Stem cells and the endocrine pancreas

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Background: Diabetes can be treated by β-cell replacement therapy but the supply of graft material from human donors is too limited to make a significant clinical impact. Substitute β-cells generated from stem cell populations offer a potential source for the large numbers of cells required.

Sources of data: Primary peer-reviewed reports of experimental studies.

Areas of agreement: Embryonic stem cells and/or induced pluripotent stem (iPS) cells are currently the most promising starting populations from which to generate large numbers of β-cells. Differentiation protocols that recapitulate in vivo development generate insulin-expressing cells in vitro.

Areas of controversy: Differentiation outcomes may depend on the source of the initial pluripotent cells. The insulin-expressing cells are not fully functional. In vivo maturation is inconsistent and not well understood.

Areas timely for developing research: Improvement of current protocols for complete in vitro differentiation to a functional β-cell phenotype. Systematic analysis to identify the most appropriate starting material. Improved purification methods to ensure safety of material for clinical transplantation.

Keywords: cell therapy/diabetes/insulin/pancreatic β-cell/adult stem cells/embryonic stem cells

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Introduction

Diabetes mellitus is emerging as a major health problem—an estimated 280 million people worldwide are currently affected by diabetes and this is predicted to increase to 440 million by 2030.1 Type 1 diabetes mellitus (T1DM) is a single-cell disorder in which insulin-secreting β-cells in pancreatic islets of Langerhans are irreversibly destroyed by an autoimmune assault, resulting in potentially fatal metabolic dysfunction as a consequence of insufficient circulating levels of insulin. The metabolic consequences of diabetes are associated with greatly increased morbidity and premature mortality, and treating people with diabetes incurs large and steadily increasing financial costs.2
Since the isolation of insulin in the 1920s the main therapeutic approach to T1DM has been insulin replacement, but excursions in blood glucose associated with intermittent insulin delivery lead to a high risk of long-term complications, which are largely responsible for the increased morbidity and mortality associated with T1DM. Interest has therefore focused on β-cell replacement therapy as a means to maintain normoglycaemia. Over the past decade advances in human islet isolation and the adoption of new immunosuppression regimens have substantially improved the clinical outcomes in studies assessing the potential of human islets transplantation as a therapeutic approach for T1DM. Current transplantation protocols are far from perfect, and the therapeutic effects are not yet permanent, but these studies offer an important proof-of-concept that cell-based treatments for T1DM can be effective. However, a major impediment to the widespread uptake of transplantation therapy for T1DM is the very limited availability of suitable transplant material. Each transplant recipient requires ~1 × 10^6 islets to achieve normoglycaemia, around the same number of islets that are found in a healthy human pancreas. However, the enzymatic isolation and purification of islets is inefficient, so islets isolated from several donor pancreases are usually required for each transplant. The only clinically acceptable source of human islets for transplantation is currently from pancreases of heart-beating, brain-dead donors. This type of organ donor is relatively rare, so human islet transplantation is unlikely to make a wide-spread therapeutic impact on T1DM unless alternative sources of insulin-secreting β-cells can be identified. Each human islet contains ~2000 β-cells, so each transplant requires at least 1 billion β-cells. To extend this transplantation therapy to the hundreds of thousands of people with Type 1 diabetes in the UK alone will therefore require access to vast numbers of functional β-cell substitutes.

**Phenotype required of replacement β-cells**

The end point of many published studies in this area is the demonstration of an ‘insulin-expressing’ phenotype by measuring levels of (pre)proinsulin mRNA, or insulin/C-peptide immunoreactivity, or both. However, a primary β-cell possesses a number of functional attributes in addition to insulin expression, so before considering potential sources of substitute β-cells it is worth considering some of the essential features of such cells. First, they must obviously be able to synthesize, process and store insulin, and to do so in sufficient amounts to satisfy metabolic demand. It is often overlooked that primary β-cells store large quantities of insulin in membrane-
bound secretory granules, with stored insulin comprising up to 10% of total cellular protein (Fig. 1). Clinically relevant substitute β-cells will require this insulin storage capacity but very few published protocols come close to achieving these levels of hormone synthesis and storage. Secondly, the cells should be able to release insulin in sufficient amounts and appropriate patterns to maintain plasma glucose in a narrow range (5–7 mM), without secreting too much insulin which may precipitate damaging hypoglycaemia. Primary β-cells use complex mechanisms to monitor and respond to changes in metabolic signals and substitute β-cells will need similar mechanisms to enable a tightly regulated release of insulin in response to environmental cues. Finally, authentic β-cells derived from human donors have an exceedingly low proliferative capacity preventing unregulated expansion of the β-cell mass which could lead to the development of hyperinsulinaemic hypoglycaemia. Ensuring limited and tightly controlled proliferation is a challenge when attempting to generate insulin-expressing cells in vitro from highly proliferative stem cell and/or precursor populations.

