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Insulin Gene Therapy for Type 1 Diabetes Mellitus: Unique Challenges Require Innovative Solutions

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Abstract

Type 1 diabetes mellitus (T1DM) is a disease characterized by chronically elevated blood glucose levels that results from the autoimmune destruction of the insulin-producing β cells of the pancreas. While treatment options exist, they all possess serious limitations. Insulin gene therapy provides a promising alternative aimed at replacing insulin production in native non- β cells. For insulin gene therapy applications to be successful in treating T1DM, a glucose-sensitive organ must be targeted for insulin expression, insulin production must be responsive to ever-changing blood glucose levels, and insulin expression must persist long term. In addition, the amount of insulin production is critical, as too little insulin would lead to poor glucose regulation and too much insulin would induce hypoglycemia, a potentially life-threatening state. Together, insulin gene therapy provides challenges that are absent with other gene therapy applications. In this chapter, we examine the challenges of insulin gene therapy and discuss how the two key components of insulin gene therapy—the insulin expression cassette and the delivery vehicle—can be tailored for the successful treatment of T1DM.

Keywords: gene therapy, type 1 diabetes mellitus, lentivirus, insulin, β cells

1. Introduction

Type 1 diabetes mellitus (T1DM) is an autoimmune disorder whereby the β cells of the pancreas are destroyed. Under physiological conditions, β cells synthesize and secrete insulin in response to changes in blood glucose levels. Insulin, in turn, acts on other cells to promote glucose uptake

from the blood and thus lower blood glucose levels to normal. Without sufficient numbers of functional β cells, insulin production becomes inadequate and unable to promptly restore normal blood glucose levels. Over time, chronically elevated blood glucose levels, termed hyperglycemia, cause numerous secondary complications, ultimately leading to widespread tissue and organ damage, as well as increased mortality. According to the National Diabetes Statistics Report, 29.1 million people, or 9.3% of the US population, have diabetes [1], with T1DM accounting for roughly 5% of all diagnosed cases in the adult population. The total economic cost of diabetes is estimated to be \$245 billion per year, with T1DM costing an estimated \$8–14 billion per year. Thus, diabetes poses a significant burden to our society. Unfortunately, no cure exists for T1DM.

1.1. Current treatment options

While there is currently no cure, several therapies exist to better control blood glucose levels. The most common form of therapy involves use of synthetic insulin, which typically requires multiple insulin injections per day. Unfortunately, this option is cumbersome and unable to restore perfect glucose control. As a result, exogenous insulin therapy delays the onset and reduces the severity of secondary complications but is unable to prevent them [2]. In addition, exogenous insulin therapy can cause hypoglycemia, a potentially life-threatening condition characterized by dangerously low blood glucose levels. The insufficiencies of exogenous insulin therapy arise from the fact that most insulin regimens are unable to mimic normal β cell secretion in response to continually fluctuating blood glucose levels. In an effort to improve upon exogenous insulin therapy and better imitate normal physiology, specialized insulin pumps, dubbed artificial pancreata, have been developed to deliver insulin when needed by continuously monitoring blood glucose levels [3, 4]. Artificial pancreata are able to improve glycemic control, but the implanted glucose sensors inevitably accumulate serum proteins that can compromise the accuracy of glucose measurements and consequently affect the precision of insulin delivered, limiting its long-term effectiveness. Ultimately, exogenous insulin therapies provide a suboptimal therapy for T1DM. Conversely, tighter glucose control can be attained through whole-pancreas transplantation [5], but this therapy is severely hampered by a shortage of donor organs and further complicated by the need for lifelong immunosuppression. Transplantation of pancreatic islets was anticipated to minimize the impact of donor shortage, as islets from one donor could be expanded *ex vivo* to a quantity sufficient for multiple recipients [6], but equivalent successes like those observed with whole-pancreas transplantation have yet to be obtained [7]. Hence, there is clearly a need for effective and broadly applicable treatment options for T1DM.

Gene therapy-based treatment options have emerged as a promising alternative. Gene therapy refers to any technique aimed at using genes to treat or prevent diseases, whether it be through delivery of a functional gene to replace a defective one or through knockdown of dysfunctional genes using gene silencing technologies. For T1DM, three primary gene therapy approaches have been explored to prevent or treat the disease. First, researchers have attempted to prevent the autoimmune destruction of β cells by modifying the immune system. Second, researchers have attempted to generate surrogate β cells from native non- β cells to replace the function of

those cells lost from autoimmune destruction. Third, researchers have taken a more straightforward approach and simply attempted to replace the primary function of β cells—insulin production—to treat T1DM. Not surprisingly, each of these approaches has their own advantages and disadvantages, which we will outline in the following sections.

