematic because many details of the transcription factor networks engaged in pancreatic endocrine cell development are unclear. In addition, promoters may be active in multiple, distinct cells types (e.g., in addition to β-cells, Nkx6.1 and insulin genes are expressed in neuronal cells), confounding the selection outcome. More importantly, the clinical efficacy of hESC differentiation involving genetic manipulations (e.g., the introduction of drug-resistance genes) is questionable. In addition to rigorous assays required to ensure that there is no risk of tumorigenicity due to the insertion of the transgene(s), the long-term effects of transgene expression in transplanted cells are difficult to assess.

Protein transduction may help to circumvent problems associated with the irreversible genetic manipulation of stem cells. Proteins can be directly delivered to cells when fused to small peptides known as protein transduction domains (PTDs). Several such peptides have been identified in naturally occurring homeodomain proteins including the antennapedia homeotic transcription factor in *Drosophila*, the VP22 protein in herpes simplex virus type 1, and the trans-activator gene product TAT of the human immunodeficiency virus. Moreover, PTDs can be found within sequences of proteins such as the transcription factors Pdx-1 and BETA2/NeuroD, facilitating their crossing of cell membranes without the addition of extraneous PTDs.[34,35] Transduction of pancreatic ductal cells with exogenous Pdx-1 protein induces insulin gene expression.[34] Also, Ngn3 fused to the TAT peptide (Ngn3/TAT) induces endocrine differentiation in mouse pancreatic explants.[36] In the same report, after incubation of mESCs with Ngn3/TAT, 100% of the treated cells stained positive for Ngn3. A detailed characterization of the differentiated cells was not reported, although the outcome is encouraging for the use of protein transduction technologies to direct the commitment of stem cells, thereby eliminating the need for genetic intervention as well as the use of viral vectors.

Most likely, further advances in the generation of ESC-derived pancreatic β-cells will require the development and optimization of methodologies for multi-step exposure to physiologically relevant stimuli. Such factors may coax the cells in a manner that recapitulates the sequential stages of embryonic development from uncommitted cells to endoderm, pancreatic anlage, pancreas endocrine cells, and eventually, mature, functional β-cells. Mouse and human ESCs have been successfully induced to definitive endoderm,[37–40] which is a first step toward pancreatogenesis. Yasunaga

*et al.*[39] stimulated the differentiation of mESCs to endoderm in a serum-free medium containing activin. Definitive and visceral endoderm cells are produced depending on the culture conditions. Cells belonging to each endoderm type can be identified based on the expression of multiple proteins, mainly goosecoid, sex-determining region Y-box 17 (Sox17), and chemokine (C-X-C motif) receptor 4 (CXCR4). In our laboratory, we have also developed a similar protocol for differentiating mESCs with activin and Wnt3a to endoderm cells expressing markers such as Sox17 and Foxa2 (Fig. 2).

Induction of hESC differentiation to definitive endoderm in medium with low concentration of serum and activin A was also reported.[40] The majority of the resulting cell population was $Sox17^{+}$ and $Foxa2^{+}$, whereas markers of other layers were absent or expressed at low levels. The cells that committed to definitive endoderm were further purified based on CXCR4 expression, and upon transplantation in SCID mice, the grafts were immunoreactive for hepatocyte-specific antigen and the intestine markers villin and CDX2. Expanding on these findings, D'Amour *et al.*[41] reported the conversion of hESCs to endocrine cells capable of synthesizing insulin, glucagon, somatostatin, pancreatic polypeptide, and ghrelin. Hormone-expressing cells appear after almost 2 weeks of treatment with factors including activin, Wnt3a, FGF10, and the sonic hedgehog inhibitor cyclopamine, among others. The insulin content of these cells is comparable with that of primary adult human islets and is the highest reported for differentiated cells. In addition to the numerous secretory granules observed, C-peptide is detected in these cells, indicating endogenous insulin synthesis. However, C-peptide secretion in response to glucose is minimal, despite pronounced stimulation by other secretagogues. Furthermore, many of the differentiated cells express multiple hormones, pointing to an immature phenotype. More experiments will be needed to answer whether an inductive or a selective mechanism underlies this differentiation procedure.

## *Adult progenitor cells*

Progenitor cells with the potential to differentiate into insulin-producing cells have been described in adult tissues such as the pancreatic duct, exocrine pancreas, pancreatic islets, liver, spleen, and bone marrow (Table 2). Until recently, adult tissues were believed to be devoid of stem cells. A long-standing notion in developmental biology has been that tissue-specific progenitor cells have limited plasticity, differentiating only to cells types of the tissue of origin. observations of tissue-residing cell transdifferentiation to developmentally distant cell types has challenged this view.

