Insulin-producing Surrogate β-cells From Embryonic Stem Cells: Are We There Yet?

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Embryonic stem cells (ESCs) harbor the potential to generate every cell type of the body by differentiation. The use of hESCs holds great promise for potential cell replacement therapies for degenerative diseases including diabetes mellitus. The recently discovered induced pluripotent stem cells (iPSCs) exhibit immense potential for regenerative medicine as they allow the generation of autologous cells tailored to the patients' immune system. Research for insulin-producing surrogate cells from ESCs has yielded highly controversial results, because many steps and factors in the differentiation process are currently still unknown. Thus, there is no consensus on common standard protocols. The protocols presently used established the differentiation from pluripotent cells toward pancreatic progenitor cells. However, none of the differentiation protocols reported to date have generated by exclusive *in vitro* differentiation sufficient numbers of insulin-producing cells meeting all essential criteria of a β-cell. The cells often lack the crucial function of regulated insulin secretion upon glucose stimulation. This review focuses on past and current approaches to the generation of insulin-producing cells from pluripotent sources, such as ESCs and iPSCs, and critically discusses the hurdles to be taken before insulin-secreting surrogate cells derived from these stem cells will be of clinical use in humans.

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INTRODUCTION

Diabetes mellitus is a major health problem currently affecting around 280 million people worldwide and predicted to increase to 440 million adults by 2030.[1] Diabetes imposes a heavy burden of morbidity and premature mortality[2] and incurs a large and steadily increasing financial cost in the health system.[3] Once lost, the function of the insulin-producing β-cells cannot be recovered, rendering the diabetic patient dependent on a life-long supplementation therapy with insulin. Transplantation of a human donor pancreas or pancreatic islets offers a cure. However, donor organs are very limited and transplantation is therefore possible only for a few severely ill type 1 diabetic patients. Therefore, much attention has been focused on the potential of bioengineered insulin-producing surrogate cells.[4–7] Several sources have been considered for the *in vitro* generation of insulin-producing cells including *ex vivo* expanded β-cells,[8] endocrine progenitor cells,[9] transdifferentiated or transduced liver or intestinal cells,[10,11] bone marrow mesenchymal stem cells,[12] and pluripotent embryonic stem cells (ESCs).[13,14] ESCs harbor great potential for future cell replacement therapy of diabetes (**Figure 1**) because they offer two unique features: availability in potentially unlimited numbers and the plasticity to generate any cell type of the body by *in vitro* differentiation.

What are the minimal requirements for an insulin-producing surrogate cell of ESC origin?

Ideally a surrogate cell should be sufficiently differentiated toward an insulin-producing phenotype to ensure expression of all structures and components required to synthesize and release insulin in response to changes in extracellular glucose over the physiological range, adequately meeting the insulin demands without the risk of hypoglycemia. Such a system should comprise a glucose transporter system to facilitate the uptake of glucose at physiological concentrations. A glucose sensor is required to translate changes in intracellular glucose into corresponding changes in metabolic fluxes to generate an adequate signal for both insulin biosynthesis and the regulated exocytosis of insulin stored in secretory granules.[15] **Table 1** addresses some of the desirable and unacceptable phenotypical characteristics of surrogate β-cells destined for β-cell replacement through implantation in patients with type 1 diabetes. The data summarized in **Table 2** depict the deficiencies of present differentiation protocols which currently prevent their use in patients with diabetes for β-cell replacement therapy.

CURRENT STATUS OF ES CELL RESEARCH—HOW CLOSE ARE WE TO A β-CELL?

A decade ago the first proof-of-concept studies describing differentiation of ESCs into insulin-producing cells were published. In an elegant approach, Soria and co-workers differentiated a mouse ES cell line in which an antibiotic resistance gene was driven by the human insulin promoter.[16] Cells differentiated from this ES cell line corrected hyperglycemia when implanted into streptozotocin diabetic mice.[16] In a later study, the insulin promoter was replaced by the β–cell-specific Nkx6.1 promoter yielding comparable

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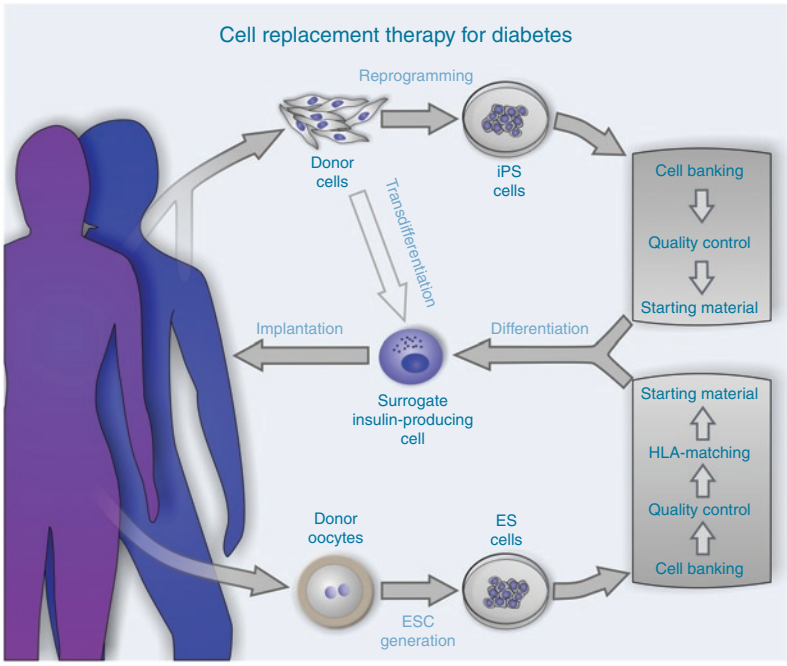

