RECENT DEVELOPMENTS IN STEM CELL THERAPY OF DIABETES: A REVIEW

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Accepted Date: 15/08/2012
Publish Date: 27/08/2012

Abstract

Diabetes mellitus is chronic metabolic syndrome. Type1 diabetes results from autoimmune destruction of pancreatic islet β-cells, also it is associated with insulin resistance. Present ways for management of diabetes i.e. oral hypoglycemic agents & insulin therapy are unable to irradicate diabetes completely. Also these therapies have several drawbacks. Stem cell therapy has the potential to dramatically change the treatment of human diseases. Stem cells are characterized by ability to renew themselves through mitotic cell division into diverse range of specialized cell types. Recent advances in stem cell biology raise possibility of complete cure of diabetes by pancreatic transplantation. Replaced β-cells can act as effectively as normal islet cells. Therefore β-cell replacement may emerge as potential therapy for both types 1 & type 2 diabetes with minimal side effects. This review highlights the recent developments taking place in stem cell therapy for diabetes.
Diabetes mellitus is a chronic metabolic syndrome characterized by increased levels of blood glucose, referred to as hyperglycemias. Type 1 diabetes generally results from autoimmune destruction of pancreatic islet β-cells, with consequent absolute insulin deficiency and complete dependence on exogenous insulin treatment. Type 2 Diabetes is associated with insulin resistance and pancreatic insufficiency and generally progresses to a state of insulin dependence. The current treatment of insulin does not represent a cure because insulin dosage is difficult to adjust. Exogenous insulin frequently fails to achieve optimal glucose control even when intensive regimens are used. Intensive therapy, which uses multiple daily insulin injections of insulin pump infusion with frequent monitoring of blood glucose, often leads to an increased incidence and severity of hypoglycemic episodes.

Studies of insulin secretion in humans at risk for type 1 diabetes show declining first phase insulin secretion years before the onset of hyperglycemia which has been interpreted as being due to declining β-cell mass. Type 2 diabetes is also characterized by a 65% decrease in β-cell mass associated with a 10-fold increase in β-cell apoptosis. In contrast to type 1 diabetes this increased apoptosis is not thought to be due to autoimmune disease. Toxic oligomers of human islet amyloid polypeptide (hIAPP) glucose and FFA-induced toxicity have all been implicated. A comparable reduction in β-cell mass in pigs, dogs and non-human primates also leads to hyperglycemia. Taken together, these data highlight the importance of β-cell mass for the maintenance of normoglycemia. Therefore, β-cell replacement is a potential therapy that might reverse rather than simply palliate both type 1 and type 2 Diabetes. Pancreas transplantation is effective, improving quality if not duration of life in people with type-1 diabetes. However, clinical success is highly dependent on the development of the following procedures:

- Transplanted cells should proliferate.
- Transplanted cells should differentiate in a site-specific manner.
• Transplanted cells should survive in the recipient (prevention of transplant rejection).
• Transplanted cells should integrate within the targeted tissue.
• Transplanted cells should integrate into the host circuitry and restore function.

Stem cell therapy has the potential to dramatically change the treatment of human disease. A number of adult stem cell therapies already exist, particularly bone marrow transplants that are used to treat leukemia. In the future, medical researchers anticipate being able to use technologies derived from stem cell research to treat a wider variety of diseases including cancer, Parkinson's disease, brain damage, infertility, multiple sclerosis, and muscle damage, amongst a number of other impairments and conditions.

Recent advances in stem cell biology raise the possibility of offering personalized therapy to people with Type 1 diabetes by applying cloning strategies to create immunologically autologous embryonic stem (ES) cells from which to generate functional pancreatic β-cells for transplantation therapy. Therapeutic cloning involves taking the nucleus from one of the patient’s somatic cells, inserting it into an enucleated human egg and allowing it to develop into a blastocyst. The inner cell mass of the blastocyst is used to generate pluripotent ES cell lines, which can be expanded in vitro to produce the billions of cells required for transplantation therapy. Alternatively, the stem cell populations may be derived by expansion of tissue stem cells from biopsy samples of the patient’s pancreas, liver, or bone marrow. Whatever their origin, the stem cells will be differentiated into insulin-producing cells, and these will be formed into islet-like structures for transplantation into the patient to cure their diabetes.