A number of suggested starting materials fail to meet one of the criteria of highly specialized secretory function and controlled proliferative capacity, and are therefore unlikely to be clinically acceptable. For example, it is relatively straightforward to engineer non-β-cells to make insulin, and this has been done in a variety of cell types including fibroblasts, skeletal muscle, neuroendocrine, kidney and ovarian cells. However, these engineered cells lack the

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**Fig. 1** Ultrastructure of a human β-cell. The image shows a transmission electron micrograph of a section through a human islet to illustrate the specialized ultrastructure of the insulin-producing β-cells. Insulin is stored in β-cells in numerous dense-cored, membrane-bound secretory vesicles (SV, arrowed) to ensure that the endocrine pancreas has sufficient stores of insulin to meet metabolic demand. N, nucleus; M, mitochondrion. Bar = 2μm. (Jones and Persaud, unpublished).
stimulus-response coupling elements used by authentic β-cells, so their insulin secretory responses are unregulated and not responsive to physiologically relevant signals. Transformed insulin-secreting cell lines can be engineered to produce regulated secretory responses, and transformed human β-cells offer one means of generating in vitro the vast numbers of cells required for transplantation therapy. However, transformed cells show unregulated proliferation and form insulinomas in vivo, and so are unlikely to offer any therapeutic benefit in the foreseeable future.

In contrast, stem cells are defined by their developmental plasticity (pluripotency) and self-renewal (proliferation) and in our view these properties make them the most likely candidates from which to generate the quantities of cells required for transplantation whilst maintaining differentiated function. The rest of this review will therefore focus on the therapeutic potential of stem cells for transplantation therapy of diabetes.

**Potential sources of replacement β-cells**

The stem-cell populations that have been used in experimental studies on generating insulin-expressing cells can be broadly subdivided as either tissue stem cells, defined as multipotent progenitor cells found in foetal and adult tissues; embryonic stem cells, defined as pluripotent, undifferentiated cells generated from the inner cell mass of a developing blastocyst; or induced pluripotent stem cells, defined as pluripotent cells generated by reprogramming differentiated adult cells by forced expression of pluripotency genes (Table 1).

<table>
<thead>
<tr>
<th>Property</th>
<th>Tissue stem cell</th>
<th>ES cell</th>
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<tbody>
<tr>
<td>Origin</td>
<td>Many adult/foetal tissues</td>
<td>Blastocyst</td>
</tr>
<tr>
<td>Function in vivo</td>
<td>Tissue repair/renewal</td>
<td>Transient, form embryo</td>
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<tr>
<td>Differentiation potential in vivo</td>
<td>Lineage restricted Host tissue/organ</td>
<td>Unrestricted All adult tissues</td>
</tr>
<tr>
<td>Differentiation potential in vitro</td>
<td>Lineage restricted: multipotent For example, osteogenic; chondrogenic; adipogenic</td>
<td>Unrestricted: pluripotent All adult cell types</td>
</tr>
<tr>
<td>Proliferation in vivo</td>
<td>Variable</td>
<td>Teratogenic</td>
</tr>
<tr>
<td>Proliferation in vitro</td>
<td>Variable</td>
<td>Unlimited</td>
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Tissue stem cells are often considered as lineage-restricted progenitor cells that mature into the differentiated cells of the host tissue, but experimental studies have suggested that progenitor cells from a wide variety of tissues may have the potential to become insulin-expressing cells. These studies have been reviewed extensively elsewhere and it is becoming apparent that some reports have proven difficult to reproduce, and that earlier promising studies using tissue stem cells have so far failed to translate into reliable protocols for generating in vitro large numbers of functional β-cells.

