In vivo gene therapy for diabetes mellitus

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Gene therapy has been hyped as a possible 'cure' for diabetes mellitus in the near future ever since insulin was first cloned and expressed in cultured cells in the late 1970s. In the past decade, however, the bar for gene therapy for diabetes has been raised because of recent advances in the clinical management of diabetes. Although current treatment modalities fall far short of a cure, they produce greatly improved, if imperfect, glycemic control. In this context, we review the latest advances in *in vivo* gene therapy and conclude that the most widely applied strategy of insulin gene transfer does not measure up to the existing treatment options, whereas the recently proved concept of induced islet neogenesis has the potential of bettering the currently available therapy. Much work remains to be done, however, before this regimen can be taken from the bench to the bedside.

There are ~17 million people in the United States and >150 million people worldwide who are afflicted with diabetes mellitus. Diabetic individuals suffer life-threatening complications including ketoacidosis, a serious acute complication, as well as chronic complications that affect essentially every organ system in the body, among them cardiovascular disease and stroke, blindness, kidney failure, neurological dysfunction, and necrosis and gangrene of extremities, which often require amputations. The discovery of insulin in 1921 revolutionized diabetes treatment and greatly reduced the acute complication of diabetic ketoacidosis. As diabetics have begun to live longer, however, the chronic complications have taken over as the principal cause of morbidity and mortality.

Advances in our understanding of the pathophysiology of diabetes in the past several decades have produced significant improvements in therapy. We have learned that there are two main forms of diabetes: type 1, which was previously known as juvenile diabetes and is caused by absolute insulin deficiency; and type 2, which occurs mainly in adulthood, is often associated with obesity, and results from a combination of insulin resistance and β-cell dysfunction, leading to relative insulin deficiency [1].

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There is now general agreement that good glucose control is paramount in the treatment of diabetes, and tight control seems to prevent or to postpone chronic complications. Medication-induced and insulin-induced hypoglycemia seems to be a limiting factor in achieving perfect glycemic control [2,3]. Nevertheless, the availability of insulin-sensitizing agents, insulin secretion enhancers and new forms of insulin has lowered the incidence of iatrogenic hypoglycemia, and glycemic control among diabetics is now better than ever. The new insulins have durations of action that range from ultrashort, as in the case of lispro and aspart insulin, to prolonged with a steady plateau and no peak, as in the case of glargine insulin. These forms of insulin and improvements in the continuous subcutaneous infusion of insulin (CSII or insulin pump therapy) allow great flexibility in insulin regimens. Recently, the development of an optimized protocol for islet transplantation has also generated much hope and excitement for a possible 'cure' for diabetes [4].

These important advances notwithstanding, perfect glycemic control is still beyond the reach of most individuals with diabetes. The main problem with insulin replacement therapy is that it is impossible to administer insulin exogenously to produce an insulin profile that exactly mimics the natural dynamics of insulin. Another problem is that when insulin is administered systemically, it reaches the liver through the hepatic artery only after it has passed through the venous and pulmonary circulation – a route that is rather different from the one taken by insulin secreted from the endocrine pancreas, which goes directly to the liver via the portal circulation. The systemic and portal routes are associated with quantitatively different physiological actions of the hormone.

Although most patients who are treated successfully with islet transplantation can be taken off insulin, the restoration of normal insulin–glucose dynamics seems to elude them [5,6]. Islet transplantation is also limited by the availability of donors, because each successful transplant requires islets from at least two, and often more, donors [4]. Furthermore, transplant patients have to receive long-term – probably lifetime – immunosuppression therapy with all its potential side-effects. Despite these reservations, the combination of insulin, other hypoglycemic agents and islet transplantation offers

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flexibility and, in general, patients with diabetes enjoy much improved glycemic control as compared with just a decade ago.

For somatic gene therapy to become a major weapon in our armamentarium against diabetes in the future, it must have the potential to be as effective as the treatment options that are currently available. Here we review the different gene therapy approaches that are being tested and examine the promise and drawbacks of each in comparison to drug and insulin therapy and islet transplantation. Although therapy directed towards the correction or amelioration of the autoimmune dysfunction of type 1 diabetes might be equally important, we limit our analysis to studies aimed at the reinstatement of regulated insulin production by the body.