STEM CELL THERAPY FOR DIABETES:
Stem cells have tremendous potential to cure human diseases; here we are focusing on the potential sources, development of embryonic stem cells, transplantation, and rate of β-cell turnover in humans.

A) Potential sources of stem cells
A variety of tissues harbour progenitor or stem cells, and if it were possible to isolate and expand these cells in vitro and then differentiate them to adopt a β-cell
phenotype, they would be a potential source of substitute tissue for transplantation. The pancreas is an obvious source tissue and a number of studies have suggested the existence of stem cells within the pancreas that can be induced to adopt some elements of a β-cell phenotype. Progenitor cells from tissues other than the pancreas have also received considerable attention. Liver and pancreas have a common embryonic origin, share many phenotype-maintaining transcription factors and both are equipped to respond to circulating glucose concentrations.

Similarly, it has been reported that stem cells derived from bone marrow can be differentiated in vitro and in vivo into insulin-expressing cells, although these progenitor cells are unlikely to be the highly proliferative haematopoietic stem cells. Recently it was demonstrated that rat neural stem cells can be expanded in vitro, and can be induced to express the insulin gene and respond metabolically to nutrients and sulphonylureas. An alternative source of highly proliferative, pluripotent cells which has received much more attention is ES cells. Derived from the inner cell mass of the blastocyst, these cells have the capacity to differentiate into all three embryonic germ layers in vitro.

B) Development of Embryonic Stem Cells in Pancreas

Various approaches have been adopted towards provision of a replenishable source of islet cells for transplantation. Here we will focus on recent advances in deriving β-cells from embryonic stem cells.

i) Islets of Langerhans: Islets of Langerhans are discrete clusters of endocrine cells scattered throughout the pancreas. Each islet contains several thousand hormone-secreting cells, comprising insulin-secreting β cells, glucagon-secreting alpha cells, somatostatin-secreting delta cells and pancreatic polypeptide-secreting PP cells. The cell which comprises about 70% of the endocrine cells in the islet is unique in its ability to express the preproinsulin. Insulin has been detected at low levels in other tissues but it is unlikely that it would be secreted in a regulated manner and we know very little about how the insulin gene is regulated in these cells. Glucose-stimulated insulin secretion (GSIS) in the β cell is driven predominantly by glucose-metabolism derived changes in the ratio of
ATP: ADP. This, along with signals from the sympathetic nervous system and incretins secreted from the gut, affects the electrical properties (KATP channel activity) of the plasma membrane leading to changes in the cytoplasmic Ca²⁺ concentration that trigger exocytosis. Between meals the pool of insulin is replenished predominantly through translational mechanisms. The insulin gene is also sensitive to nutrients with changes in insulin mRNA levels that occur over longer time periods.

There are important differences between rodent and human islets that should be emphasized. In rodent islets the β-cells are clustered in the core of the islet surrounded by a mantle of α, σ, and PP cells. The islet is highly vascularised, and while the pancreatic artery supplies both islets and the surrounding exocrine tissue, the islets receive up to 20 times more blood flow than the acinar tissue. Within the islet the blood flows in the direction β-cell to alpha cell to delta cell. This presumably ensures that the β-cells of the central core are protected from the powerful inhibitory effects of glucagon and somatostatin. Recent data, however, have prompted a shift in ideas concerning intraislet cellular interactions in humans. In humans and non-human primates the various cell types are scattered throughout the islet. The majority (71%) of β-cells are found in direct contact with other endocrine cell types, suggesting a more important role for paracrine interactions in the human. These differences between rodent and human islets are raised because of their implications for the derivation of insulin-secreting cell clusters from mouse and human ES cells. Other differences include the glucose transporter GLUT2, which is highly abundant in rat islets, but in human islets is present at very low levels. The relevance of this is unclear but may reflect differences in glucose sensing mechanisms. There are also differences in the way islet cell mass are maintained in rodents and humans. Rodents appear to have a substantial capacity for β-cell replication, whereas in humans, where the ability to measure these parameters is much more limited, β-cell replication does not appear to be common. This may be relevant to approaches that might be used to scale up the production of ES cell-derived β-cells.