*Duct and islet precursor cells.* During embryo development, cells in the pancreatic anlage migrate from the ducts while differentiating to form clusters that will eventually become islets.[42] Islet cells are also observed in close proximity to the ducts in patients with type I diabetes[43] and after partial pancreatectomy in rodents.[44] The postnatal pancreatic duct may harbor islet precursor cells, which could be expanded and stimulated *ex vivo* to obtain insulin-producing cells.

Duct cells isolated from murine[45] or human[46] pancreata have been grown in culture, yielding hormone-producing cells organized in islet-like aggregates. These cells express multiple islet proteins and respond to glucose stimulation by releasing insulin, albeit less than native islets. Nonetheless, diabetic non-obese diabetic (NOD) mice receiving the duct-derived cells experience almost normal blood glucose levels for more than 3 months. The ability of adult duct cells to contribute to islet regeneration *in vivo* is still debatable. Dor *et al.*[47] employed a lineage-tracing methodology to show that new pancreatic β-cells originate from

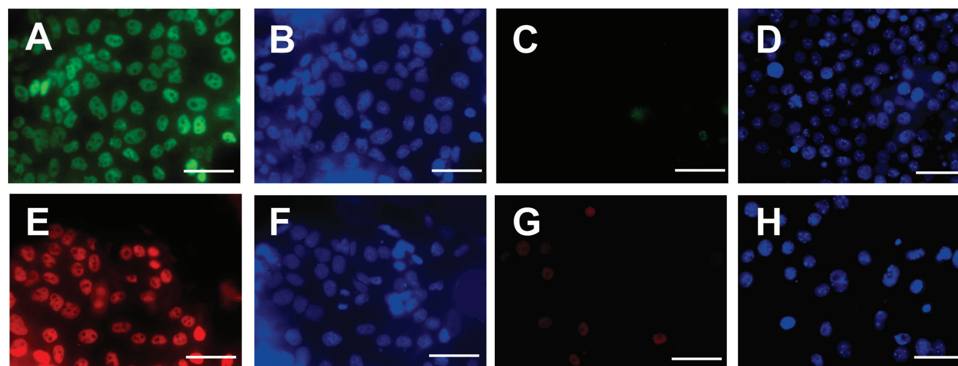


**FIG. 2.** Embryonic stem cells (ESCs) differentiate to cells expressing definitive endoderm markers. Mouse ESCs after a 5-day treatment with activin (100 ng/mL) and Wnt3a (25 ng/ml) express (**A**) Sox17 and (**E**) Foxa2. (**B**) and (**F**) depict the same fields as (**A**) and (**E**) stained with 4′,6-diamidino-2-phenylindole (DAPI). Mouse ESC controls (cells cultured without activin and Wnt3a) do not express (**C**) Sox17 or (**G**) Foxa2. (**D**) and (**H**) represent the DAPI-stained fields corresponding to (**C**) and (**G**), respectively. Bar: 50 μm. Color images available online at www.liebertpub.com/ten.

TABLE 2. *IN VITRO* DIFFERENTIATION OF ADULT PROGENITOR CELLS TO INSULIN-PRODUCING CELLS

| *Adult progenitor cell sources* | *Markers* | *Glucose-stimulated insulin secretion* | *Reversal of diabetes (rodents)* | *References* |
|---|---|---|---|---|
| Human ductal cells cultured in ITS, nicotinamide, KGF | Cytokeratin-19, Insulin, glucagon, somatostatin, PP | Yes | N/A | 46 |
| Rat acinar cells, EGF, LIF | Insulin, Pdx1, C-peptide, GLUT-2 | Yes | Yes | 59 |
| Rat hepatic cells, ITS, vitamin C, dexamethasone, HGF, EGF, adenoviral transduction with Pdx-1 | Insulin 1 & 2, Pdx1, Ngn3, NeuroD, Isl-1, Pax6. | No* | N/A | 62 |
| Rat hepatic oval cells, high glucose | Insulin 1 & 2, Pdx1, GLUT-2, Nkx2.2, Nkx6.1, Pax4, Pax6, glucagon, somatostatin | Yes | Yes | 63 |
| Rat marrow mesenchymal cells, nicotinamide, 2-mercaptoethanol | Insulin 1 | No** | Yes*** | 69 |
| Human adipose tissue-derived mesenchymal stem cells, nicotinamide, exendin-4, activin A, HGF, B27, N2, pentagastrin | Isl-1, Pdx1, Ngn3, Pax6, Insulin, C-peptide, glucagon, somatostatin | N/A | N/A | 79 |
| Human peripheral blood monocytes ($CD14^+$, $CD45^+$), nicotinamide, EGF, HGF | Ngn3, Nkx6.1, NeuroD, Pax6, HNF3β Nkx2.2, Insulin, C-peptide | Yes | Yes | 81 |
| Human neurosphere cell lines, N2, EGF, nicotinamide, IGF1, bFGF, retinoic acid | Pdx1, Insulin, Foxa3, GK, GLUT-2, Nkx6.1 | Yes | No**** | 83 |