Figure 1 Strategy to obtain insulin-producing surrogate cells from pluripotent cell sources. The cell replacement therapy of diabetes with differentiated pluripotent cells requires either human embryonic stem cells generated from fertilized donor oocytes or reprogrammed somatic cells, induced pluripotent stem cells (iPSCs), as starting material. The intended therapeutic use presupposes "clinical grade" cell lines as seed stocks for the *in vitro* generation of insulin-producing cells. Cell banking systems assure that a uniform population of pluripotent cells is preserved, stocked, and made available to facilitate high quality and standardized research and later clinical use. To improve the chances of a successful implantation the banked cell lines would be analyzed for human leukocyte antigen (HLA)-antigen presentation and minimum matching levels must be defined in order to choose appropriate starting material for the patient. This step can be circumvented when patient-specific iPSCs are used. The differentiation of pluripotent cells recapitulates the pancreas development and organogenesis of islets of Langerhans. A purification step would be needed to enrich the endocrine β-cell population which must be devoid of contaminating cells harboring teratogenic potential. Before implantation, rigorous *in vitro* testing would determine whether the cells meet the functional criteria such as glucose-induced insulin secretion and other parameters as mentioned in **Table 1**. Directed transdifferentiation of somatic cells into insulin-producing surrogate cells, as recently shown for neurons, blood progenitors, and cardiomyocytes, may render the pluripotent intermediate expendable.

results.[17] Though in principle attractive, this approach has not been adopted in later studies for the generation of insulin-producing surrogate cells. The strategy of selecting insulin-expressing (and thus antibiotic resistant) cells after essentially spontaneous differentiation resulted in relatively low yields when compared to directed differentiation protocols, and the genetically altered phenotype would preclude their use in clinical studies (**Table 1**).

In a study that initially appeared ground breaking Lumelsky and co-workers[14] published a five stage, directed differentiation protocol which differentiated mouse ESCs (mESCs) into islet-like clusters composed of glucagon-, somatostatin-, and insulin-positive cells releasing insulin in response to glucose. The concept underlying this work was primarily based on a previous study in which functional neurons were generated from mESCs[18] and the suggestion that β-cells and neurons share a common progenitor cell expressing the neural stem cell-specific neurofilament nestin.[19] Various groups reproduced this protocol in both mouse and human ESCs, with variable degrees of success.[20–29] The origin of the protocol and the variable results obtained by other groups raised questions about the nature of the obtained cells with the Lumelsky protocol. It was suggested that these cells were primarily of a neuronal phenotype with a low level of insulin gene expression.[27] It became clear that cells generated with this protocol were highly prone to apoptosis or necrosis and they adsorbed significant amounts of insulin from the differentiation medium supplemented with a very high concentration (25 μg × ml^{-1}) of insulin.[22,25,26]

It became apparent that progress in the design of efficient protocols for generation of pancreatic β-cells from ESCs would depend on application of knowledge about the normal developmental mechanisms of the pancreas. Intensive study of the development of the endocrine pancreas, greatly assisted by the use of gene deletions in mice, has provided detailed information about the sequence of developmental events driving cells from foregut endoderm into fully differentiated β-cells.[30] Some of the key stages are shown in **Figures 2** and **3**. Mapping these events onto ESCs maintained in culture should allow the recapitulation *in vitro* of the normal *in vivo* developmental process. However, it is worth noting that the *in vitro* directed differentiation protocols do not exactly map onto normal *in vivo* development for human β-cells (**Figure 2**). Thus, the most influential directed differentiation protocols for human ESCs (hESCs)[31–33] allow ~20 days to differentiate into mature endocrine islet cells, although all available evidence suggests that insulin-expressing cells are first detected in the human fetal pancreas at around 8 weeks of development.[34] It remains to be seen whether some of the problems with current *in vitro* protocols reflect this artificial compression of the developmental process. It would be interesting to determine whether

Table 1 Functional phenotype of surrogate β-cells for transplantation therapy of type 1 diabetes