One important question concerns whether transplanted ES cell-derived β-cells would...
function as well, in the absence of alpha and delta cells, as would human islets. It is difficult to answer this question. The only available data are from flow cytometry sorted rat β-cells, which appeared to function in rats rendered diabetic following treatment with streptozotocin almost as well as intact islets. Transplanted β-cell will function as equal potential to normal β-cells. In excessive amounts insulin can be fatal. It is absolutely essential therefore that the sophisticated mechanisms that regulate insulin production and secretion are recreated in all their aspects in any ES-derived β-cell.

ii) Initial studies on ES-derived insulin-secreting cells: ES cells are lines derived from the inner cell mass of preimplantation embryos that have been allowed to reach the blastocyst stage. They can be expanded in culture indefinitely while retaining the functional attributes of pluripotent cells of the embryo, i.e. the ability to differentiate into any cell type in the body. A cell trapping strategy, in which the gene conferring resistance to neomycin was placed under the control of the insulin promoter, was used to select for insulin-expressing clones. The selected cells were able to normalize blood glucose levels when placed under the kidney capsule of mice rendered hyperglycemic following treatment with streptozotocin. This was a seminal study in so far as it was the first to describe the differentiation of ES cells into insulin-expressing cells. However, the efficiency of generation of insulin-expressing clones was extremely low, they did not survive well, and the animal studies have been criticized on several counts including the failure to demonstrate that removal of the kidney containing the grafted cells would reverse the beneficial effects of the graft on hyperglycaemia.

A number of other studies have described the derivation of insulin-secreting cells from mouse ES cells. Several of these were based on protocols that had been developed to differentiate mouse ES cells towards neurons. The approach involved generating a highly enriched population of nestin positive cells from embryoid bodies (EBs). Nestin is a filament protein that was originally identified as a marker for neuroepithelial progenitors but subsequently found to be up-regulated in progenitor cells of other lineages. The rationale for deriving insulin-secreting cells...
from a nestin-enriched population was based on the reported presence of nestin in adult islets and in the developing pancreas. However, although nestin appears to be present in many cell types in the developing pancreas there is no convincing evidence that it directly affects the differentiation of islet cells. It has also been argued that the results of the studies using Lumelsky protocol have been misinterpreted due to potential artefacts resulting from the uptake of insulin from the medium. The message from these studies was that C-peptide biosynthesis and excretion should be demonstrated to substantiate claims that insulin-expressing cells can be derived from ES cells and that pre-selection via a nestin-enriched population, involving the use of ITSFn and FGF2, should be avoided, since even if bonafide insulin expression was detected it could likely come from insulin-expressing neurons.

The weaknesses in these early studies served to emphasize the need to recapitulate the normal series of events that occur during embryonic development. There is no doubt that insulin-secreting cells can arise spontaneously from mouse EBs by a process that fails to mimic pancreatic development and extra-embryonic tissue may be the source of some of these cells. It is unlikely; however, that these cells will ever become β cells. Since the pancreas is derived from endoderm it would make more sense to focus efforts on inducing the in vitro formation of this germ layer.

iii) Developmental biology of the pancreas:

