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MINI REVIEW

Beta-cell replacement strategies for diabetes

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ABSTRACT

Diabetes is characterized by elevated levels of blood glucose as a result of insufficient production of insulin from loss or dysfunction of pancreatic islet β-cells. Here, we review several approaches to replacing β-cells that were recently discussed at a symposium held in Kyoto, Japan. Transplant of donor human islets can effectively treat diabetes and eliminate the need for insulin injections, supporting research aimed at identifying abundant supplies of cells. Studies showing the feasibility of producing mouse islets in rats support the concept of generating pigs with human pancreas that can serve as donors of human islets, although scientific and ethical challenges remain. Alternatively, *in vitro* differentiation of both human embryonic stem cells and induced pluripotent stem cells is being actively pursued as an islet cell source, and embryonic stem cell-derived pancreatic progenitor cells are now in clinical trials in North America in patients with diabetes. Macro-encapsulation devices are being used to contain and protect the cells from immune attack, and alternate strategies of immune-isolation are being pursued, such as islets contained within long microfibers. Recent advancements in genetic engineering tools offer exciting opportunities to broaden therapeutic strategies and to probe the genetic involvement in β-cell failure that contributes to diabetes. Personalized medicine might eventually become a possibility with genetically edited patient-induced pluripotent stem cells, and the development of simplified robust differentiation protocols that ideally become standardized and automated. Additional efforts to develop a safe and effective β-cell replacement strategy to treat diabetes are warranted.

INTRODUCTION

Diabetes mellitus is characterized by chronic hyperglycemia as a result of insufficient levels of the hormone, insulin, often accompanied by insulin resistance. Without appropriate treatment and care, the disease results in devastating complications including diabetic nephropathy, retinopathy and neuropathy, as well as cardiovascular and cerebrovascular diseases, all of which substantially impair the quality of patients' lives. There are several types of diabetes[1], of which in higher income countries 87–91% are type 2 diabetes, 7–12% type 1 diabetes and 1–3% are other forms[2]. Treatment strategies vary depending on disease type.

Asia is becoming the global epicenter for diabetes[2]. In 2015 there were ~153 million adults with diabetes in Asia, and by 2040 that number is projected to rise to 215 million, with related deaths surging by 46%. Asian diabetes is primarily characterized by impaired insulin secretion in the absence of obesity[3]. Therefore, strategies are being investigated to preserve pancreatic β-cell mass and function, with the goal of preventing diabetes onset, progression and its complications. In addition, although incident rates of type 1 diabetes among children in Asian countries are relatively low, rates are increasing similarly to other parts of the world. Therefore, strategies for β-cell replacement therapies have been gaining much attention in this region (Figure 1).

CELL-BASED INSULIN REPLACEMENT

As diabetes results from inadequate production of insulin, many patients rely on daily insulin injections or insulin infusions by pump for survival. Although these technologies save lives, such means of exogenous insulin replacement do not typically prevent the onset of debilitating complications, as it is virtually impossible to replicate the dynamic insulin production achieved by pancreatic β-cells. We believe that the re-establishment of regulated insulin production from within the body will ultimately provide

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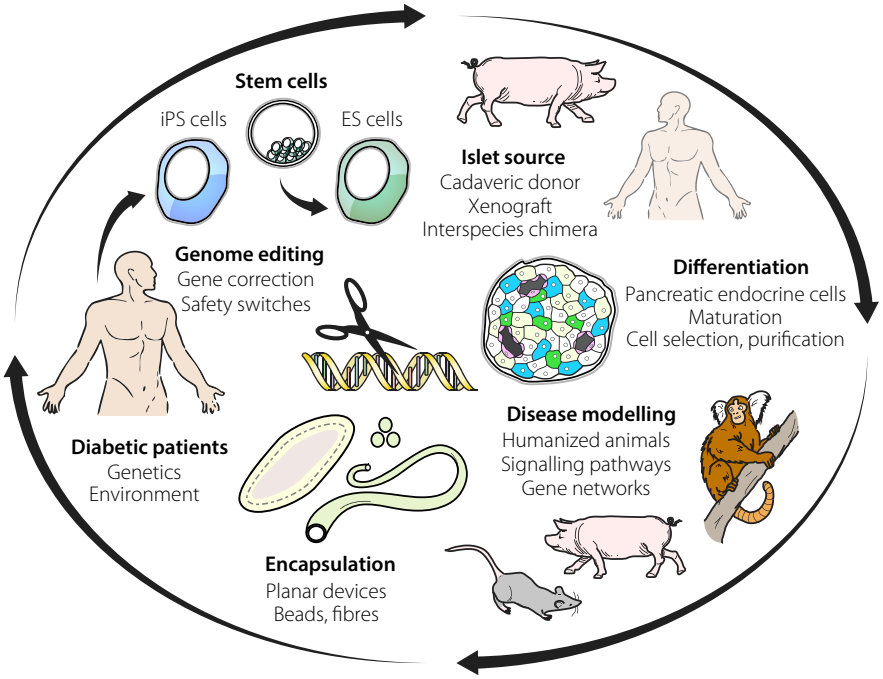