Optimal transplant material phenotype	Minimum acceptable phenotype	Unacceptable phenotype
Homogenous insulin-expressing population	Heterogenous population enriched with endocrine islet cells, but containing other endodermal cells (exocrine pancreas, hepatocytes); no undifferentiated ESCs	Heterogeneous population containing cells from all three germ layers; undifferentiated ESCs with potential for teratoma/teratocarcinoma formation
Full complement of β-cell-specific genes	Pdx-1, proinsulin, response elements (see below); full complement of β-cell-specific genes only after ***in vivo*** maturation	No/low expression of Pdx-1 and other β-cell specific genes; no improvement in gene expression after ***in vivo*** maturation
Processing of proinsulin to insulin and C-peptide	Significant levels of proinsulin, insulin and C-peptide	No processing of proinsulin; no C-peptide
Insulin content similar to mature β-cells; appropriate secretory ultrastructure	Lower insulin content, reduced number of secretory granules; appropriate secretory ultrastructure	Very low insulin content, few or no secretory granules, constitutive insulin release
Expression of glucose-response elements (Glut, GK, Kir6.1, Sur1, VDCC) at levels similar to mature β-cells	Expression of all glucose-response elements but at lower levels	Failure to express some or all essential response elements
Response to nutrients (glucose, amino acids, fatty acids)	Insulin secretion responsive to glucose	Poor or no responsiveness to physiological stimuli
Secretion potentiated by receptor-operated stimuli (incretins, neurotransmitters, etc.)	No potentiation by neurotransmitters or incretins	Inhibition of secretion in response to neurotransmitters
Good graft survival: tolerance to hypoxia, rapid revascularization, resistant to cytokine assault	Partial graft failure after implantation, susceptible to cytokine toxicity	Graft failure; necrosis, apoptosis.
Long-term maintenance of normoglycemia in animal models of diabetes; normal glucose tolerance	Reverse diabetic hyperglycemia but abnormal glucose tolerance remains	Failure to reduce hyperglycemia ***in vivo***
Hypo-immunogenic; not recognized as β-cell by diabetic host immune system	β-Cell immunophenotype; recipient requires immunosuppression	Hyperimmunogenic; rapid and aggressive immune response leads to graft rejection
No altered genotype	No foreign genes	Randomly inserted foreign genes

ESCs, embryonic stem cells.

extending the *in vitro* protocols to accurately reflect *in vivo* development would improve their efficiency and explain the current requirement for an *in vivo* maturation stage, as discussed below.

The observation that mESCs can be forced into the germ layer of definitive endoderm (DE) by treatment with activin A in the absence of fetal bovine serum offered an important first stage in directed differentiation protocols.[35] Activin A is a member of the tumor growth factor-β superfamily acting by Nodal signaling on the tumor growth factor-β pathway. Supplementation of the differentiation medium with high concentrations of activin A yielded 60% DE-cells from mESCs[35] and up to 80% from hESCs.[36] The effectiveness of this treatment can vary between different ES cell lines, consistent with reports that human ES cell lines differ in their differentiation propensities.[37,38] D'Amour *et al.* at NovoCell subsequently developed an *in vitro* differentiation protocol that guided hESCs through the key developmental steps of the pancreas from a pluripotent cell into DE, foregut, pancreatic endoderm, endocrine progenitor, and finally into a pancreatic endocrine cell by treatment with a sequential cocktail of growth factors and bioactive small molecules.[31] The cells produced with this differentiation protocol had an insulin content comparable to that of human islets and were positive for insulin and C-peptide as proof of *de novo* synthesis of insulin rather than passive uptake from the culture medium. The cells released insulin in response to various secretagogues, although not in response to glucose.[31] Moreover, the cells were double-positive for insulin and glucagon or for insulin and somatostatin suggesting improper endocrine specification (**Table 1**, unacceptable phenotype). Cho and coworkers reproduced this differentiation protocol for hESCs but added β-cellulin and nicotinamide to the final differentiation medium.[39] This allowed a sustained expression of PDX1, an important transactivator of the insulin promoter.[40] However, the results were not fully comparable with those of D'Amour *et al.* and the reported effectiveness of the protocol was considerably lower.[39] This may reflect differences in the differentiation potential of the initial hESC lines, emphasizing the requirement for a systematic evaluation of the cell lines used by different groups working in this area to identify the most suitable starting material from which to differentiate functional β-cells.[41]

Shim *et al.* used a shorter differentiation protocol and treated hESCs sequentially with fetal calf serum, activin A, and retinoic acid (RA).[42] After activin A treatment they obtained a $SOX17^+$ cell population which was committed to DE and by further treatment with RA they generated a $PDX1^+/FOXA2^+$ population. It was concluded that RA may convert gut tube endoderm into pancreatic endoderm, thereby confirming the results of D'Amour *et al.*[31] Nonetheless, further *in vitro* differentiation did not yield a significant fraction of insulin-producing cells, suggesting that additional cues are necessary to drive *in vitro* generated pancreatic endoderm toward endocrine progeny. An alternative approach by Jiang *et al.* started with the derivation of DE by treatment with a combination of activin A and sodium butyrate.[43] The developmental trigger

