REVIEW

Gene Therapy Progress and Prospects: Gene therapy for diabetes mellitus

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Diabetes mellitus has long been targeted, as yet unsuccessfully, as being curable with gene therapy. The main hurdles have not only been vector-related toxicity but also the lack of physiological regulation of the expressed insulin. Recent advances in understanding the developmental biology of β -cells and the transcriptional cascade that drives it have enabled both in vivo and ex vivo gene therapy combined with

cell therapy to be used in animal models of diabetes with success. The associated developments in the stem cell biology and immunology have opened up further opportunities for gene therapy to be applied to target autoimmune diabetes.

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In brief

Progress

- *In vivo* gene therapies aimed at reducing immune reaction or inducing immune tolerance have produced encouraging results in rodent models of autoimmune diabetes.
- *In vivo* gene therapies aimed at insulin gene expression ectopically have yielded generally inadequate results for potential human applications.
- *In vivo* gene therapy aimed at inducing ectopic islet neogenesis has been demonstrated with proof-of-concept experiments.
- Adult stem/progenitor cells that play a role in islet and β -cell production and regeneration are being identified.
- *Ex vivo* gene therapy and cell therapy for diabetes are in their very nascent stages.

Prospects

- Advances in vector technology would make toxicity negligible and cell/tissue-specific homing an easier task.
- Identification of islet stem cells and β -cell progenitor cells would make cell-based therapy a viable alternative.
- Further knowledge of the differentiation and development of islets would facilitate the development of protocols for *in vitro* differentiation of pluripotent adult stem cells or totipotent embryonic stem cells into islets.
- *Ex vivo* gene therapy in combination with cell therapy would become a realistic alternative to current islet transplantation protocols.
- Advances in immune tolerance and limiting immune destruction could abort the development of autoimmune diabetes; these are also essential for the success of cell and gene therapy for treating autoimmune type I diabetes.

Introduction

Diabetes is caused by an absolute (type I) or relative (type II) insulin deficiency. In type I diabetes, there is almost universally an autoimmune destruction of the β -cells, while in type II diabetes, the β -cells are unable to

keep up with the body's insulin requirements, which are increased due to insulin resistance. Although there have been important advances in our understanding of both the pathogenesis and management of both forms of diabetes, treatment options, which include injectable insulin and oral agents, remain unsatisfactory. Injectable insulin is not physiologically regulated and has been associated with patient noncompliance resulting in suboptimal control and ensuing diabetic complications. In addition, frequent hypoglycemia, due to the lack of physiological regulated insulin delivery, leads to significant morbidity and even mortality. Islet transplantation has opened up the possibility of returning physiological control to insulin replacement therapy;

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however, donor shortage and the necessity of adjuvant immunosuppression, despite recent advances,¹ have limited its wide applicability. However, these limitations can be potentially circumvented with cell/gene therapy. Indeed, advances in cell and gene therapy have rekindled the possibility of not only ameliorating but also potentially curing diabetes. In this article, we provide an overview of the recent advances in this field and speculate on the prospects for their application to the treatment of diabetes. This review deals mainly with therapy for type I diabetes, in which the primary goal is restoration of normal insulin production and secretion, although some of the principles are also applicable to type II disease as β -cell dysfunction is also an underlying defect in the latter.

Gene therapy strategies for diabetes can broadly be divided into three categories: preventive, adjunctive and curative. While preventive and adjunctive strategies primarily address the autoimmune pathogenesis of type I diabetes and the immune response to islet grafts post-transplantation, respectively, curative strategies involve restoring insulin production and secretion by effecting insulin transgene expression or by inducing islet neogenesis ectopically.

In vivo gene therapies aimed at reducing immune reactions or inducing immune tolerance have produced promising results in rodent models of autoimmune diabetes

Primary prevention involves identifying patients at risk and instituting measures to prevent the autoimmune destruction of β -cells such as immunomodulatory gene therapy. This has been shown to work in rodent models.²⁻⁷ This area has been reviewed recently (see Bottino *et al.*⁸). Briefly, these strategies are aimed at inducing tolerance or hyporesponsiveness to islet autoantigens by either increasing tolerance inducing regulatory T lymphocytes (IL-10 gene therapy²), suppressing reactive T lymphocytes (immunogene therapy using recombinant vaccinia virus expressing glutamic acid decarboxylase³ or targeting these cells for apoptosis⁶) or inducing tolerance in target cells (by overexpression of 'suppressor of cytokine signaling-1'⁴ or by inducing antigen-presenting cells to express proinsulin, an autoantigen⁵). However, the translation of the animal experiments to human trials is constrained by the lack of markers with acceptable positive and negative predictive values to unequivocally identify and select appropriate patients who are at risk for preventive therapy. Yet, conceptually, this is the area that will need to be explored if our ultimate goal is the primary prevention of type I diabetes. Preventive strategies have currently focused on secondary prevention, that is, measures to prevent the further loss of β -cells once diabetes has set in. These are instituted optimally at diagnosis or in the honeymoon period following the initial acute presentation as often seen in type I diabetes.^{9,10}