Figure 1 | Strategies being explored for the development of novel therapies for diabetes. Embryonic stem (ES) and induced pluripotent stem (iPS) cells combined with genome editing technologies are valuable tools to probe disease mechanisms, engineer in desired attributes such as safety switches and upon differentiation to use as potential replacement cell sources. Other potential cell sources are pigs, possibly with human pancreas. Such 'humanized' animals might also prove valuable for disease modeling, as will other genetically modified species. To accompany cell sources some form of immunoprotection will be required, such as retrievable planar, bead or fiber encapsulation devices.

superior glycemic control, freeing patients from the burden of self-management of insulin replacement and resulting in superior health outcomes.

In 1997 the Japanese Society for Pancreas and Islet Transplantation organized a Working Group to construct a system for clinical islet transplantation in Japan, including registration of recipients through The Japanese Islet Transplant Registry, procurement of pancreas tissue for islet isolation and transplantation of isolated islets. The first islet transplant in Japan was carried out in 2004, following the 'Edmonton Protocol'[4]. Subsequently, a multicenter analysis of 34 transplantations of pancreatic islets in 18 Japanese participants with diabetes revealed graft survivals (defined as a C-peptide level ≥0.3 ng/mL) of 72.2, 44.4 and 22.2% at 1, 2 and 5 years, respectively, and all recipients became free of severe hypoglycemia unawareness while graft function was maintained[5]. Likewise, a phase 3 clinical trial at eight centers in North America involving 48 participants who together received 75 islet infusions showed that transplanted human islets provided glycemic control (median glycated hemoglobin level was 5.6% at both 1 and 2 years), restoration of hypoglycemia awareness and protection from severe hypoglycemic events[6]. In Vancouver, a multiyear study on progression of diabetes complications showed that islet transplantation yields improved glycated hemoglobin and resulted in less progression of retinopathy compared with intensive medical therapy during 3-year follow up[7]. Collectively, these studies highlight both the feasibility and the tremendous benefits of treating diabetes by islet cell replacement.

LARGE ANIMALS AS MODELS AND A SOURCE OF ISLETS

As with most organ transplants, a limiting factor for islet transplantation is supply. With advancements in genetic engineering tools, there has been a resurgence of the concept of using pigs as an alternative source of cells for transplant[8]. Porcine islets function similarly to human islets, and historically, humans were treated with purified porcine insulin until recombinant human insulin became available. However, one concern with the use of pigs as islet donors is the presence of many dormant porcine endogenous retroviruses (PERVs), which could pose a risk to recipients. Using CRISPR-Cas9, all 62 copies of the PERV *pol* gene were inactivated, resulting in a >1,000-fold reduction in PERV transmission to human cells[9], and PERV-inactivated pigs were successfully generated, addressing this safety concern for clinical application of porcine-to-human xenotransplantation[10]. Genome editing can also be used to reduce the expression of antigens that typically promote aggressive immune responses to xenografts.