Pancreatic stem cells are one of the more promising candidates. Although the proliferative capacity of adult β-cells is very low, it is well established that the β-cell mass increases during development, in pregnancy and with obesity. The origins of these new β-cells is uncertain but lineage tracing studies in mice suggest that they can arise by the slow self-renewal of existing β-cells, from glucagon-expressing α-cells and from proliferative human progenitor cells that have been localized previously to the exocrine pancreas, to pancreatic ducts and to endocrine islets. Isolating progenitor populations from human pancreas, expanding them ex vivo and differentiating them into functional β-cells is an attractive therapeutic option, but it remains to be demonstrated whether this is technically feasible.

Bone marrow (BM) stem cells (haematopoietic and/or mesenchymal) are also attractive candidates for clinical use because they are already used therapeutically and offer the potential for autograft transplantation therapy. Several studies have reported that BM stem cells can be driven towards an insulin-expressing phenotype, either after in vivo administration, by selective culture conditions in vitro, or by the forced expression of β-cell transcription factors. However, other studies in rodent models suggest that BM stem cells reverse experimental diabetes in vivo by enhancing the regeneration and survival of endogenous β-cells rather than re-populating the islets with trans-differentiated β-cells.

Other tissues have been suggested as potential sources of cells from which to generate graft material. For example, in vivo studies have demonstrated that liver cells can be induced to adopt some functional aspects of β-cells by forced over-expression of the β-cell transcription factor PDX-1, although these observations are reportedly difficult to reproduce. In a more recent study, the over-expression of another endocrine transcription factor, ngn-3, was sufficient to induce lineage-switching in hepatic progenitor oval cells to generate functional insulin-expressing cells in mouse liver. In a similar approach, the over-expression in vivo of three pancreatic transcription factors...
factors—ngn-3, Pdx-1 and mafA—was sufficient to drive mouse exocrine pancreas cells towards an insulin-expressing β-cell phenotype. It remains to be seen whether these interesting observations in mouse models will translate to human tissues with sufficient efficiency to generate substantial amounts of material for therapeutic purposes. Progenitor/stem cells from other tissues—including cells localized to central nervous system, intestinal epithelium, dermis, spleen, salivary gland and blood monocytes—have also been reported to differentiate into insulin-expressing cells but there is little convincing evidence that these cells are capable of the in vitro expansion required to generate significant amounts of tissue for effective transplantation therapy.

Embryonic stem cells

At present, the consensual view is that embryonic stem (ES) cells have the greatest clinical potential as a source material for cell replacement therapy of diabetes because of two important and intrinsic qualities of all ES cells—they have, at least in theory, a limitless capacity to expand in vitro so they can provide the vast numbers of cells required for transplantation therapy; and their developmental plasticity means that they can, again in principle, generate any cell type of the body including pancreatic β-cells.

The first studies describing differentiation of mouse ES (mES) cells into insulin-expressing cells were reported over a decade ago, and used a strategy in which an antibiotic resistance gene driven by the insulin promoter allowed selection of cells that spontaneously differentiated to an insulin-expressing phenotype. Soon afterwards it was shown that human ES (hES) cells also differentiated spontaneously to generate insulin-expressing cells, albeit at a very low frequency, with only ~1% of cells expressing insulin. Although these studies provided important proof-of-concept data, this experimental strategy was not adopted by other groups partly because spontaneous differentiation resulted in very low yields of insulin-expressing cells. Attention soon became focused on driving ES cell differentiation using a variety of directed differentiation protocols. An important early directed differentiation study adapted a five-stage culture protocol that was originally designed to generate neurons from mES cells, and reported high levels of insulin expression in the differentiated cells. However, subsequent application of similar protocols to both mouse and hES cells demonstrated that the differentiated cells were highly apoptotic, and that they adsorbed significant amounts of insulin from the differentiation medium rather than synthesizing insulin de novo.
These early directed differentiation protocols may have been flawed but they focused attention on the experimental approach of driving the pluripotent, undifferentiated ES cells towards a β-cell phenotype through a series of developmental decisions. Parallel advances in our understanding of the developmental biology of the endocrine pancreas over the past decade, often through the use of gene knockout mice, have produced a detailed knowledge of the sequence of events and developmental cues in the formation of the endocrine pancreas. This information has been applied in more recent studies to devise differentiation protocols based on sequential exposure of hES cells to growth factors and mitogens. These protocols are designed to recapitulate in vitro the important in vivo signals that drive pluripotent cells first towards definitive endoderm, then to form posterior foregut cells, then to pancreatic endoderm, then to an endocrine progenitor phenotype and finally to fully differentiated pancreatic endocrine cells. Measurement of important staging markers, usually transcription factors, enables an assessment of the effectiveness of the differentiation protocol in driving differentiation down the appropriate lineages (Fig. 2).