Strategies for *in vivo* diabetes gene therapy

Somatic gene therapy uses one of two gene delivery methods: *ex vivo*, whereby tissues are removed from the patient and transduced with a therapeutic gene *in vitro* and then reimplanted back in the patient; and *in vivo*, whereby the gene therapy vector is administered directly to the patient, for example, by intravenous, subcutaneous or intrabronchial routes, or via local injection.

The goal of the *ex vivo* approach is to generate cells *in vitro* that have the properties of β cells – that is, insulin-producing cells that are responsive to glucose (for example, see Ref. [7]). *Ex vivo* gene therapy has been also used to expand normal β cells *in vitro* for transplantation. *Ex vivo* strategies have been reviewed elsewhere [8–10] and thus are not discussed in this article.

In vivo gene therapy is the preferred delivery method because of its simplicity and convenience in that the therapeutic transgene is administered directly to the patient like any other pharmaceutical. But the development of safe and effective vectors for *in vivo* gene therapy is demanding. The main concern with *ex vivo* gene therapy lies in the mechanics of surgically removing host cells for transduction *in vitro* and reimplanting the genetically modified cells in the host; for *in vivo* therapy, toxicity related to the vector is often the limiting factor.

There are three strategies for *in vivo* gene therapy for diabetes, which all have the aim of lowering blood glucose. These strategies involve the delivery of genes encoding proteins that facilitate glucose utilization and/or inhibit hepatic glucose production, genes encoding glucose-regulatable insulin, or genes encoding developmental/transcription factors that induce the production of β cells in the liver.

Gene transfer of non-insulin 'glucose-lowering' genes

Two types of non-insulin transgene have been used to lower blood glucose: those that inhibit glucose production in the liver, and those that enhance glucose utilization by the liver or skeletal muscle. In the first category, glucokinase (Gck) gene transfer in rodents has been carried out by many different groups [11–15]. Although Gck has been categorized as a transgene that lowers glucose production in the liver [16], hepatic glucose production has not been measured directly in any of these studies and it is likely that a principal downstream effect of Gck is an increased utilization of glucose [12]. High-dose Gck gene transfer causes hyperlipidemia and fatty liver [12,15], and Gck gene transfer is best used as adjuvant treatment (a debatable role for gene therapy) to complement insulin therapy [15].

Gene transfer of the Gck regulatory protein produces an effect very similar to that of Gck gene transfer [17]. A mutant form of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase has been used to activate phosphofructokinase-1 and, simultaneously, to inhibit fructose-1,6-bisphosphatase to downregulate gluconeogenesis. Its overexpression has been shown to downregulate glucose-6-phosphatase and upregulate Gck, which stimulates glucose disposal and inhibits hepatic glucose production in a mouse model of type 2 diabetes [18].

Another way to downregulate hepatic glucose production is to divert glucose to glycogen by overexpressing 'protein targeting to glycogen' (PTG) [19,20]. This protein is a member of the family of glycogen-targeting subunits of protein phosphatase-1 that regulate glycogen metabolism. Adenovirus-mediated transfer of PTG stimulates glycogen synthesis in the liver and lowers blood glucose in rats, and thus represents a potential therapeutic approach to diabetes gene therapy [19].

As alluded to above, hepatic overexpression of Gck seems to enhance glucose utilization, and thus Gck is also one of the potential therapeutic genes in the second category that simulate glucose disposal. Notably, overexpression of Gck in skeletal muscle also stimulates glucose disposal and protects against hyperglycemia in streptozotocin-induced diabetic mice [21]. Similarly, other transgenes, including the mutant 6-phophosfructo-2-kinase/fructo-2,6-bisphosphatase and PTG, seem to affect both glucose utilization and glucose production in the liver [19,20].

In summary, there are different strategies to modulate blood glucose by targeting genes (other than insulin) that affect glucose production and utilization. Most of these represent adjuvant therapy, however, and seem to be better targets for small molecular weight compounds (for example, see Ref. [22]) than for gene therapy.