As an alternative to using modified porcine organs, it is conceivable to combine gene knockouts in key developmental genes and interspecies chimeras to produce pigs with complementing human organs that can be harvested for transplant. As proof of concept for chimera complementation, Nakauchi *et al.* first created mice with rat pancreas[11], and subsequently rats with mice pancreas[12]. These remarkable feats were achieved by injecting rat pluripotent stem cells into early-stage mouse embryos that lacked the *Pdx1* gene, or mouse pluripotent stem cells into early-stage rat embryos that lacked the *Pdx1* gene, respectively. Furthermore, islets isolated from rats with mouse pancreas were able to successfully reverse diabetes in recipient mice for >1 year, in the absence of chronic immunosuppression. These data provide compelling evidence for the therapeutic potential of stem cell-derived islets generated by blastocyst complementation in a xenogeneic host. As a next step towards the generation of pigs with human pancreas, knockout pig embryos were created with an apancreatic phenotype. Complementation of these embryos with allogenic blastomeres then created functioning pancreata in the vacant niches[13]. Ethical issues and regulations in Japan currently preclude testing the feasibility of reconstituting pancreas from human pluripotent stem cells in these animals.

Aside from being a source of cells for transplant, large animals with severe combined immunodeficiency could be very useful models to test the safety and efficacy of cell-based strategies to treat diabetes, before clinical trials. For instance, using messenger ribonucleic acid-encoding zinc-finger nucleases, the interleukin-2 receptor gamma (*IL2RG*) gene was knocked out in porcine fetal fibroblasts, and *IL2RG* knockout pigs were subsequently generated using these cells through somatic cell nuclear transfer[14]. The resulting *IL2RG* knockout pigs completely lacked a thymus, and were deficient in T and natural killer (NK) cells, but not B cells. A similar approach was used to generate and *IL2RG* knockout marmosets with a phenotype similar to humans with severe combined immunodeficiency[15]. Recombination activating gene (*RAG*)2 knockout pigs were produced by conventional gene targeting of somatic cells followed by somatic cell nuclear transfer, resulting in pigs that lacked T and B cells, but not NK cells[16]. Double IL2RG/RAG2 knockout pigs lacked T, B and NK cells altogether, producing an animal model that can bridge the gap between rodents and humans, and provide a human-scale model of preclinical research on stem cell therapies[16]. Single-stranded oligodeoxynucleotide-mediated knock-in approaches with CRISPR-Cas9 were recently used to replace large segments of the rat genome with the corresponding human sequences, showing a simplified 'humanization' of animal models by genome engineering[17]. Humanized animals are likely to become valuable model systems for probing disease mechanisms and testing novel therapeutic approaches. Large animal models can also be produced to model diabetes and its complications; for example, pigs carrying a mutant version of human hepatocyte nuclear factor-1α, which is responsible for maturity-onset diabetes of the young type 3[18]. Ongoing advances in genetic engineering will facilitate the generation of additional useful models.

INSULIN-PRODUCING CELLS FROM HUMAN STEM CELLS

The quality of glycemic control that can be achieved by islet transplant has fueled efforts to develop a readily available supply of insulin-producing cells to replace the current reliance on cadaveric tissue. Importantly, the success of islet replacement provides clear clinical evidence that a cell-based treatment of diabetes is possible. In theory, human stem cell-derived β-cells should be able to generate the same results, if not better than isolated human islets. Islet tissue is highly variable, and compromised by the quality of the donor organ and digestion process required to liberate islets that are scattered throughout the exocrine tissue. In contrast, laboratory-grown β-cells or their progenitors can be cultivated under optimal standardized conditions to purity and in vast quantities as a readily available cell source.

Efforts to differentiate pluripotent stem cells, whether embryonic stem (ES) cells or induced pluripotent stem (iPS) cells, into β-cells have been guided by decades of studies unraveling the processes by which islet cells normally develop[19]. A great deal of effort has been required to optimize the culture conditions, particularly the concentrations of media constituents and timing of the activation or inhibition of key signaling pathways to obtain stepwise differentiation of the cells through normal developmental pathways. For example, it is possible to selectively differentiate iPS cells into anterior or posterior domains of definitive endoderm by simply using different concentrations of CHIR99021, a small molecule Wnt/β-catenin pathway activator[20]. Other screens identified erythropoietin as a facilitator of the differentiation of stem cells into definitive endoderm through the activation of extracellular signal-regulated kinase signaling[21], and interestingly, mild electrical stimulation with heat shock potentiates the differentiation of ES cells into definitive endoderm[22].