In vivo gene therapies aimed at ectopic insulin gene expression have yielded positive but grossly inadequate results to justify human applications

Many studies in the last decade have used induced ectopic insulin secretion, primarily in liver and skeletal muscle, as a therapeutic strategy. There are three key

components to insulin transgene therapy, first the tissue specificity of expression, second the level of transgene expression and third its regulation; that is, insulin secretion must be exquisitely regulated by changes in glucose concentrations. To regulate insulin transgene expression, investigators have made use of glucose-responsive promoters such as the insulin promoter, glucose 6-phosphatase promoter, fructose 1,6-bisphosphatase promoter¹¹ or glucose-inducible response elements such as those based on S14 gene, a gene involved in lipogenesis that is regulated by glucose, fatty acids and thyroid hormone as well as other factors.¹² This last step of according glucose responsiveness has been the most difficult to achieve primarily because in the normal β -cell glucose-regulated insulin secretion is biphasic¹³ and is attained through an immediate first phase insulin secretory response that is dependant on the release of preformed insulin secretory granules, a step that has been impossible to replicate with insulin transgene therapy, and a more sustained second phase that is regulated both at the transcriptional and post-transcriptional levels. Furthermore, insulin secretion must be rapidly turned off when blood glucose drops to prevent the occurrence of hypoglycemia. Most strategies for insulin transgene therapy have exploited control of insulin transgenes only at the transcriptional level. Hence, although glucose-dependent insulin response has been demonstrated *in vitro* and *in vivo* in diabetic animal models, this approach is doomed to failure due to an inability to accomplish optimal control of hyperglycemia, normal glucose tolerance and freedom from hypoglycemia.¹⁴

In vivo gene therapy aimed at inducing ectopic islet neogenesis has been demonstrated with proof-of-concept studies and is poised for further development

Compared to ectopic insulin secretion brought about by insulin gene transfer, that produced by induced islet neogenesis offers a more attractive option. When the strategy works properly, the newly formed islets are endowed with the appropriate mechanisms that regulate insulin production and secretion, which are intrinsic to the pancreatic islets. The newly formed ectopic islets also take advantage of paracrine factors that normally operate in intact pancreatic islets.¹⁵ Recent studies in non-autoimmune insulin-deficient rodent models of diabetes have explored this possibility with some success.^{16,17}

Ferber *et al.*¹⁸ delivered an islet transcription factor involved in the differentiation of both exocrine and endocrine pancreas, Pdx-1, using a first-generation adenovirus vector, and demonstrated the appearance of insulin-producing cells in the liver of streptozotocin (STZ)-induced diabetic mice. However, work carried out in our laboratory using helper-dependant adenovirus (HDAD) delivering Pdx-1 to the liver of diabetic mice induced insulin-producing cells, which coexpressed trypsin, a digestive enzyme produced by pancreatic exocrine cells that is downstream of PDX-1, resulting in severe hepatotoxicity.¹⁷ The Pdx-1-induced expression of exocrine pancreatic digestive enzymes in the liver was subsequently confirmed by the Ferber laboratory.¹⁶ We further showed that the HDAD-mediated delivery of NeuroD/BETA2, an islet transcription factor downstream of Pdx-1, led to the generation of clusters of

insulin and other islet hormone-producing cells in subcapsular regions of the liver, leading to a complete reversal of hyperglycemia in STZ-induced diabetes in mice for more than 4 months with negligible toxicity.¹⁷ Further studies utilizing other transcription factors and exploration of this strategy in other type I diabetes models are underway. A combination of this approach with further improvements in gene delivery vectors and methods could lead to acceptable therapies in the future.