Table 2 Overview of the differentiation protocols used for mouse and human ESCs

Differentiation protocol	Approach	Obtained cell type	Adopted by	Main findings
Assady *et al.*[13] Shiroi *et al.*[113] Houard *et al.*[114]	Random differentiation	Mixed cell population. Disorderly differentiated pancreatic endocrine cells.	—	Proof of principle that pluripotent cells can differentiate toward a pancreatic, endocrine phenotype.
Soria *et al.*[16] León-Quinto *et al.*[17]	Selection by "gene trap"	Spontaneously differentiated insulin-expressing cells.	Not yet reproduced.	Selection of insulin-producing cells via promoter coupled selection with antibiotics. Reversal of normoglycemia in STZ-diabetic mice.
Lumelsky *et al.*[14]	Nestin selection	Neuronal cell type with very low endogenous insulin gene expression but significant uptake of insulin from the medium.	**Confirmed:** Hori *et al.*[23] Blyszczuk *et al.*[21] Moritoh *et al.*[24] Miyazaki *et al.*[115] Segev *et al.*[29] Bai *et al.*[20]	Differentiation via a nestin-positive cell precursor toward an insulin-positive cell type organized in islet-like structures.
			Qualified: Rajagopal *et al.*[26] Hansson *et al.*[22] Sipione *et al.*[27] Paek *et al.*[25] Naujok *et al.*[28] Naujok *et al.*[49]	Passive uptake of insulin from the differentiation medium into apoptotic / necrotic cells.
D'Amour *et al.*[31]	Directed development	Heterogeneous cell population enriched with pancreatic p rogenitors and polyhormonal islet cells.	**Confirmed:** Kroon *et al.*[2] Jiang *et al.*[43]	Differentiation through the key developmental steps of the pancreas into a polyhormonal endocrine cell type. Further *in vivo* maturation after implantation into immunocompromised mice. Reversal of normoglycemia in STZ-diabetic mice.
			Qualified: Matveyenko *et al.*[50] Courtney *et al.*[41]	Limited *in vitro* differentiation. Inconsistent maturation *in vivo*.
Borowiak *et al.*[53] Chen *et al.*[52] Zhu *et al.*[51]	Small molecules	Heterogenous cell population directed into pdx1$^+$, early pancreatic cells.	Not yet reproduced.	Usage of small chemical molecules to manipulate cell signaling pathways. Very efficient differentiation into a pdx1$^+$ population reminiscent of pancreatic endoderm. Further *in vitro* differentiation yielded large exocrine and small endocrine cell populations.

using RA was replaced in this protocol by a combination of EGF, bFGF and Noggin, and endocrine specification was induced by the addition of nicotinamide and IGF-II to the culture medium. The differentiated cells displayed a glucose-responsive C-peptide release. However, the partially abnormal expression of pancreatic transcription factors during the differentiation and the detection of polyhormonal cells positive for both C-peptide and glucagon, or for C-peptide and somatostatin suggest that these cells were not typical mature β-cells. Another approach which comprised treatment with activin A, RA and finally bFGF and nicotinamide resulted in islet-like structures with distinct insulin-, glucagon-, and somatostatin-positive monohormonal cells.[44] These cells showed low levels of glucose-stimulated release of insulin and C-peptide when cultured in adherence, but this was significantly enhanced when the differentiated cells were transferred to a suspension culture system.[44] A similar effect has been reported for the immortal insulin-producing MIN6 cell line, where responsiveness to nutrients is greatly enhanced by culture as nonadherent islet-like structures.[45,46].

In vitro vs. *in vivo* differentiation—maturation effects *in vivo*

Advances in the understanding of the differentiation potential of hESCs have raised expectations that large numbers of functional surrogate β-cells maybe produced by *in vitro* differentiation. Despite the promising results and the proof-of-principal studies demonstrating that mouse and human ESCs can give rise to a pancreatic endocrine progeny, most published protocols yielded populations of functionally restricted insulin-producing cells. These had either a polyhormonal phenotype,[31,42,43] lacked