Studies with isolated pancreatic progenitors obtained from ~9-week-old human fetal pancreas showed that cells at that stage of development, marked by the transcription factors PDX1 and NKX6.1, are capable of finishing maturation to β-cells once transplanted[23,24]. Therefore, stem cell differentiation protocols have focused on inducing the expression of these two key markers. Aside from media components, the induction of $PDX1^+/NKX6.1^+$ cells is affected by cell density in adherent cultures, and markedly improved with cell aggregation cultures[25] and inhibition of Rho-associated kinase or non-muscle myosin II[26]. Protocols have been developed to efficiently mass produce cells marked by PDX1 and NKX6.1, including in three-dimensional suspension bioreactor systems, and these cells are able to mature into islet cells and effectively lower blood glucose in immunodeficient mice[27–30]. ViaCyte Inc. has advanced this approach into clinical trials, and thus far ~20 participants have received implants of human ES-derived

pancreatic progenitors in Canada and the USA, but detailed results of the trials have not yet been released[29,31]. This important milestone, first in human trials in patients with diabetes, paves the way for additional clinical testing of stem cell-derived implants.

One theoretical caveat of the strategy to implant pancreatic progenitor cells as opposed to mature β-cells is the potential that patient variables can impact cell maturation in unpredictable ways. For instance, human pancreatic progenitor cells seem to mature differently in mice vs rats[32], and altered levels of thyroid hormone can impair the formation of β-cells[33], indicating that the tissue environment can influence pancreatic progenitor maturation. More advanced scalable differentiation protocols have been developed[34–42], and cells generated, while not fully mature β-cells, can more rapidly reverse diabetes in mice compared with pancreatic progenitors[35]. Additional studies are required to verify if the cells function more consistently in variable host conditions in order to safely and effectively regulate blood glucose levels.

Considerable efforts are underway to further improve on current differentiation protocols, including reducing the reliance on expensive growth factors, and deriving fully mature insulin-producing β-cells from pancreatic progenitors. In this regard, small molecule screens have identified a previously unappreciated pathway regulated by vesicular monoamine transporter 2, which when inhibited releases a monoamine-dependent suppression of pancreatic progenitor cell differentiation[43]. In another study, sodium cromoglicate was found to increase the percentage of insulin$^+$ cells by almost threefold, at least in part by the inhibition of bone morphogenetic protein 4 signaling[44]. Others have engineered new tools to facilitate the identification of compounds that promote β-cell formation. For example, dual reporter human iPS cells were created expressing the fluorescent proteins, Venus and mCherry, under the control of intrinsic insulin and neurogenin 3 promoters[45]. Tracking reporter expression during high-throughput screening of chemicals identified a specific kinase inhibitor of fibroblast growth factor receptor 1 that acted in a stage-dependent manner to promote the terminal differentiation of pancreatic endocrine cells, including β-cells, from the intermediate stage of pancreatic endocrine progenitors while blocking the early development of pancreatic progenitors. Finally, a novel assay using dissociation culture of adult islets showed that dopamine D2 receptor antagonization with domperidone repressed apoptosis and dedifferentiation, leading to enhanced proliferation and increased β-cell mass[46]. It is likely that a combination of both guided protocol optimization and unbiased screening with empirical testing will ultimately be successful in yielding robust protocols for expansive production of mature β-cells from pluripotent stem cells.

CELL ENCAPSULATION

Current islet transplant protocols require the use of chronic immunosuppression to protect cells from immune-mediated destruction. In an effort to eliminate the need for such drugs, which can place patients at risk of complications and opportunistic infections, approaches are being investigated that encapsulate implanted cells to isolate them from the immune system, while maintaining permeability to nutrients and secreted products including insulin. Multiple studies have documented the ability of stem cell-derived pancreatic progenitor cells or endocrine cells to survive and function within macro-encapsulation devices that are implanted subcutaneously[28,39,47]. However, the long-term survival and function of macro-encapsulated stem cell derivatives in humans has not yet been reported. Micro-encapsulated clusters of differentiated human stem cells in a derivative of the natural material alginate were effectively protected long term from immune attack when implanted into the intraperitoneal cavity of mice[48]. Smaller capsules such as these might provide better oxygenation for the highly metabolically active β-cells within, compared with macro-encapsulated cells. However, microcapsules are not readily retrievable, a disadvantage compared with subcutaneously placed larger devices. As a potential means to harness the immunoprotective capacity of alginate, or other polymers, yet achieve retrievability, a method was developed to encapsulate islet cells within meter-long microfibers that can be weaved into macroscopic structures with various spatial patterns, implanted and subsequently retrieved[49]. It remains to be seen if such innovative approaches will prove to be superior to macro-encapsulation strategies that are currently being tested clinically[31].