Adult stem/progenitor cells that may play a role in islet and β -cell regeneration have been identified

An exciting advance in the last few years is the demonstration of adult stem cell plasticity (reviewed by Martin-Rendon and Watt¹⁹ and Verfaillie *et al*²⁰). This has been demonstrated with many cell types derived from not only the bone marrow (BM) but also other organs and tissues. One cell type is called the multipotent adult pleuripotent cell (MAPC), which appears to be a subset of BM mesenchymal stem cells.²¹ The MAPC subpopulation has been demonstrated to have both pluripotency and the ability to replicate in culture for at least 120 population doublings and has been reported to differentiate into ectodermal, endodermal and mesodermal tissues when injected into blastocysts. In addition, MAPC-derived cells have differentiated into hepatocytes, chondrocytes, osteocytes, endothelial cells as well as neurons.^{21–25} A different progenitor cell termed ‘side population cell’ (SP cell), isolated based on its rapid efflux of a DNA-binding dye, Hoechst 33342, has been shown to be capable of not only hematopoietic differentiation but also differentiation into muscle, liver and heart under reparative conditions, often through cell fusion.^{26–31} This property of dye efflux has been attributed to the preferential expression of the ATP-binding cassette transporter protein ABCG2 (also known as breast cancer resistance protein 1, Bcrp1) in these progenitor cells^{32,33} attesting to ABCG2 being a marker of early progenitor cells. In other experiments, BM-derived cells have been shown not only to reconstitute the BM but also contribute to muscle cells in transplantation experiments.^{34,35} The adult pluripotent cells, including the MAPC and the SP cells, have been shown to also reside in tissues other than the BM.^{22,31} These cells are distinct populations of adult stem cells that seem to possess pluripotency and play a role in tissue repair and regeneration. SP cell phenotype and ABCG2 expression have been demonstrated in human islet-derived nestin-positive cells by Lechner *et al*³⁶ (see below). However, stable transformation of these cells *in vivo* into a pancreatic islet cell type has not been reported.

Bone marrow/spleen stem or progenitor cells contribute to islet neogenesis. Ianus *et al*³⁷ studied the potential contribution of BM-derived cells to islets and β -cells by transferring tagged BM cells into myeloablated mice and demonstrated that the donor BM cells contributed to insulin-positive cells in the otherwise normal islets of the adult recipient mice. No evidence of cell fusion between the donor and recipient cells, which could have contributed to the insulin positivity of the donor cells, was found in this study. However, the donor cells contributed to only a very small fraction (1.7–3%) of

the islet cell population in the 4- to 6-week study period. The role of BM stem cells in injury repair and regeneration of pancreatic islets was explored by Hess *et al*,³⁸ who performed BM transfer into myeloablated STZ-diabetic mice and showed that the maneuver reversed hyperglycemia by inducing islet neogenesis. However, the authors found little evidence of donor BM cell contribution to islet neogenesis. Instead, the donor BM cells (especially the c-kit-positive cell fraction) contributed to cells surrounding the islet including some expressing endothelial markers that appeared to facilitate islet neogenesis.³⁸ In a study in which β -cell injury was produced by low-dose STZ after BM transplant, Choi *et al*³⁹ found that the transplanted cells did *not* contribute to the regenerating islets. These investigators treated the donor BM cells with 5-fluorouracil (an antimetabolite drug), which led to enrichment with high proliferative potential colony-forming cells. The lack of contribution of BM-derived cells in islet neogenesis was consistent with the observations of two other reports.^{40,41} Kojima *et al*⁴⁰ induced islet injury after BM transplantation and found that rare BM-derived cells migrated to the acinar region, not the endocrine cells of the pancreas of the diabetic animals. Lechner *et al*⁴¹ performed BM transfer using tagged cells and found no significant transdifferentiation of donor BM cells to islet cells. In agreement with this conclusion, Li *et al*⁴² demonstrated that BM cells play a role in β -cell maintenance under conditions provoking β -cell turnover while BM-derived cells themselves do not seem to contribute to the islet cell population.

In a further report, splenocyte transfer from nondiabetic mouse donors into nonobese diabetic (NOD) mice (a naturally occurring mouse model of autoimmune diabetes) not only leads to a decrease in the autoimmune response to β -cells in the recipient NOD mice but the splenocytes themselves also contribute to the regenerating islets and duct cells.¹⁰ In this study, the reversal of diabetes and islet neogenesis were optimal when the recipient mice were first made normoglycemic (by temporary islet transplantation under the kidney capsule) and were pretreated with complete Freund’s adjuvant, an immunomodulator. There was also a reversal of the underlying autoimmunity in the recipient mice as evidenced by a decrease in the insulinitis and peri-insulinitis in their islets. Of note, in this study, even transfer of irradiated donor splenocytes to euglycemic NOD female mice led to islet neogenesis, although to a lesser extent, raising the possibility that the modification of recipient autoimmunity and prolonged euglycemia in combination may be contributing factors to islet neogenesis.