**Fig. 2** Directed differentiation protocol for generating β-cells from ES cells. The schematic shows (A) the sequence of in vivo developmental events involved in the normal development of the endocrine pancreas, with biological signals known to be involved in specific developmental stages (mitogens, growth factors) shown on top (green) and transcription factors known to be involved in developmental transitions shown below (red). (B) A typical in vitro directed differentiation protocol designed to recapitulate in vivo developmental cues, showing the sequential application of mitogens and growth factors (top, green) and the transcription factors used as staging markers shown below (red).
Studies of this type have produced encouraging results. Culture conditions that promoted differentiation of hES cells into definitive endoderm\textsuperscript{31–33} offered a starting point from which to progress to islet endocrine precursors and hormone-expressing cells.\textsuperscript{32–40} However, cells generated by these \textit{in vitro} protocols were generally functionally restricted, showing polyhormonal phenotypes and/or poor nutrient-induced insulin secretory responses.\textsuperscript{35,39,40} A number of options for improving the differentiation process are currently being explored. Several studies have demonstrated improved phenotypes in pancreatic progenitors derived from hES cells after transplantation into the \textit{in vivo} environment in mice, including enhanced differentiation and glucose-responsive insulin secretion.\textsuperscript{35,39} The basis of this effect remains unknown but the improved performance of the cells after exposure to the \textit{in vivo} environment suggests that something is lacking from the current \textit{in vitro} protocols. Identification of the factors involved in the \textit{in vivo} maturation may inform the last stages of an entirely \textit{in vitro} differentiation protocol for functional β-cells. Interactions between different cells and tissues are known to be important in the development of the endocrine pancreas \textit{in vivo},\textsuperscript{30} so the introduction of other cell types into the \textit{in vitro} protocols may increase the extent or efficiency of the differentiation process.\textsuperscript{41} Another important factor may relate to the timing of \textit{in vivo} development of the human endocrine pancreas, in which insulin-expressing cells are first detected at around 8 weeks of foetal development.\textsuperscript{42} In contrast, most directed differentiation protocols for hES cells allow only \textasciitilde20 days \textit{in vitro} culture for pluripotent hES cells to differentiate to mature islet endocrine cells, so some of the problems with current \textit{in vitro} protocols may reflect this artificial compression of the developmental process.

Despite the progress over the past few years there are important caveats to an over-optimistic interpretation of hES cell studies using directed differentiation protocols. Thus, similar \textit{in vitro} protocols are reported to have very different differentiation efficiencies, without obvious reasons. One likely cause is the differences in the differentiation potential between hES cell lines, emphasizing the importance of a systematic evaluation of available hES cell lines to identify the most suitable starting material. Similarly, inconsistencies have been reported recently in the \textit{in vivo} functional maturation of partially differentiated hES cells, with a failure of hES cell-derived pancreatic progenitors to differentiate further on implantation in nude rats.\textsuperscript{43}
Induced pluripotent stem cells

Induced pluripotent stem (iPS) cells generated from the patient’s own fibroblasts or other cell types offer an alternative, pluripotent starting material to ES cells for differentiation into functional β-cells, with the obvious benefits of an autologous cell population. The application of directed differentiation protocols similar to those applied to hES cells has been reported to generate insulin-expressing cells from human iPS cells derived from normal fibroblasts and from fibroblasts from a patient with type 1 diabetes. These studies suggest that, as for hES cells, current protocols demonstrate the possibility of deriving insulin-expressing cells from iPS cells, but improved differentiation protocols are required to generate functionally competent β-cells.