Gene transfer of the glucose-responsive insulin gene

Most publications on diabetes gene therapy involve the delivery to liver cells of variants of the insulin gene that (i) have been modified either to make the proinsulin expressed susceptible to processing into mature insulin or to obviate the need for processing, or (ii) have been modified to render gene expression responsive to changes in blood glucose concentration [23,24]. Because liver cells do not produce the islet prohormone convertases PC1/3 and PC2, many investigators have introduced new proteolytic cleavage sites into the pro-insulin molecule that are recognized by furin, a protease that is present in many tissues including liver cells [25–27]. Alternatively, the insulin gene can be modified to encode single-chain insulin [28], which has 20–40% of the activity of normal mature insulin [29].

The most challenging part of insulin gene therapy is to confer glucose responsiveness to expression of the insulin

transgene. Normal β cells are very smart: they respond to changes in glucose concentration by producing an almost instantaneous (in less than a minute) burst of insulin. The different glucose-responsive promoters used by various investigators, such as promoters from the phosphenolcarboxykinase (PEPCK) gene [30], elements from the L-pyruvate kinase gene [31], the glucose-6-phosphatase gene [32] and other genes (reviewed in [23,33]), allow the insulin transgene to be regulated by changes in glycemic levels within 1–2 h at the transcriptional level and within 3–4 h at the protein secretion level. They also take a long time to be turned off when blood glucose is normal or low.

Because of the lag in the secretory response, glycemic control by transcriptionally regulated insulin transgenes is often erratic and hypoglycemia is a major complication. Another approach is to control secretion at the level of the endoplasmic reticulum by drug-induced protein disaggregation [34]. Any manipulation requiring pharmacological agents defeats the purpose of gene therapy, however, because there is considerable flexibility in the wide array of pharmacological therapy using the different forms of insulin that are currently available.

Gene therapy aiming at induced β cell neogenesis in the liver

The potential problems with the approaches summarized above can be circumvented by inducing the formation of β cells or islets in the liver. To prevent the development of diabetes, Ferber *et al.* [35] used first-generation adenovirus (FGAd) to deliver the *pancreatic duodenal homeobox 1* (*Pdx1*, also known as *Ipf1*) gene or the gene encoding β-galactosidase to the liver of mice 2 days after streptozotocin injection [35]. The experiment was terminated 8 days later. Compared with FGAd–β-galactosidase controls, FGAd–*Pdx1* had caused a significant lowering of blood glucose at day 8; in addition, a plasma insulin response and intrahepatic insulin-positive cells were detected in the *Pdx1*-treated mice.

A recent report by Kojima *et al.* [36] indicates that the premise of Ferber *et al.* [35] is correct: that is, it is possible to induce endocrine cells in the liver by delivering islet-specific transcription factors. Kojima *et al.* studied the hepatic delivery of two different transcription factor genes, *Pdx1* and *Beta2* (also known as *Neuro* D, designated *NeuroD*/*β2*), to streptozotocin-induced diabetic mice. Instead of FGAd, they used a helper-dependent adenovirus (HDAd) to deliver the genes in their experiments. In FGAds E1 is deleted, which prevents the virus from unbridled replication that would otherwise overwhelm the recipient host. FGAds contain genes expressing all other adenoviral proteins, however, and these are co-expressed with the therapeutic transgene in the target cell [37–39]. As a consequence, FGAds stimulate a strong inflammatory response in the host animal [38,39] and, when they are used to deliver genes to the liver, the recipient animals develop severe hepatitis. Furthermore, the host will mount an immune response that prematurely terminates transgene expression within weeks of treatment. Thus, less toxic vectors that lead to prolonged transgene expression are better suited for testing the true and long-term potential of a therapeutic gene.