*Glucose-stimulated insulin secretion data are not available, but insulin was secreted in response to tolbutamide, GLP-1.
**Supernatant insulin is detected but no glucose challenge results are available.
***Results lack statistical analysis.
****Cells were transplanted to immunocompromised mice and responded to a glucose challenge (IPGTT) by releasing human C-peptide.

proliferating β-cells already residing in the adult islets. These results cast doubt on the hypothesis that stem and progenitor cell differentiation is responsible for renewal of adult islet mass, although mechanisms of islet regeneration after injury (e.g., partial pancreatectomy, duct ligation) or neonatal islet formation were not examined. In fact, recent data[48] suggest that between days 20 and 31 the β-cell number in rats triples and that 30% of the new β-cells are not derived from replicating β-cells.

However, markers for the identification of islet cells capable of generating hormone- producing progeny remain elusive. As mentioned above, the intermediate filament nestin, a marker of neural stem cells,[49] is also expressed in cells within human and rodent islets as well as within ducts and centroacinar regions.[50] $Nestin^+$ cells from human fetal pancreata are hormone-negative, exhibit extensive proliferative capacity, and can be coaxed *in vitro* to form hepatic and endocrine pancreas cells. The latter cells express insulin, glucagon, and the transcription factor Pdx-1 and reverse hyperglycemia[51] in diabetic mice after implantation.

Several groups have questioned the use of nestin as a marker of pancreatic stem cells.[52,53] For example, precursor cells derived from islet and duct populations contain $nestin^+$ and $nestin^-$ cells. In culture, these precursors give rise to multiple neural and pancreatic endocrine lineages.[54] The differentiated progeny is positive for insulin (and nestin) and secretes insulin (less than primary islets) in response to glucose and other secretagogues. A sensible approach is to include additional markers in the identification and isolation of pancreatic precursors, considering that nestin is found in multiple types of intra- and extrapancreatic cells. Also, the fraction of precursor cells isolated from the pancreas is small and heterogeneous, posing a major hurdle for potential clinical use of these cells.

*Exocrine cells.* In addition to duct cells, other exocrine pancreatic cells are considered capable of transdifferentiation to insulin-producing cells. Acinar cells may regress to a more immature phenotype with subsequent re-differentiation to duct cells and endocrine pancreas.[55,56] Implementing a *Cre/loxP*-based lineage tracing system, Minami *et al.*[57] showed the transition of $amylase^+$/$elastase^+$ acinar cells to insulin-secreting cells resembling β-cells. In a follow-up study,[58] acinar cells isolated from diabetic rats gave rise to insulin-producing cells *in vitro*, but these cells did not secrete insulin after a glucose challenge. Transdifferentiation of acinar cells to islet cells has also been reported during *in vitro* treatment of exocrine cells with epidermal growth factor (EGF) and leukemia inhibitory factor,[59] a process that involves activation of Ngn3.[60]

*Liver cells.* During embryo development, hepatic and pancreatic cells arise from the endoderm layer. This has led to the hypothesis that the liver may harbor cells with the capacity to dedifferentiate and subsequently commit to pancreatic cell lineages. Zalzman *et al*.[61] immortalized human fetal progenitor liver cells while introducing a cassette for activation of *Pdx-1*. The induction of *Pdx-1* in these liver progenitors is concomitant with the expression of multiple β-cell genes. When the differentiated cells were transplanted into hyperglycemic immunodeficient mice, normoglycemia was restored and maintained for more than 70 days. A major difficulty in interpreting these results stems from the absence of expression of GLUT-2, glucokinase, and the SUR1 genes, which are required for glucose-regulated insulin secretion. In related work,[62] *Pdx-1* overexpression boosted the release of insulin by hepatic progenitor cells responding to glucose and other secretagogues, but Pdx-1– differentiated hepatic progenitors exhibited a similar insulin secretion profile.