There remain other barriers to the clinical use of cells derived from iPS cells. The initial re-programming protocols used clinically unacceptable integration of known oncogenes, but a number of alternative non-integrating reprogramming strategies have been reported recently. There is also evidence of environment-dependent epigenetic modifications of iPS cells. A recent study used whole-genome profiling of epigenetic modification to demonstrate that all iPS cell lines investigated contained genomic regions that were aberrantly modified, causing alterations in gene expression. In addition, all iPS cell lines showed significant reprogramming variability compared with both hES cells and other iPS cells. These issues were compounded by the parallel publication of two other independent studies demonstrating that the generation of iPS cells is associated with copy number variation and with the accumulation of somatic coding mutations. These observations emphasize the requirement for a systematic evaluation of the genetic and epigenetic stability, and the differentiation potential, of iPS cells derived from different somatic cells to identify the most suitable starting material from which to generate clinical-grade material.

Safety issues

The pluripotency and proliferative potential of stem cell populations raises the undesirable possibility of uncontrolled cellular proliferation and formation of teratomas after transplantation into graft recipients, as has been reported in mouse recipients of cells derived from hES cells. The issues associated with the teratogenic potential of stem-cell derived material are not confined to cell replacement therapy for diabetes, but it is a particularly important point when considering clinical transplantation of human islets through the currently favoured route of intra-portal administration. This ensures an excellent distribution of
the islets throughout the liver, which is a major target organ for insulin, but also renders the transplanted material essentially irretrievable in the event of an adverse outcome. The distribution of $5\times 10^8$ potentially teratogenic cells throughout the liver is a major safety issue, and the future clinical usefulness of substitute β-cells derived from stem cells will depend not only on their functional competence but also on the development of fool-proof methods of ensuring their safety after transplantation.

**Conclusions**

Transplantation therapy offers a novel treatment for diabetes but it remains a restricted, experimental treatment because of severe limitations in the supply of graft material from donor human pancreases. The availability of unlimited amounts of functionally competent graft material from other sources would allow islet transplantation to evolve into a more widely available therapy, and recent experimental studies suggest that stem cells have the potential to provide the starting material from which to generate clinically useful β-cell substitutes. A number of different stem cell populations are currently being studied, each with inherent advantages and disadvantages.

Autologous grafting of insulin-secreting cells derived from the patient’s own tissue stem cells is attractive, but experimental studies have not yet translated into clinically useful material, mainly because of problems with restricted proliferative capacity, low levels of insulin expression and poor, or non-existent, insulin secretion.

ES cells and iPS cells have the required proliferative capacity *in vitro*, and a series of recent studies has demonstrated the differentiation of hES and iPS cells to an insulin-expressing phenotype without using clinically unacceptable genetic modification. There remain a number of technical obstacles before experimental studies using ES/iPS cells can translate into clinical treatments of diabetes, none of which appears to be insurmountable at present:

(i) Assessment of the differentiation potential of hES and iPS cell populations to allow selection of the most appropriate starting material.

(ii) Refinements of current directed differentiation protocols to allow the generation of fully functionally competent β-cells entirely *in vitro* under defined culture conditions.

(iii) Validation of purification methods of sufficient stringency to allow the transplantation of the differentiated β-cells while ensuring the absolute exclusion of potentially teratogenic, pluripotent cells.
(iv) Development of techniques to scale-up laboratory based protocols to generate the many billions of cells required for clinical use.

Developments in this fast-moving area of research over the past decade have provided proof-of principle of generating insulin-expressing cells from stem cells, with the real possibility of generating unlimited numbers of functional β-cells for transplantation therapy of diabetes. However, the adoption of a stem cell-based therapy for diabetes will depend on it being demonstrably as safe and effective as the current therapy of administration of exogenous insulin, which has now been used for almost a century.

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References

Generating insulin-secreting β-cells from stem cells