A recent study⁴³ monitored for up to a year the fate of β -cells tagged by an inducible genetic marking system (a tamoxifen-inducible Cre recombinase and a double reporter gene one of which was flanked by loxP sequences) in a pulse-chase experiment wherein the β -cells of mice were differentially marked, before and after the tamoxifen pulse. The authors demonstrated that most, if not all, of the β -cells arise from pre-existing β -cells in adult mice under normal conditions and also under regenerative conditions after partial pancreatectomy. As the contribution of ductal cells in models of β -cell injury is well documented by other laboratories,^{44,45} the findings and interpretation in the different studies will need to be reconciled.

Figure 1 Schematic representation of the key aspects of cell-based gene therapy protocols for diabetes therapy.

Ex vivo cell and gene therapy for diabetes is in its infancy

Vector-associated toxicity has been a major concern in human trials in recent years (reviewed by St George⁴⁶). Cell therapy strategies (Figure 1) could circumvent many of the problems of *in vivo* gene therapy, but would depend on the identification of islet progenitor cells and coaxing them to differentiate to mature islets or β -cells *in vitro* prior to transplantation. Success depends on identifying the specific islet progenitors as well as optimizing the *in vitro* differentiating conditions. One approach is to coax pleuripotent cells to differentiate into islets or β -cell by *ex vivo* gene transfer of appropriate development-related gene(s).

Ex vivo conversion of cells into functional β -cells.

Some recent groundbreaking studies have demonstrated the potential of *ex vivo* conversion of non- β -cells into either insulin-producing β -cells or insulin-producing islet-like cell clusters. Generation of insulin-positive cells *in vitro* from pancreatic duct cells, of both human and rodent origin, has been reported by many groups in the past. Lechner *et al.*³⁶ meanwhile, isolated progenitor cells from adult rat and human islets that expressed the neural stem cell marker nestin, an intermediate filament, cultured them *in vitro* for about 8 months and coaxed them to differentiate into cells expressing hepatocyte-specific, pancreatic exocrine- or endocrine-specific markers. They also succeeded in generating cells that expressed insulin. These studies require further confirmation, as other investigators found that nestin was a marker for non-endocrine cells.^{47,48} Seaberg *et al.*⁴⁹ reported clonal identification of multipotent pancreatic precursors from adult mouse pancreas that differentiate *in vitro* into both islet endocrine and neural lineages. Interestingly, these islet lineage clones display glucose-responsive insulin secretion.

Yang *et al.*⁵⁰ demonstrated conversion of hepatic oval cells into insulin-producing cells with islet markers by culturing them in a high-glucose medium. These 'transdifferentiated' cells synthesize and secrete insulin. Progenitor cells have also been isolated from salivary glands after ductal-ligation, cultured *in vitro* on collagen, and demonstrated to express hepatic markers such as

α -fetoprotein and albumin, and islet hormones, including glucagon and insulin.⁵¹ In a recent study, Oh *et al.*⁵² showed that unfractionated BM cells differentiate into clusters of insulin-producing cells *in vitro* when they are cultured in a high-glucose medium. Transplantation of these BM-derived insulin-producing cell clusters reversed the hyperglycemia of diabetic NOD-*scid* mice, although they did not report the basal and glucose-stimulated insulin secretory response in the recipient animals. Their data, however, indicate the relative ease with which insulin gene expression can be turned on when BM cells are exposed to high-glucose medium, an observation that corroborates the observations of Kojima *et al.*⁴⁰ in mice *in vivo*. Embryonic stem (ES) cells are totipotent. There are recent reports on the differentiation of ES cells into insulin-producing islets *in vitro*,^{53,54} although caution on the interpretation of such data has been raised by some investigators.⁵⁵

Ex vivo gene therapy as adjunctive therapy for islet transplantation. Investigators have attempted to use different factors such as glucagon-like peptide-1 and hepatic growth factor to expand islet cell mass *in vitro* prior to transplantation in animals. Transfer of the gene for these proteins increased islet numbers, size and insulin content at least partly through decreased apoptosis.^{56–58} This approach aims at enhancing islet yield for transplantation and could be an adjunctive measure for optimizing cadaveric islet transplantation. Clinical trials on this approach have not been reported.