Shortly after fertilization of the egg the blastocyst, a spherical structure of about 50–60 cells, forms. This consists of an outer cell layer, the trophoderm surrounding a cluster of cells called the inner cell mass and a hollow cavity known as the blastocoels (Fig. 1). The trophoderm gives rise to the yolk sac and the placenta while the ICM generates the embryo. The ICM forms a bilaminar structure in which the cells closer to the blastocoel are known as the hipoblast or visceral endoderm (VE) and contribute only to the formation of extra-embryonic tissue, whereas the cells closer to the trophoderm are known as the epiblast or primitive ectoderm and give rise to the entire embryo. Around embryonic day 6.5 (e6.5) in the mouse the epiblast undergoes gastrulation, whereby a region of proliferating and migrating cells (the primitive streak), gives rise to the three
germ cell layers, ectoderm, mesoderm and definitive endoderm (DE). The pancreas is formed from the DE. Mesoderm and DE originate from an intermediate population of bipotential cells called the mesendoderm. One of the challenges of stem cell research has been to identify robust markers that can distinguish ectoderm, mesendoderm, mesoderm, VE and DE and to identify the factors involved in their formation. Several families of growth factors, including fibroblast growth factors (FGFs) and the transforming growth factor β (TGF β) super family regulate gastrulation in the mouse. TGFβs are particularly important in the generation of DE through an indirect effect on the production of mesendoderm, while the Sry-related HMG box gene Sox17 plays a determinant role in the formation of DE. At the end of gastrulation the DE is an undetermined sheet of cells. It then forms a primitive gut tube, which becomes regionalized along its anterior–posterior axis in response to retinoic acid (RA) and FGFs released from the lateral plate mesoderm\textsuperscript{27}. At around e8.5 signals from the adjacent notochord and mesenchyme induce patterning of the forward region of the gut tube resulting in the formation of the dorsal and ventral pancreatic buds\textsuperscript{28}. During the next 10 days of foetal development the pancreatic buds expand, the two lobes fuse, and individual cells of the branching epithelial network differentiate into acinar and ductal tissue of the exocrine pancreas as well as the islets of Langerhans. These events are controlled by the sequential activation of transcription factors, most of which function as positive activators but some, such as Nkx2.2 can act as inhibitory factors. Strategies towards recapitulating these events in vitro have been based on our understanding of the role played by specific transcription factors in establishing cell lineages, and the identification of the growth factors and signalling molecules emanating from the surrounding mesoderm and mesenchyme that regulate their activity.
Figure 1. Gastrulation in the mouse.

The morula (16 cell stage embryo) forms a blastocyst which contains the inner cell mass (ICM) and the trophoectoderm. The cells of the ICM then start to delaminate into hypoblast and the epiblast (early gastrula). The epiblast gives rise to the ectoderm, mesoderm and endoderm (late gastrula). An intermediate stage involves delamination of the epiblast cells and formation of a bipotential intermediate cell called the mesendoderm.

IV) Recapitulating pancreatic development in ES cells: During embryogenesis cells pass through a series of checkpoints in their progress towards a specific lineage (Fig. 2). For islet cells these checkpoints, starting from undifferentiated ES cells, include definitive endoderm (DE), posterior foregut (PF), pancreatic endoderm (PE), islet precursors, and differentiated islet cells. Each checkpoint would be expected to express a specific set of genes that would serve as specific markers. One of the bottlenecks in driving ES cells towards a pancreatic lineage has been the lack of robust markers for DE. The reason for this is that several of the genes used as markers of DE are also expressed by visceral endoderm (VE). The fact that DE but not VE originates from a Mixl1+/Bry+ mesendoderm
population has been important in devising and monitoring differentiation protocols. In the absence of single unique markers the following combinations have proved useful: DE, Sox17, Foxa2, SOX7 & Bry; VE, Sox17 (lo), Foxa2, Sox7+, Bry--; and mesoderm, Sox17−, Foxa2−, Sox7−, Bry+. Markers for primitive foregut include Foxa2 (Hnf1β) and HNF4 alpha, while Pdx1 and Nkx6.1 serve as markers for pancreatic endoderm, and Ngn3 as a marker for islet precursor cells. A fully differentiated β-cell would express insulin, Pdx-1, islet amyloid polypeptide (IAPP) and MafA, while the other hormone-secreting islet cells could be identified on the basis of specific hormone expression.

Figure 2. Step-wise differentiation of ES cells towards an islet phenotype.

ES cells are cultured as a monolayer and treated with various growth factors and inhibitors over a period of time that can vary from 15 to 35 days to generate the intermediate cell populations. Progress through the pathway can be controlled by the addition to the culture medium of: (1) activin A and wnt3a (to drive ES cells towards mesendoderm and from there to DE); (2) FGFs (that are known to affect patterning of the primitive foregut); (3) the Hh inhibitor cyclopamine (to induce formation of Pdx1+ cells) and RA (to affect further patterning of the foregut cells); (4) The Notch (gamma secretase) inhibitor DAPI (to induce formation of Ngn3+ islet progenitor cells); and (5) factors known to affect differentiation of islet cells, including nicotinamide, exendin 4, IGFs and HGF.