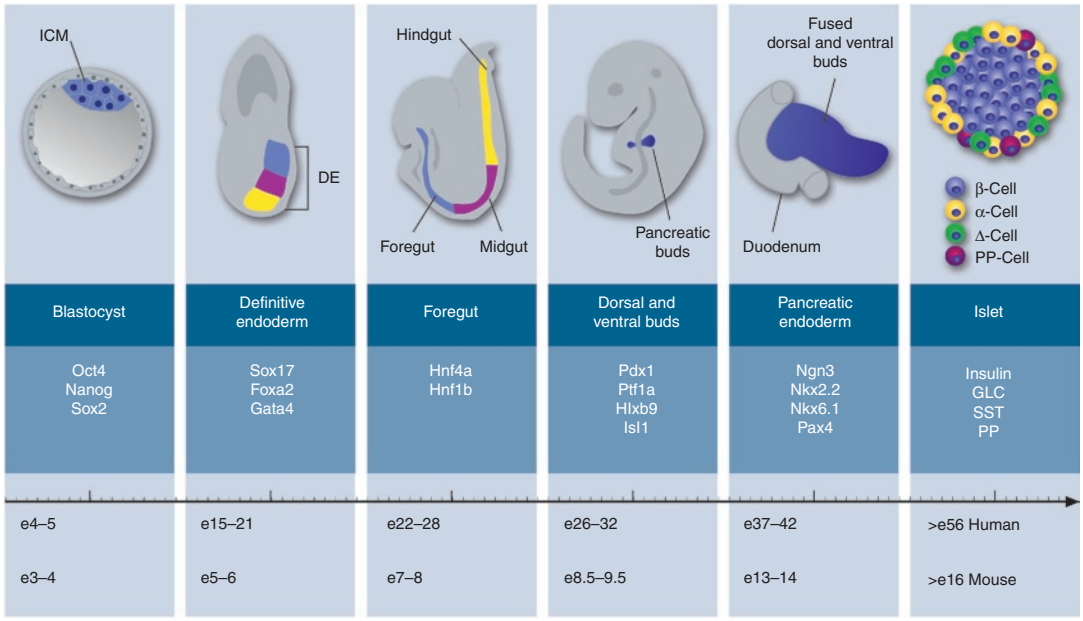

Figure 2 Simplified schematic presentation of the pancreas development in mice. The inner cell mass (blue) of the blastocyst, sometimes referred as embryoblast, gives rise to the three germ layers in the process of gastrulation. The definitive endoderm is then formed by the recruitment of epiblast cells through the primitive streak via a mesendodermal progenitor with the latter cells of the foregut (blue), midgut (purple), and hindgut (yellow). Morphogenesis of the primitive gut is a result of an invagination movement by which the layered definitive endoderm becomes a tube structure. The pancreas formation begins with the independent budding of the dorsal and ventral buds at the posterior region of the foregut. These two buds grow into the surrounding mesenchyme, branch in a tree-like structure and eventually fuse after rotation of the gut to form the definitive pancreatic endoderm. This predifferentiated epithelium grows in size with distinct endocrine and exocrine differentiation. The endocrine cells are organized in islets which are embedded in exocrine tissue and are composed of four major hormone-secreting cells types. Insulin is secreted by β-cells (blue), glucagon by α-cells (yellow), somatostatin by Δ-cells (green), and pancreatic polypeptide by PP-cells (purple). The timeline plots these key events for mouse. For comparison only, comparable stages of human β-cell development have been mapped on the timeline. Several markers characteristic of each developmental step are listed. DE, definitive endoderm; GLC, glucagon; ICM, inner cell mass; PP, pancreatic polypeptide; SST, somatostatin.

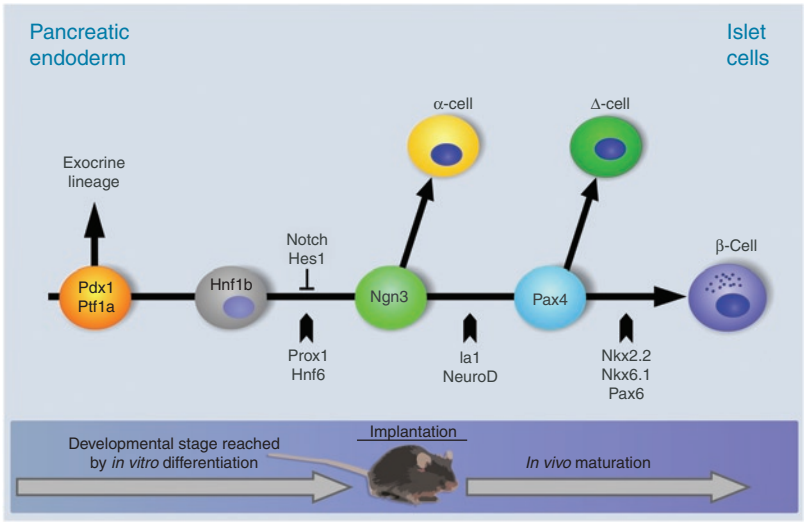

Figure 3 Simplified schematic presentation of the transcriptional hierarchy of essential factors and signaling pathways during endocrine specification of the pancreatic endoderm. The pdx1$^+$/ptf1a$^+$ population (orange) is the earliest cell type defining the pancreatic lineage beginning with the evagination of the pancreatic buds. With the fusion of the dorsal and ventral buds pdx1 and other transcription factors are downregulated (though still detectable) and endocrine cells that are marked by expression of ngn3 (green) become visible in the epithelium of the ducts. An intermediate cell type, presumably an hnf1b+ precursor duct cell (gray) is considered to be an ngn3-precursor. Proliferation of ngn3$^+$ cells is negatively regulated by the notch signaling pathway via the repressor Hes1. Pax4$^+$ cells (pale blue) are an intermediate cell type directly regulated by ngn3 and function as a switch to specify between β-cells (blue) and Δ-cells (dark green). Arrows point to the developmental step at which indicated transcription factors play an essential role. The time line along the bottom of the diagram shows the differentiation stages which have been achieved by *in vitro* differentiation alone or by a combination of *in vitro* differentiation and *in vivo* maturation.

appropriate glucose-induced insulin secretion,[31] or were contaminated with other cell types, including undifferentiated ESCs that could give rise to teratomas after implantation (**Table 1**, unacceptable phenotype).[21,32,47,48] Thus, it remains an unresolved question whether insulin-producing cells can be solely produced by *in vitro* differentiation.