Prospects

Advances in vector technology that reduce toxicity and increase efficiency would be a boon to *in vivo* diabetes gene therapy

Advances in gene transfer vector development in the last few years have led to significant improvements in toxicity profiles, such as use of transposons flanked by *Flp* motifs combined with a second vector carrying the *Flp* and *Sleeping Beauty* recombinases resulting in random integration of the gene of interest,⁵⁹ achieving site-specific integration of adeno-associated virus (AAV)

combined with an efficiency approaching that of HDAD. These advances in vector development have been recently reviewed in detail;^{46,60} additional studies are needed to see if the projected advantages of the new vectors are realized in disease models *in vivo*. Further, cell and tissue specificity and engineered tissue-specific homing are being addressed to improve further the safety profile of gene transfer vectors. Regulated expression of the targeted gene using cell-type-specific promoters has been around for a long time and remains a valuable tool; however, tissue-specific regulation is often limited by a lack of cell-type-specific tropism of the vector. In this regard, some recent studies have held out promise by modifying the capsid proteins of AAV and other vectors with cell-type-specific peptides to target these vectors to desired cell types⁶¹ or modifying the coxsackie-adenoviral receptor (CAR) specificity of adenoviral constructs to improve target tissue specificity.⁶² Although there are no β -cell-specific surface markers that have been reported to date, identification of such markers will greatly facilitate efficient targeted gene therapy for diabetes. The identification of c-met, receptor for hepatocyte growth factor (HGF), as a possible marker for pancreatic stem/progenitor cells by Suzuki *et al*⁶³ is an encouraging development.

Identification of islet stem cells and β -cell progenitor cells would make cell-based therapy a viable alternative

Studies to isolate islet and β -cell progenitor cells have focused on identifying possible markers for selection from regenerating islets^{64–68} and pancreatic duct cells.⁶⁹ Isolation and differentiation of these cells and the ability to expand them *in vitro* would mark a major advance in islet biology and diabetes therapy.

ES cells have been touted to be able to differentiate into multiple cell lineages including the pancreatic endocrine cell lineages.^{54,70,71} Notwithstanding the ethical and current legal controversies regarding the use of ES cells, there are significant potential advantages and some disadvantages of using stem cells for cell therapy for diabetes. ES cells by nature are totipotent and in theory could reconstitute the entire islet and thus be the ideal replacement therapy for type I diabetes. In addition, they may have the highest replicative potential and the lowest tendency for early senescence. On the flip side, ES cells, being totipotent, could potentially also differentiate or dedifferentiate into other cell lineages under different *in vivo* conditions. Current work has mainly focused on conversion of ES cells into insulin-producing cells. Much work, however, remains to be performed on the methods of converting ES cells into specific cell lineages, and on the underlying mechanisms of differentiation and possible dedifferentiation so that we could harness the full potential of these cells. Obviously, access to human ES cell lines would greatly enhance and accelerate this process.

*Further knowledge of the differentiation and development of islets would facilitate the development of protocols for *in vitro* differentiation of pluripotent adult stem cells into islets*

With the identification of pluripotent cells with a potential to differentiate into cells of ectodermal,

endodermal and mesodermal origin^{21–25,72} as well as advances in understanding the transcriptional pathways involved in islet development,^{73–75} one could envision the possibility of coaxing these cells into becoming functional β -cells or pancreatic islets in the near future. The more we understand the underlying differentiation process, the better we are equipped to take advantage of the process in producing functional β -cells *in vitro*. The knowledge would greatly facilitate the generation of autologous progenitor cell-derived islets differentiated *ex vivo*, or islets derived from differentiation of ES cells *in vitro*. Thus, continued research into islet growth and development is an important component of the entire cell and gene therapy enterprise.

Advances in immune tolerance and limiting immune destruction to prevent autoimmune diabetes will allow long-term survival of transplanted or newly formed β -cells and islets

Assuming that we can induce islet neogenesis *in vivo*, or efficiently expand β -cells *in vitro*, further advances in our understanding of the autoimmune process in type I diabetes^{76–79} will help us to design strategies to prevent the autoimmune process from destroying the newly formed or transplanted islets or β -cells. Thus, secondary prevention as adjunctive therapy would be necessary for cell and gene therapy to succeed in reversing type I diabetes.

In conclusion, gene and cell therapy for type I diabetes is still in its formative years. However, exciting advances are being made and the field is poised to make an impact on different fronts. We are hopeful that applications of these important advances to human trials will be forthcoming in the not too distant future.

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