Hepatic "oval" stem cells[63] also form islet-like aggregates in culture and express pancreatic islet cell differentiation markers such as Pdx-1, Pax4, Pax6, Nkx2.2, Nkx6.1, and islet hormones. These aggregates secrete insulin when challenged with glucose and reverse diabetes when transplanted in STZ-treated NOD-SCID mice. Similarly, correction of hyperglycemia was reported[64] in diabetic mice injected with a rat hepatic cell line, which was cultured in high-glucose medium and expressed an active form of *Pdx-1* (and the reporter gene RIP-enhanced green fluorescent protein (eGFP) to facilitate cell selection). Despite the observed improvement in mice that underwent transplantation, the cells failed to secrete insulin when stimulated by glucose, and their insulin content was less than 1% of that of native β-cells.

Liver cells infected with a NeuroD-carrying adenovirus and treated with the mitogen betacellulin have also been shown to reverse STZ-induced diabetes.[65] Although these studies have suggested that liver cells may ultimately serve as a potential source for β-cells, important questions must be addressed regarding which liver cell(s) can commit to a pancreatic endocrine cell fate and the minimum set of genes required to be activated or inactivated for transdifferentiation to a fully functional β-cell.

*Bone marrow cells.* Stem cells derived from the bone marrow are also considered a prospective source for pancreatic β-cells.[66,67] Rat bone marrow mesenchymal cells differentiate in low-serum or serum-free media toward cells exhibiting secretory-like granules and expressing β-cell genes. However, insulin secretion by these cells, compared with that of isolated islets, has been reported as low[68] or high enough to reverse hyperglycemia in diabetic rats.[69] This discrepancy may be attributed to differences in the isolation and differentiation protocols described in these studies. The potential of bone marrow cells to adopt an insulin-producing cell phenotype was further illustrated using transplantation of bone marrow cells isolated from male (insulin II gene promoter-CRE x ROSA-stoplox-eGFP) mice into sublethally irradiated female mice.[70] Four to 6 weeks after transplantation, cells positive for the Y chromosome were detected in the pancreas and other tissues. However, eGFP-positive cells were found only in the pancreatic islets and constituted 1.7% to 3% of all islet cells. The authors also conducted experiments that ruled out the *in vivo* occurrence of cell fusion. Other groups could not reproduce these results,[71,72] which warrant further investigation into whether bone marrow progenitors are capable of adopting a pancreatic β-cell fate.

*Umbilical cord blood cells.* Umbilical cord blood (UCB) is also known to contain progenitor cells, especially hematopoietic cells.[73] However, the presence of mesenchymal stem cell markers[74] may be indicative of a greater plasticity of UCB cells than previously believed. Cord blood can be readily available in sufficient amounts with low risk for graft rejection[75]. Pessina *et al*.[76] cultured human UCB cells under conditions that promoted the expression of Isl-1, Pdx-1, Pax-4, and Ngn3, which are present in endocrine cell commitment. Transplantation of human UCB cells to diabetic mice led to correction of hyperglycemia, presumably because of *in vivo* differentiation of the cord cells to insulin-producing cells.[77] The detection of human C-peptide after intraperitoneal glucose tolerance tests further supported this claim.

Other tissues have also been investigated for the presence of progenitor cells that can transdifferentiate to insulin-producing cells. Cells isolated from the spleen,[78] adipose tissue,[79] salivary glands,[80] blood,[81] amniotic epithelium,[82] and central nervous system[83,84] express β-cell markers upon differentiation *in vivo* or *in vitro.* In particular, injection of splenocytes combined with Freund's adjuvant improved diabetes in diabetic NOD mice.[78] It was suggested that the splenocytes became insulin-producing cells, whereas the adjuvant minimized the onset of autoimmunity. Subsequent reports,[85–87] however, found no evidence of allogeneic spleen cell differentiation to new islet β-cells and attributed the increase in islet mass to the blocking of autoimmunity, allowing the repopulation of islets with regenerating β-cells.