COMBINING GENETIC ENGINEERING WITH STEM CELLS

The combination of modern genome engineering tools and iPS cells has poised the field to improve both transplant outcomes and safety. Unlike using adult islets, current *in vitro* stem cell differentiation protocols do not fully recapitulate *in vivo* maturation and lineage restriction, thus leading to concerns over potential tumorigenic growth of progenitors or residual undifferentiated cells. To date, the limited number of ES or iPS cell-derived therapies that have reached clinical trials have undergone careful scrutiny and have raised no apparent need for concern[50], yet measures to ensure monitoring and control of transplanted cells remain advantageous. Lentiviral integration of transgenically encoded 'safety switches,' such as chemically inducible caspase-9, allow the selective ablation of transplanted cells and have proven efficacy *in vitro* and in teratomas[51], and more recently using *in vivo* mouse models of spinal cord injury for selective and regulated cell ablation[52]. Transgene targeting into the adeno-associated virus integration site 1 locus, or other genetic 'safe-harbor' loci – which show no known phenotype from disruption and enjoy a privileged epigenetic signature – permits reliable gene expression and avoids the potential mutagenic load of random lentiviral integration. Therapeutic transgene delivery to the albumin locus using zinc-finger nucleases, which has thus far been proven *in vivo* in mice[53] and recently received US Food and Drug Administration approval as an orphan drug for gene therapy, results in transgene expression from an endogenous promoter, and reveals an

option for cell type-specific transgene responses. The adeno-associated virus integration site 1 locus shows consistent expression in a variety of differentiated cell types[54], making it suitable for tracking cells using visible luciferase reporters after transplantation, and an obvious candidate for housing safety switches. However, as the field moves towards integration-free iPS cells without permanent transgenic modification, methods such as selective elimination of residual undifferentiated cells by metabolic restriction of methionine[55,56] or transient delivery of potent apoptotic regulators limited in their activity by the presence of stem-cell specific micro-ribonucleic acids[57] might form a new standard.

Although animal models have utility in improving our understanding of pancreatic development and testing the efficacy of stem cell-derived β-cells, fundamental differences in physiology could confound their use for studies of human disease and development. Using human pluripotent stem cells as *in vitro* models for human development, researchers have probed the function of key transcription factors by gene knockout; for example, showing conserved haploinsufficiency for PDX1, as well as a potentially divergent role of neurogenin 3 between mice and humans in pancreatic β-cell differentiation[58]. Patient iPS cells provide a valuable resource to decipher disease mechanisms, such as the discovery that nonsense-mediated decay is the underlying mechanism for *HNF1β* mutations in mature-onset diabetes of the young[59]. Gene-corrected isogenic iPS cells provide experimental validation of causative diabetes mutations. Furthermore, combined with data from genome-wide association studies, gene editing has helped to reveal more complex genetic interactions, such as those shown for ER stress in response to CDKAL1 dysfunction, exposing novel therapeutic solutions[60]. Thus human islet cells derived from differentiated human pluripotent stem cells also provide a platform to screen for new drugs to treat diabetes. Furthermore, as autologous β-cells derived from diabetes patient iPS cells would require gene correction before therapeutic use, precision genome editing could eventually permit a truly personalized approach to regenerative medicine. More economically feasible banking of human leukocyte antigen-matched and validated iPS cell derivatives[61] also stands to benefit from genome engineering applications that reduce immune rejection and improve broader patient compatibility, thus facilitating transplant therapy with 'universal' cells.

CONCLUSION

In summary, there continue to be many exciting advances in β-cell replacement strategies for diabetes, including the recent creation of model systems and tools that will facilitate new discoveries, some of which will hopefully progress to clinical trials (Figure 1). Ultimately, one of the approaches discussed here could develop into a practical way to effectively restore regulated endogenous insulin production and normal glucose homeostasis, addressing the critically unmet need to better control blood glucose levels in patients with diabetes.

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