HDAd is one such vector because it is totally devoid of adenoviral protein genes [40–44]. It has negligible toxicity in mice, and transgenes delivered by an HDAd are usually expressed for many months [45–48], even for life [49], after a single treatment. HDAd-mediated delivery of *Pdx1* to the liver of streptozotocin-induced diabetic mice led to transient partial lowering of the blood glucose in these animals [36]. The *Pdx1*-induced hypoglycemic response was dose-dependent, but at a dose of *Pdx1* that led to the complete reversal of hyperglycemia the treated mice became sick, stopped eating and died. Necropsy showed the presence of insulin-producing cells in close proximity to portal triads in the liver. There was also evidence of severe hepatitis, which normally does not occur with HDAd treatment.

Further analysis showed that the mice also expressed trypsin, a digestive enzyme produced by the exocrine pancreas [36]. Insulin and trypsin were co-produced by the same cells in these mice, which accounted for the transient nature of the hypoglycemic response. The cells that expressed insulin died as a result of autodigestion by the coexpression of trypsin, which severely limited the duration of the therapeutic effect. In retrospect, the exocrine and endocrine-promoting actions of *Pdx1* are well known. Mice and humans born without *Pdx1* function lack a pancreas – that is, both exocrine and endocrine pancreas are missing [50,51] – and it is known that *Pdx1* has differentiating functions towards both the exocrine and endocrine pancreas lineages throughout embryonic development [52].

NeuroD/*β2* functions downstream of *Pdx1*. Because mice and humans with defective *NeuroD*/*β2* function develop diabetes and markedly disorganized islets, Kojima *et al.* [36] examined *NeuroD*/*β2* as their next therapeutic gene. HDAd-mediated delivery of *NeuroD*/*β2* to the liver of diabetic mice led to a significant reduction of the hyperglycemia. Addition of an islet growth factor, betacellulin [53], led to the complete reversal of diabetes in these animals. The mice started producing normal levels of plasma insulin, and an intraperitoneal glucose tolerance test showed an essentially normal response.

Mice treated with HDAd-*NeuroD*/*β2* started producing insulin transcripts and protein in the liver. Immunohistochemical analysis detected the presence of islet clusters in the liver. Most of these clusters were located immediately underneath the liver capsule [36]. The cells in the liver islets produced insulin, as well as the other principal islet hormones glucagon, somatostatin and pancreatic polypeptide. They produced and secreted mature insulin because the prohormone convertases PC1/3 and PC2 were also expressed, in addition to the ATP-sensitive potassium channel subunits Kir6.2 and SUR1. Notably, immunoelectron microscopic analysis detected the presence of insulin granules inside vesicles in endocrine cells that lacked features of hepatocytes. In short, *NeuroD*/*β2* gene delivery to the liver led to the reversal of diabetes in mice by the induction of islet neogenesis in the liver.

Insulin gene therapy versus islet neogenesis

We have reviewed the recent data on insulin gene therapy and induced islet neogenesis as therapeutic options.

Although both approaches can lead to the reversal of hyperglycemia, there are significant differences between them. To understand the requirements for successful gene therapy for diabetes, we have to examine how the β cell regulates insulin production [54] (Figure 1).

To be effective, the insulin-producing cell must possess a glucose-sensing mechanism. By using the liver as a target organ, this requirement is partially solved, because, like the β cell, the liver cell has its own glucose-sensing mechanism that can be borrowed by the cell transduced with the insulin gene. Nonetheless, there are differences between the glucose-sensing mechanism of the liver and that of the β cells, and only islet cell neogenesis has the potential of re-establishing the authentic β cell-specific glucose-sensing mechanism in the target cell.

Mature insulin is the most active form of the hormone; it is also the predominant form that is secreted from the β cell. After the hepatic delivery of insulin cDNA as the transgene, the hormone is produced as pro-insulin, which has only ~5% of the activity of mature insulin. This situation can be corrected by inserting into the transgene construct proteolytic cleavage sites for furin, a fairly ubiquitous protease that is also produced in the liver. The mutant pro-insulin protein product is cleaved by furin present in the host liver cell and is subsequently secreted as mature insulin [25–27]. Alternatively, a single-chain insulin transgene construct can be used as the therapeutic gene. The mildly reduced potency of the single-chain insulin (20–40% that of mature insulin [29]) is of little consequence and normalization of hyperglycemia can be readily achieved [28]. Therefore, the absence of the prohomone convertases in the liver can be circumvented by these maneuvers, although the possibility of an immunogenic mutant form of insulin remains.