## REGENERATION OF PANCREATIC β-CELLS

Postnatal β-cells proliferate under normal physiological conditions, albeit at a low rate.[88,89] Faster turnover has been documented in response to insulin resistance,[90,91] type 1 diabetes,[78] pancreas injury,[92,93] and pregnancy.[94] β-cell apoptosis, which is observed even in non-diabetic individuals, also supports the turnover of existing β-cells as a mechanism for maintaining islet mass.[91] *In vivo* stimulation of β-cell regeneration with molecular therapeutics or *ex vivo* induction of β-cell expansion may be used to produce large

quantities of functional (and possibly immunocompatible) β-cells.

Multiple compounds have been investigated for their potential to augment β-cell proliferation or limit β-cell apoptosis. Glucagon-like peptide-1 (GLP-1) and its long-lasting analog exendin-4 are capable of stimulating β-cell insulin secretion, inhibit apoptosis, and increase islet mass in diabetic and non-diabetic rodents.[95–98] In fact, type 2 diabetes patients treated with exendin-4 in clinical trials experienced an improvement in glycemic control associated with no weight gain.[99,100] GLP-1 analogs may also be combined with molecules that minimize degradation of these analogs, thereby enhancing the regeneration of β-cells. For example, dipeptidyl peptidase IV inhibitors have been investigated for the treatment of patients with type 2 diabetes.[101,102]

Other molecules stimulating the proliferation of β-cells include members of the regenerating gene protein family,[103,104] especially the islet neogenesis gene-associated protein,[105] hepatocyte growth factor,[106–108] insulin-like growth factor,[109,110] betacellulin,[111] and the combination of EGF and gastrin.[112] Many of these factors have been shown to increase β-cell proliferation and expand β-cell mass, mainly in rodents. A deeper insight into the effect of these factors on the human β-cell cycle and signaling will be essential before considering their use for therapeutic purposes. In addition to achieving extensive regeneration to realize sufficient amounts of β-cells, altering the immunological profile of these cells may be necessary, especially for type 1 diabetes remedies.

## THE CHALLENGES AHEAD

Published studies on the differentiation of ESCs and APCs and the regeneration of primary pancreatic β-cells have sparked great hope that a cell replacement therapy for diabetes is possible. Nonetheless, these studies also attest to significant challenges that must be addressed before translating laboratory findings to clinical reality. Low efficiency and the production of highly heterogeneous cell populations thus far characterize *ex vivo* derivations of insulin-producing cells. Broadening of our knowledge of pancreas development and progenitor cell renewal and differentiation will be essential in the improvement of methods for the generation of islet cells.

For the use of ESCs in therapies, the risk of tumorigenicity is of major concern. ESCs transplanted in mice form teratomas[113] or teratocarcinomas present a potential danger if undifferentiated ESCs are inadvertently present in the cell population intended for transplantation. Potential karyotypic abnormalities that have been observed in some hESC lines after prolonged culture can further compound this problem.[114–116] The development of tools suitable for assessing the quality of the initial stem cell population intended for differentiation and the resulting cells will be necessary. Current lineage selection protocols entailing the introduction of selectable marker genes to ESCs have significant drawbacks. Long-term effects of ESC gene manipulation are not well understood. Furthermore, no single drug-resistance enrichment procedure generates a completely pure population of insulin-producing cells. This is important because teratomas result not only from the transplantation of ESCs but of ESC-derived, partially differentiated cells as well. On the other hand, most selection methods entail a procedure for sorting cells or islet-like structures (e.g., fluorescence activated cell sorting[117,118]). Current cell-sorting technologies require further improvements for processing large quantities of cellular material. Thus, the development of high-throughput platforms capable of meeting clinical demands is expected to play a key role in future stem cell–based therapies.

In addition to oncogenicity, the immunocompatibility of hESC-derived cells should be scrutinized. In the case of type 1 diabetes, which is an autoimmune condition, the matter of transplanted cells' immunocompatibility requires the consideration of multiple factors, many of which are also germane to immune system cells and are beyond the scope of this review.[119,120] However, there are still gaps in our knowledge regarding the immunostimulatory potential of hESCs. Evidence[121,122] suggests that hESCs have low immunogenicity, a property that depends strongly on the transplantation setting (e.g., allogeneic, xenogeneic).[123] Reduction of host reactivity to allogeneic ESC-derived transplants could be achieved through immunosuppression, which unfortunately is associated with serious side effects. Alternatively, the islet cell surrogates can be protected from responses of the immune system when encapsulated in biocompatible matrices. Furthermore, the cells can be engineered to cause minimal stimulation of immune response. For example, cell banks can be established for the storage of hESC lines modified to display particular human leukocyte antigen (HLA) profiles, although generating a sufficient number of HLA-isotyped hESC lines will require enormous effort and resources. Immune rejection of implanted cells may also be avoided by eliminating immune attack-triggering agents such as proteins of the major histocompatibility complex,[124] but the long-term effects of such knock-outs are unknown.