The NovoCell group used hESCs to generate a population one step before terminally differentiated insulin-producing β-cells.[32] These cells were characterized by expression of the transcription factors NKX6.1, NGN3, and NKX2.2, a phenotype comparable to fetal 6–9-week-old pancreatic epithelial cells. Upon implantation into immunocompromised mice, the expression of human C-peptide was detected after 30 days and human insulin was readily detected 94 days postimplantation, demonstrating that triggers in the *in vivo* environment facilitated the final maturation into islet cells of the *in vitro* generated endocrine progenitor cells (**Table 1**, minimum acceptable phenotype).[32] The beneficial effect of the *in vivo* environment was also reported for mESCs[49] and for hESCs differentiated using a protocol different from that of NovoCell.[42] The basis of this effect remains unknown and seems to be independent from the implantation site being used, but the identification of the biochemical and physical factors involved in this process might provide the missing information required to generate fully matured β-cells by *in vitro* differentiation. In a recent study using implantation in nude rats Matveyenko *et al.*[50] reported inconsistent maturation of insulin-producing cells generated by NovoCell from hESCs according to their differentiation protocol.[32] These problems with reproducibility led Matveyenko and colleagues to conclude that "the extent of islet formation and its function is not yet sufficiently reproducible to be clinically useful."[50] Thus, it remains unclear, whether *in vivo* maturation alone can solve the problem of incomplete *in vitro* differentiation.

Role of small molecules during differentiation

Differentiation protocols for ESCs have so far been based on combinations of recombinant growth factors, supplements, and bioactive molecules. Nonetheless, the *in vitro* differentiation of ESCs is often poorly controlled probably because of differences in the activity of recombinant proteins, their half-lives, and their concentration dependency with respect to the particular stem cell line being used, all of which may affect the reproducibility of the differentiation protocols. To address this issue efforts have been made to identify membrane-permeable small molecules that can control cellular processes and induce endodermal and subsequently pancreatic differentiation.[51–53] The use of these molecules should offer a more controllable and reproducible approach because they can be chemically synthesized in high purity allowing standardization between laboratories. Recently a number of small molecules have been reported to direct mESCs and hESCs into the DE lineage, including the compounds IDE1/2[53] and the staurosporine family member stauprimide.[51] IDE1/2 induced a SOX17$^+$/FOXA2$^+$ cell population, reminiscent of DE, earlier and more effectively than the activin A/Wnt3a combination via the same tumor growth factor-β/Nodal signaling pathway. Stauprimide possibly interacts with NME2, a transcription factor controlling c-MYC expression, which is required by ESCs to maintain their pluripotent state. Upon binding of stauprimide to the NME2, its nuclear localization is inhibited and c-MYC expression is downregulated thereby priming ESCs for differentiation. In combination with small amounts of activin A, 60% of DE-cells were generated from mESCs and 80% of DE-cells using hESCs.[51] ESCs committed to DE can be manipulated to further develop via the foregut stage into pancreatic endoderm by treatment with (−)-Indolactam V.[52] This treatment generated populations with 20% pdx1$^+$ cells from DE-committed ESCs, and this was further enhanced when (−)-Indolactam V was combined with FGF-10, resulting in up to 46% pdx1$^+$ cells.[52] (−)-Indolactam V is a broad spectrum agonist of novel and classical PKCs.[54,55] But the novel PKC isoform Δ is a target of the RA-signaling pathway, providing evidence for an analogous effect of (−)-Indolactam V to that of RA on ESCs.[56,57] On the other hand, neither knock out of classical PKCs[58,59] nor knockout of novel PKCs [60,61] had a negative effect on mouse pancreatic, endocrine development as would be expected if PKC did play a critical role. Additional work is needed to fully understand the role of PKC-signaling and its isoforms during pancreas organogenesis.

However, these findings are a major contribution to the field, since the discovery of additional small molecules (reviewed in refs. 62,63) exerting their effects on this particular developmental stage would drive ESCs to the ngn3$^+$ endocrine progenitor stage, the developmental step prior to the mature islet cell (**Figure 3**).

PATIENT-SPECIFIC STEM CELLS

Human ESCs are derived from embryos and although it is beyond the scope of this review, the use of embryos for this purpose remains controversial. In addition to the obvious ethical concerns about the use of hESCs, one of the major barriers to their use for cellular replacement therapy for type 1 diabetes is the challenge of immune rejection of the transplanted cells. This issue has yet to be addressed using somatic cell nuclear transfer technology to derive patient-specific hESC lines and as a result, the search for an alternative source of autologous stem cells has continued to receive significant attention.

In 2006, Yamanaka and co-workers reported a significant advance in this area. From an initial pool of 24 candidate transcription factors they demonstrated that the ectopic expression of just four of these factors (Oct3/4, Klf4, Sox2, and c-Myc) was sufficient to reprogram mouse embryonic fibroblasts into pluripotent stem cells, which were termed induced pluripotent stem cells (iPSCs).[64] These cells were shown to be morphologically similar to ESCs, to possess a normal karyotype, to express ESC-marker genes and to maintain the developmental potential to form teratomas of all three germ layers when injected into nude mice. Within a year, two independent groups had successfully reprogrammed adult human fibroblasts to produce human iPSCs.[65,66] Besides fibroblasts, iPSCs have now been derived from a range of somatic cell types including neural progenitor cells,[67] keratinocytes,[68] peripheral blood cells,[69] pancreatic β cells,[70] and hepatocytes.[71]