It had been previously shown that human ES cells could spontaneously differentiate
into insulin-secreting cells\textsuperscript{30}, and that this could be improved by grafting the differentiated cells along with dorsal pancreatic rudiments under the kidney capsule of immunocompromised SCID mice\textsuperscript{31}. With the success in generating a highly enriched population of DE cells the way was open to extend the differentiation of human ES cells towards a β-cell fate using a more systematic step-wise approach (Fig. 2). This involved sequential exposure of the cells in monolayer culture to: (i) activin A and wnt3a to form mesendoderm followed by activin A in low serum (0.2%) to form DE; (ii) FGF10 and the Hh inhibitor cyclopamine (CYC) to form primitive gut; (iii) RA, CYC and FGF10 to form posterior foregut; (iv) the Notch (gamma-secretase) inhibitor DAPT and exendin 4 (Ex4) to form pancreatic endoderm; (v) Ex4, IGF1 and HGF to form islet hormone expressing cells. The protocol was performed over 18 or so days and generated a population of cells, of which around 12% stained positive for insulin as assessed by flow cytometry. The insulin content of the purified insulin-expressing cells was around 14–208 pmol/micro g DNA which is roughly similar to the insulin content of adult human islets. There were many positive outcomes to this study in that during the differentiation process the cultures tended to recapitulate expression in the correct sequence of key endodermal and pancreatic markers\textsuperscript{32}.

C) Transplantation

i) Pancreas / Islet transplantation: Pancreas transplantation has potential for a cure with total independence from insulin injections. It minimizes use of immunosuppressant as well as risk associated with surgery. Despite of this pancreas transplantation was used in few patients because of high cost, morbidity and scarcity of suitable donors. Because of this islet transplantation has preferred in recent times.

Islet transplantation using Edmonton protocol achieved great success\textsuperscript{33}. This protocol combined steroid free immunosuppression with at least two separate islet mass to liver to achieve insulin independence in the majority of recipients. Islet transplantation therapy is technically easier, has lower morbidity and permits storage of islet graft in tissue culture or cryopreservation for banking. Islet transplantation can be promisingly used for correcting diabetes in young
patients including children. In spite of progress achieved, islet transplantation does not offer an adequate solution for permanent cure of hyperglycemia.

Islet transplantation has significant side effects due to accompanying immunosuppressive therapy. Number of islets required to achieve insulin independence is very high and resources of human donor organs to provide islet grafts are limited. Thus, achieving successful single donor islet transplantation is currently a major challenge.

ii) Xenotransplantation: Availability of xenogenic β-cells, like porcine islets of Langerhans and their similarity to human pancreatic islets render the utilisation of xenografts in diabetic patients very attractive. However, the immune reaction against xenotransplants, which is a consequence of the natural immunological inter-species barrier, is of great disadvantage. One way to avoid this phenomenon is to use cells that do not express xenogenic surface antigens, such as those developed in transgenic pigs. Alternatively, islets can be embedded in an alginate microcapsule that protects them from T-cell immunological reactions. On the other hand, it is also possible to influence the recipient’s immune system. For example, immunosuppressive therapy targeting T cells led to long-term survival of intraortally transplanted wild-type porcine islets in diabetic nonhuman primates. An unresolved issue is the risk of transmission of zoonoses to the human species.

D) Rates of β-cell turnover in humans: Successful use of the approach of suppression of β-cell apoptosis to increase β-cell mass from endogenous β-cell regeneration requires there to be sufficient new β-cell formation. Finegood et al attempted to quantitatively assess β-cell turnover by using the frequencies of BrdU or thymidine incorporation in β-cells in rats. Based on these data, a turnover rate of 2% β-cells per day was calculated in adult rats. However, using continuous long-term BrdU labeling in adult mice, only 1/1,400 β-cells underwent replication per day. Assuming no additional input from new islet formation, transdifferentiation or other potential sources, this would correspond to a proliferation rate of 0.0701% per day. Thus, even assuming a 0% rate of β-cell death, recovery of a 50% deficit in β-cell
mass would be expected to occur after 1,429 d, a time period that far exceeds the typical life span of a mouse. In humans, similar calculations are difficult to perform, since BrdU labeling cannot be used for obvious reasons, but based on the reported frequencies of Ki67 labeling, the turnover rate of β-cells seems to be even slower. On the other hand, the increase in β-cell mass observed in humans during pregnancy implies that this turnover rate can be increased by several-fold under certain conditions even in adult humans.