Generation of autologous hESCs has also been proposed through the transfer of somatic nuclei of the patient to enucleated human eggs, an approach known as therapeutic cloning. Although current technologies for successful and efficient transfer of human cell nuclei require further development, the use of human oocytes for the generation of autologous donor cells encumbers non-trivial ethical and legal problems. For this purpose, alternative methods (e.g., nuclear reprogramming,[125,126] parthenogenetically derived embryos[127]) for deriving hESCs have been proposed that may ultimately eliminate the need for human embryos and oocytes.

Such ethical concerns are not germane to the derivation of adult stem cells. Moreover, no evidence exists of tumor formation in APC grafts. However, relying on adult stem

cells as a source for β-cell surrogates presents other challenges. First, tissue-residing stem cells are isolated in small numbers as part of heterogeneous populations, and standard protocols for the isolation and purification of these cells are lacking, which also makes difficult the interpretation and comparison of different studies on APCs. Second, unlike ESCs, most APCs (with exceptions, e.g., multipotent APCs[128]) have limited proliferation potential, greatly impeding efforts to scale up APC culture systems. Culture conditions allowing for prolonged proliferation of these cells are highly desirable, but factors promoting APC proliferation are not fully known. In many cases, progenitor cells are maintained in culture media supplemented with mitogenic factors (e.g., EGF), which may contribute to the appearance of genetically abnormal strains over extended cell passaging. Third, many tissue-residing progenitors possess limited plasticity, which makes their differentiation to distant cell types uncertain. In fact, evidence to support the proposal for transdifferentiation (i.e. the adoption of a substantially different fate by cells already committed to a specific lineage) has been controversial, and for some studies, alternative explanations may become more attractive.[129,130] Fourth, APCs present a model of autologous transplantation with the donor and the recipient of the cells being the same patient. This attribute of APCs may be a boon for the treatment of other diseases but not of autoimmune diabetes. The potential use of APCs in diabetes will require altering their immunogenic profile to minimize the prospect of autoimmune rejection.

With the exception possibly of *in situ* drug-stimulated regeneration of β-cells, insulin-replacement therapies will necessitate the development of scalable bioprocesses for the expansion of cells to clinically relevant quantities. *In vitro* stem cell differentiation and β-cell regeneration have been conducted in static cultures, which are difficult to scale up. Because the number of cells grown is proportional to the surface area available for growth, an impractically large expanse would be necessary for generating the cell quantities needed for therapies. The lack of tight control and continuous monitoring of culture variables increases the risk of spontaneous differentiation to non-β-cell lineages, further exacerbating this shortcoming.

Bioreactor systems may help to tackle such limitations. Stirred suspension vessels have been used for the expansion of undifferentiated mESCs on microcarriers or as aggregates and their differentiation.[131–133] In conventional stirred-tank cultures, concentrations of $10^6$ to $10^7$ cells/mL are common. To put this in perspective, the minimum number of human islets required for insulin independence after transplantation is approximately 9,000 islet/kg,[134] or approximately $6\times10^5$ islets for a 70-kg recipient. This translates to approximately 1.3 billion β-cells for each procedure. Therefore, suspension culture vessels with working volumes of a few hundred mL to a few L will be sufficient, although problems related to the respective efficiency of differentiation (or regeneration) and that of downstream processes should be considered as well. Furthermore, stirred-suspension conditions can promote the organization of insulin-producing cells into islet-like clusters. This may enhance the cell secretory function, considering the intimate link between islet-structure and β-cell function. Development of such systems will require better understanding of the determinants underlying stem cell self-renewal and differentiation.

## CONCLUSIONS

Insulin-producing cells derived from stem cells display phenotypic similarities with native β-cells but significant differences as well. Regeneration of β-cells has been demonstrated, but yields are still less than adequate to satisfy clinical needs. Generating large quantities of *bona fide* β-cells suitable for therapies may not be feasible in the immediate future. However, the data reviewed here undoubtedly prove that stem and progenitor cells and regenerating islet cells will play a key role as renewable sources of β-cell surrogates. The rapid advances and the extensive ongoing research effort in this field suggest that the challenges lying ahead can be addressed effectively and probably sooner than previously thought.

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