In addition to the obvious benefits of an autologous cell population, these cells have the major advantage that their derivation does not require the use of human embryos or oocytes, making their use less controversial than hESCs, both ethically and politically. However, in spite of this, there remain significant barriers to the clinical use of iPSCs. Genes that are known or suspected to

be oncogenes must be omitted from the reprogramming protocol. Thus, reports have demonstrated that iPSCs can be generated, albeit at lower efficiency, in the absence of the known oncogenes c-myc and Klf4.[72] Furthermore, although the initial reports describing the derivation of iPSCs used lentivirus or retrovirus to introduce the exogenous reprogramming factors, the potential for insertional mutagenesis using this approach renders it unlikely to lead to the generation of clinically useful cell populations. As a result, a number of alternative nonintegrating reprogramming strategies have been described, including the use of expression plasmids,[73] episomal vectors,[74] "piggyBac" transposition,[75] Cre- or Flp-recombinase-based excisable viruses,[76,77] and most recently membrane-soluble protein-induced methods,[78,79] although the efficiency of these protein-based methods is very low. The introduction of these more clinically acceptable reprogramming strategies meant that research into the generation of differentiated cell types for replacement therapy has gathered momentum. Indeed, this has included reports describing the differentiation of insulin-producing cells from human iPSCs generated from normal human fibroblasts[33,80] and fibroblasts from patients with type 1 diabetes.[81] These studies are based on differentiation protocols that begin with the induction of DE using activin A and then proceed with the subsequent stepwise differentiation into precursor populations similar to those found during *in vivo* pancreatic development. The authors demonstrated, using RT-PCR and immunofluorescence analysis, the appearance of markers of the pancreatic lineage. These include, amongst others, PDX1, HNF6, HNF4a, and NKX6.1 and the islet hormones insulin and glucagon. In addition, these iPSC-derived, insulin-expressing cells responded to elevations in glucose concentrations with either modest increases in C-peptide release[33,80] or in the case of one study,[81] increases that were at least fivefold over basal levels, suggesting the presence of functional glucose-sensing cells. However, although all three studies concluded that it was possible to derive insulin-expressing β-like cells from iPSCs, they universally added the caveat, that until differentiation protocols are improved, it will not be possible to directly compare iPSC-derived insulin-expressing cells to pancreatic β-cells. As discussed above, this is very similar to current thinking with respect to the differentiation of hESCs into pancreatic β-cells. Indeed, in reports where differentiation toward a β-cell phenotype has been compared between hESCs and iPSCs, much of the data are largely comparable.[33,80,81]

It is clear that the minimum requirements for insulin-producing cells derived from hESCs will also apply to cells derived from iPSCs. These cells will therefore have to exhibit absolutely no tumorigenic potential, be xenogen-free, have a high degree of cellular homogeneity and exhibit appropriate insulin content and secretion in response to physiological concentrations of glucose and, ideally, to other relevant stimuli. However, there are additional considerations when deriving insulin-producing cells from iPSCs, because full concordance of the iPSC-genome, transcriptome, and methylome with ESCs has yet to be demonstrated. Epigenetic markers, such as DNA-methylation, have been identified as a barrier to full reprogramming[82] and some somatic cell types, particularly somatic stem cell populations such as neural and myeloid stem cells, may be more amenable to reprogramming. Guenther *et al.* reported minimal differences in the chromatin structure and gene expression of human iPSCs compared to hESCs,[83] but there is now evidence that iPSCs may retain the epigenetic signature associated with their somatic cell type of origin.[84,85] Several studies revealed substantial epigenetic differences, reprogamming variability and somatic memory,[86–89] which are transmitted to iPSCs derived from somatic cells,[89] and which may increase variability and affect the differentiation potential of the iPSCs.[90,91] Recently gene copy number variations in early passage iPSCs[92,93] have been reported.[85,86] Some iPSC lines cumulated somatic coding mutations[94] and chromosomal aberrations,[95] raising concerns about an increased disease risk. Additionally, the rate of teratoma formation after implantation into immunodeficient mice has been shown to be increased in iPSCs generated from adult cell types compared to hESCs or iPSCs generated from mouse embryonic fibroblasts.[96] The therapeutic benefit of iPSCs compared to ESCs as a renewable, autologous cell population has been questioned by a recent report where T-cell-mediated immune-rejection of mouse iPSCs was observed in syngenic recipients.[97] These results encourage to classify iPSCs as overall similar, but not identical to ESCs and, thus, it will be important to identify the most appropriate starting population for iPSC-derivation and to carefully analyze the genetic, epigenetic, and immunogenic status of any derived iPSC-line.