Type 1 diabetes is difficult to cure, because cells are destroyed when body’s own immune system attacks and destroys them. This autoimmunity overcomes during pancreatic transplantation therapy. That means type 1 diabetes is now curable by pancreatic transplantation therapy. Stem cell offers potential starting material to generate large number of cells required. There is need to know more about molecular mechanisms and signaling pathways that control expansion and differentiation of stem cells. In case of diabetic therapy it is especially true for pancreatic β-cells.

Major achievements in isolation culture and targeted differentiation of ES cells prompt hopes that it will be possible to replenish β-cell mass in patients with diabetes using ES cells derived insulin producing cells. That means tremendous progress has been made but there are many challenges that should be overcome. In following section some of these challenges are discussed.

A) Variation between human ES cell lines: The efficiency of methods to generate specific cell type varies between human ES cell lines and often between laboratories growing the same line. In addition, some lines appear to prefer to differentiate into derivatives of a specific germ layer. These observations are often based on anecdotal reports and are rarely discussed in published reports. This issue has been systematically addressed in a comparative study performed on 59 independently derived human ES cell lines from 17 laboratories worldwide. Gene expression profiling of differentiated cells showed marked differences between the various lines. Thus, a major challenge is to develop
a universal protocol for the derivation of human ES cells and chemically defined culture media that can be applied to all human ES cell lines\textsuperscript{37}.

**B) Xeno-free and chemically defined culture conditions:** All the currently available protocols use animal products (foetal bovine serum, mouse feeder cells and bovine serum albumin) or unknown components that are present in Matrigel\textsuperscript{TM}, Serum Replacements and conditioned media. Importantly, the serum and the feeders used in these protocols cannot be substituted by serum albumin or by artificial matrices, suggesting that uncharacterised factors are required for the differentiation of human ES cells to DE. These factors obscure analysis of developmental mechanisms and potentially render the resulting tissues incompatible with future clinical applications. The development and validation of Xeno-free and chemically defined culture conditions for achieving specification of hESCs into DE and beyond therefore remains a major challenge.

**C) The criteria for a functional β-cell:** A fully differentiated functional β-cell should: express insulin (C-peptide) at levels equivalent to those seen in an average human β-cell; contain storage granules as detected by EM microscopy; efficiently process proinsulin to insulin; and exhibit GSIS, i.e. an acute 3-fold stimulatory response to glucose. In vivo studies should include: the ability to detect human Cpeptide in the blood of SCID mice for periods up to 4–6 weeks following engraftment; an increase in blood C-peptide upon administration of glucose following engraftment; normalization of blood glucose levels in a diabetic animal model; and no formation of teratomas\textsuperscript{38}.

**D) Generation of mature β-cells:** Maturation of pancreatic progenitors in vitro represents a key step towards the generation of fully functional β-cells from human ES cells. The protocols currently available allow for the production of β-cells with the characteristics of immature cells, i.e. low insulin secretion and co-expression of several islet hormones. The factors and mechanisms that control the final maturation stage are not well understood.

**E) ES cell-derived β-cells required for a single transplant:** Based on the Edmonton Protocol, each transplant would require
about 600,000 islet equivalents (IE). Since each IE contains about 1000 β cells, this would mean that roughly 1 billion (assuming some loss following engraftment) ES-derived β-cells would be required. It is likely that the differentiation protocol will contain a stage at which the cells are expanded. More research is required on the proliferative potential of the various sub-populations however based on data from the developing mouse; the Sox9 enriched population might be best suited for this purpose.

F) Avoidance of immunosuppression:
Human ES cells and their differentiated derivatives express human leukocyte antigens (HLA) and major histocompatibility complex (MHC) molecules and are likely to be rejected by the immune system after transplantation. One method to avoid immunosuppression of islet transplant recipients has been to encase the islet grafts in selectively permeable microcapsules made of sodium alginate or poly-l-ornithine\(^3\). Preliminary studies in humans have proved that microencapsulation represents the future for stem cells in the treatment of diabetes. Other possible ways to avoid the need for immunosuppression include reducing expression of MHC molecules by genetic modification of human ES cells mixed chimerism, or the use of dendritic cells\(^4\).

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