The one aspect of insulin biogenesis and control that is difficult, if not impossible, to mimic is the acute control of insulin secretion. In response to a rapid increase in blood glucose, such as occurs after a meal or a sugar-containing drink, the β cell produces bursts of insulin almost instantaneously because insulin secretion is effected by regulated exocytosis [54]. By contrast, secretion of insulin from the liver after insulin gene transfer occurs via the constitutive pathway and is thus unregulated. Similarly, turning off insulin production in such cells is also delayed.

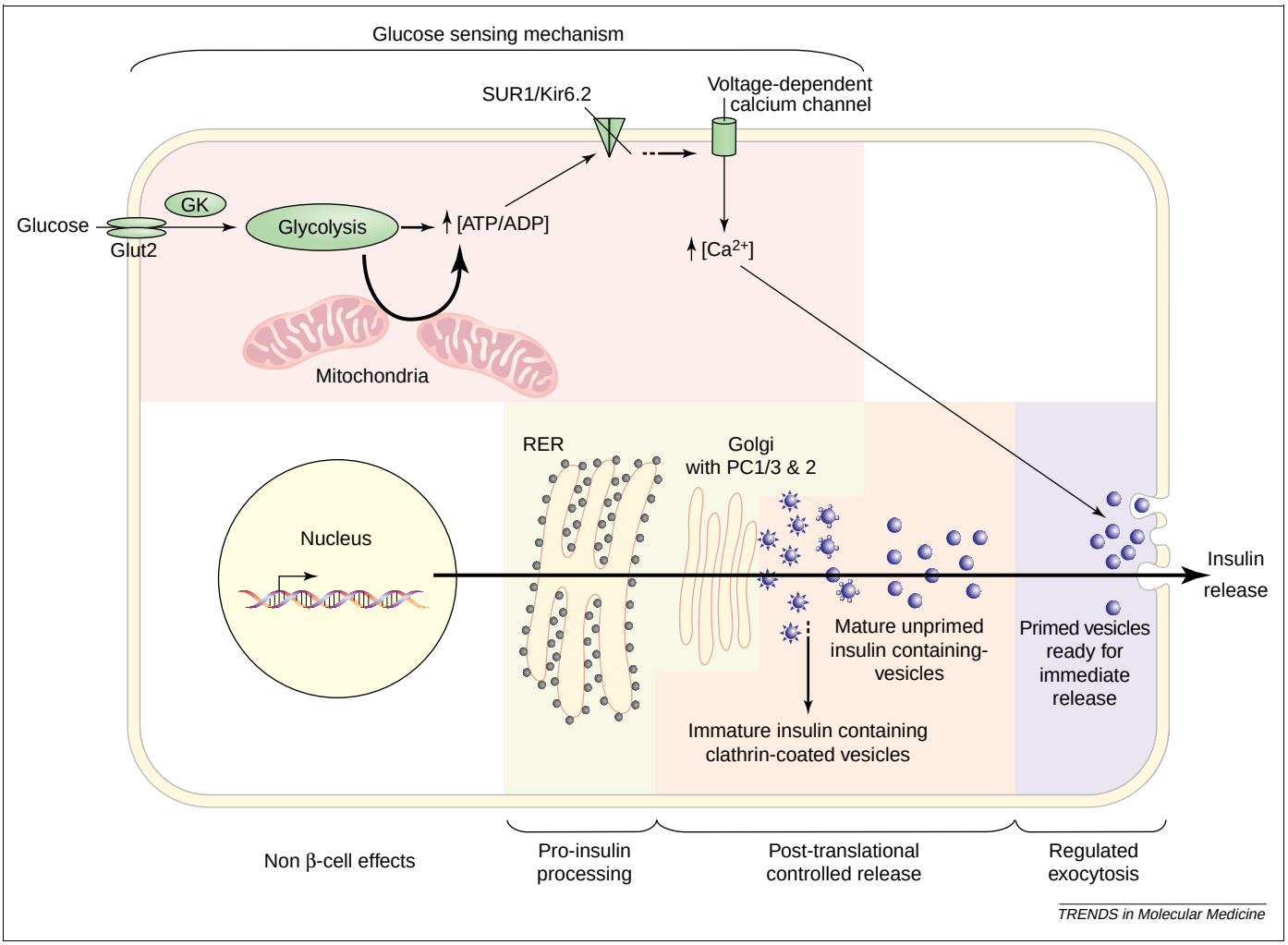

Figure 1. Factors that regulate acute secretion of insulin from β cells. The most important factors that allow the β cells to respond to acute changes in blood glucose are (i) a glucose-sensing and metabolic signaling mechanism; (ii) pro-insulin processing, which requires the combined action of the prohormone convertases PC1/3, PC2 and carboxypeptidase H; and (iii) post-translational controlled release, culminating in (iv) regulated exocytosis. Insulin production is also regulated at the transcriptional and translational levels, but the secretory response to acute changes in blood glucose is controlled mainly at the post-translational level. (v) Finally, β cells are in close contact with non-β islet cells, whose secretions modulate the secretory response of β cells but also have a direct effect on glucose homeostasis.

This is the most serious drawback of insulin gene therapy. The marked delay in the insulin secretory response results in erratic blood glucose control, with hyperglycemia immediately after meals and hypoglycemia several hours later and during exercise.

Finally, it is important to stress that β cells do not occur in isolation. They are surrounded by and in close contact with non-β islet cells, whose hormone products are capable of modulating the insulin secretory response, as well as directly regulating blood glucose themselves. How important such interactions are in normal insulin secretion is unclear at present. In general, induced islet neogenesis displays all of the characteristics of insulin secretion from normal β cells. Whether ultimately it will be the regimen of choice for gene therapy for diabetes will depend on future developments.

Future prospects