Although pluripotent cells hold enormous promise for cell therapy of type 1 diabetes, both iPSCs and ESCs share the adverse capability of uncontrolled cellular proliferation and formation of teratomas upon implantation into a host organism.[98,99] The crucial issue of tumourigenicity of ESCs and iPSCs has been reviewed recently.[100,101] Though principally benign, teratomas not only pose a risk to the graft[47] but also a serious health risk to the patient. The exclusion of such potentially dangerous cells from transplant material is a prerequisite before pluripotent cells will ever become acceptable as a source for cell replacement therapy in regenerative medicine (**Table 1**, unacceptable phenotype). To achieve this several options have been considered: implantation of mature cells without contaminating residual undifferentiated cells, sorting techniques, positive selection utilizing resistance genes[16,17,102,103] and selective ablation of undifferentiated cells.[104–106] Thus, effective techniques to remove potentially teratogenic cells from mixed populations have been reported, but they have not yet been combined with protocols for differentiation of pluripotent cells into insulin-producing β-cells. This prevents pluripotent cells from clinical use at present.

Finally, recent work describing the directed conversion of fibroblasts to functional neural cells,[107] similar reports for directed conversion of human fibroblasts into multilineage blood progenitors,[108] mouse fibroblasts into cardiomyocytes[109] and mouse fibroblasts into functional hepatocyte-like cells[110] may render both hESCs and iPSCs redundant for regenerative medicine applications. Indeed, similar studies investigating the direct *in vivo* reprogramming of fully differentiated pancreatic exocrine cells into cells that closely resemble pancreatic β-cells by transient adenoviral overexpression of the three transcription factors Ngn3, Pdx1, and MafA, suggest that this direct reprogramming approach may offer an attractive alternative therapeutic strategy.[111]

CONCLUSION

The derivation of β-cells from human pluripotent cells for cell replacement therapy of diabetes remains an unresolved issue.

While possible in principle, current differentiation protocols yield insufficient numbers of insulin-producing cells which do not yet meet the functional criteria of genuine β-cells. The cells obtained so far were heterogeneous populations of mixed phenotypes and harbored teratogenic potential (**Table 1**, unacceptable phenotype). Progress has been made by applying development principles to *in vitro* differentiation protocols. However, much of that what is known today about the development of the endocrine pancreas has been obtained by studying gene functions in knockout mice. One of the key challenges will be to address the question whether the transcriptional networks driving pancreas development in mice also play a crucial role in human pancreas development.

ESCs can be forced via the DE-stage into a pdx1$^+$ cell population reminiscent of fetal pancreatic endoderm, but further *in vitro* differentiation toward authentic β-cells has been hampered by low efficiencies and a high exocrine to endocrine ratio of the generated cells.[50] This can be overcome, at least in part, through an *in vivo* incubation period after implantation of the cells into small laboratory animals, where extensive maturation into islet-like structures with a robust insulin and C-peptide release has been reported. Transplant material generated by this *in vivo* approach is obviously not suitable for therapeutic use in humans, so alternative approaches must be devised for driving ESCs from pdx1$^+$ progenitor cells toward fully functional β-cells. In future studies additional parameters to insulin content or release will have to be taken into account. Functional criteria for an authentic β-cell phenotype must be assessed, *e.g.*, whether the differentiated cells express the full complement of β-cell-specific transcription factors, amongst them NEUROD1, MAFA, ISL1, the expression of structural genes necessary for glucose-responsive insulin secretion at similar levels to mature β-cells, including glucose transporters, glucokinase, KIR6.1/SUR1, and voltage-dependent calcium channels (**Table 1**, optimal transplant material phenotype). Moreover, long-term studies in large animal models are required to demonstrate that implanted insulin-producing cells generated from ESCs or iPSCs engraft successfully and maintain their function over a time-span of years. Whether implanted insulin-producing surrogate cells of ESC/iPSC origins will present a target for autoimmune cell destruction in patients with type 1 diabetes remains unknown. If so, patient-specific, pluripotent cell lines (such as iPSCs) may not be the most appropriate starting material for differentiation and later implantation. Complete identity between donor and recipient, as well as complete discordance between them may cause subsequent problems,[112] so a more effective therapeutic option to ensure long-term function might be to use insulin-producing cells generated from an human leukocyte antigen-matched hESC-line with mild immunosuppression after implantation. Alternatively, it may be possible to engineer cells to resist the potential autoimmune attack, but this would require genetic manipulation, thereby raising concerns about insertional mutagenesis. Finally, implantation of cells derived from pluripotent stem cells entails the risk of tumor formation and this safety issue will need to be addressed to enable the translation of pluripotent stem cell-based therapies into clinical treatment of diabetes.

In summary, this is an exciting and fast-moving area of research and recent experimental studies have provided proof-of-concept that pluripotent stem cells can be driven to differentiate into insulin-expressing cells. There remain several obstacles to translating these observations into clinical treatments of diabetes, but none of these appears to be insurmountable in the future. These include (i) assessment of the differentiation potential of pluripotent stem cell populations to select the most appropriate starting material; (ii) refinements of current protocols to enable the generation of functionally competent β cells entirely *in vitro* under defined culture conditions; (iii) validation of purification methods of sufficient stringency to ensure the absolute exclusion of potentially teratogenic, pluripotent cells; (iv) development of techniques to scale-up laboratory based protocols to generate the large numbers of cells required for clinical use.

Finally, the wide-spread adoption of any new therapy will depend on it being shown to be at least as effective and as safe as the well-tested current choice of administration of exogenous insulin. It remains to be seen how the current obstacles to therapeutic translation can be overcome.

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