Experimental gene therapy for diabetes is still in its infancy. The simplicity of insulin gene therapy has made it a popular approach in many laboratories. During the past decade, research in this area has concentrated on recreating the proper glucose responsiveness to the insulin transgene by trial and error using different glucose-responsive promoters. Unfortunately, the control of insulin production and secretion is complex, and our understanding of the process is rudimentary [54]. It is now apparent that attempts to imitate the normal insulin secretory response by adding control elements to the insulin transgene are doomed to failure, because we cannot reproduce regulated exocytosis by insulin gene transfer. In comparison, induced neogenesis seems to be a much more promising approach towards a 'cure' for diabetes, assuming that we can solve the problem of autoimmunity associated with type 1 diabetes.

The fact that the strategy holds promise does not mean that we are anywhere close to its clinical application. There are many issues that need to be addressed. Most of the islet cells induced by *NeuroD/β2* gene therapy produce several islet hormones, which suggests that the newly formed islets are immature. Other than *Pdx1*, *NeuroD/β2* is the only transcription factor that has been examined as a potential therapeutic gene to induce islet neogenesis and other transcription factors upstream and downstream of *NeuroD/β2* should be tested. One or more of them might induce more mature islets than *NeuroD/β2*. Perhaps a combination of factors will work better than any one alone, as has been shown for combination therapy using *NeuroD/β2* and the islet growth factor betacellulin.

Another interesting issue is whether the islets produced by *NeuroD/β2* gene transfer are 'permanent'. A recent study by Ber *et al.* [55] suggests that *Pdx1*-induced insulin-producing cells can persist for a long time after the FGAd–*Pdx1* vector has disappeared. Although further understanding of the process of islet neogenesis might not be absolutely necessary for its successful application in humans, it is always welcome because it could point to alternative approaches.

The principal limiting factor for clinical trials at this time seems to be the availability of a safe and efficacious gene delivery vector. Fortunately, as the lack of an ideal vector is a major hurdle for *in vivo* gene therapy in general, the development of safe and efficient gene transfer vectors is a chief objective of many gene therapy laboratories around the world [56]. It is conceivable that rapid progress in vector development, coupled with continued progress in the development of islet neogenesis regimens, will enable us to move gene therapy for diabetes from animal experiments to clinical trials in the foreseeable future.

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