1.2. Prevention of autoimmune β cell destruction

The most logical therapy for T1DM would be to take preemptive measures and prevent the development of autoimmunity that causes β cell destruction in susceptible individuals. Not surprisingly, many researchers have pursued this strategy as a potential alternative to current treatment options, and to do so, various genetic modifications to both β cells and immune cells have been tested in experimental models of T1DM. First, researchers have sought to induce immune tolerance so that β cell antigens are no longer recognized as foreign. French and colleagues sought to induce immune tolerance by driving intrathymic expression of proinsulin under the control of the major histocompatibility complex (MHC) class II promoter and found that it prevented the onset of T1DM. Similarly, Tian and colleagues transduced autologous bone marrow hematopoietic stem cells of non-obese diabetic (NOD) mice with diabetes-resistant MHC class II I-A β chain molecules to examine whether this could prevent the development of diabetes. Indeed, they found that expression of this diabetes-associated allele prevented the development of autoreactive T cells by intrathymic deletion and protected the mice from developing insulinitis and diabetes [8]. Second, groups have aimed to modulate the immune system through cytokine-based approaches. For example, gene transfer of transforming growth factor- β and calcitonin gene-related peptide have been shown to prevent the onset of diabetes in NOD mice [9, 10]. Lastly, groups have attempted to modify residual β cells so that they can resist autoimmune destruction, an event that generally occurs through apoptosis. Liu *et al.* overexpressed the antiapoptotic gene, *bcl2*, in β cells to increase the survival of β cells without affecting their function [11].

Although these preventative options have shown promise, they are hampered by several limitations: (1) These strategies rely on the early detection of diabetes. This is difficult because individuals often do not become symptomatic until they have already lost greater than 80% of their β cells. Thus, efforts to protect the remaining β cells would still leave the patient hyperglycemic. (2) T1DM is a multifactorial disease, making it nearly impossible to accurately predict who from the general population will succumb to the disease [12]. Thus, early intervention can be risky and perhaps even accelerate the progression of the disease. (3) The immune system is highly evolved and its complexities are not well understood. Further, innumerable functional redundancies exist that allow the immune system to compensate for the loss of any single factor or pathway. At our current level of understanding, it seems unlikely that selectively targeting a specific component of the immune system could prevent the autoimmune destruction of remaining β cells. As a result, other gene therapy strategies have been explored.

1.3. Reprogramming non- β cells into β cells

The goal of reprogramming non- β cells into surrogate β cells is to create replacement cells that are as similar to native β cells as possible, including the ability to not only synthesize insulin but also store it within secretory granules and secrete it instantaneously upon elevations in blood glucose levels. The most common way that researchers have done so is by overexpressing β cell-specific transcription factors in non- β cells. Transcription factors are DNA-binding proteins that modulate the rate of transcription of specific genes in a cell type-specific manner. During development, transcription factors play a critical role in executing differentiation programs by driving the expression of cell type-specific genes, and during adulthood, transcription factors are important for maintaining the differentiated status of somatic cells. For β cells, the transcription factors PDX1, NeuroD1, Neurog3, Pax4, Pax6, Nkx6.1, Nkx2.2, and MafA are among the many transcription factors ultimately responsible for directing and/or maintaining the β cell fate.

With the aforementioned knowledge in mind, researchers have attempted to overexpress these transcription factors in a variety of cell types with the hopes of reprogramming them into β cells, including pancreatic exocrine cells [13, 14], keratinocytes [15], hepatic oval stem cells [16], adipose-derived stem cells [17], and hepatocytes. Of these, hepatocytes have been most commonly targeted due to the fact that they are closely related developmentally to β cells and easy to target. PDX1, which regulates early pancreas morphogenesis during development and controls glucose-dependent insulin expression in β cells, has been shown to be indispensable for the conversion of non- β cells into β cells. For example, Ferber *et al.* expressed PDX1 in the livers of diabetic mice and observed insulin expression and secretion, as well as restoration of normoglycemia [18]. Expression of NeuroD1 has also been shown to be a potent inducer of insulin expression in both primary duct cells and hepatocytes [19, 20]. However, ectopic expression of Neurog3 and Nkx6.1, which are also associated with β cell development, was unable to generate surrogate β cells. For example, despite the ability of Neurog3 to activate the persistent expression of NeuroD1, the use of Neurog3 in β cell engineering is not sufficient to generate surrogate β cells [21–25]. However, co-delivery of Neurog3 with PDX1 and MafA was successful in converting pancreatic exocrine cells into surrogate β cells [25]. Similarly, Gefen-Halevi *et al.* overexpressed Nkx6.1 in liver cells but found that it alone was unable to induce insulin expression. However, when co-expressed with PDX1, it promoted reprogramming of liver cells to β cells capable of glucose-stimulated insulin secretion [26]. Together, these studies validate the utility of transcription factor-mediated production of surrogate β cells in animal models and underscore its potential for treatment of T1DM.

Despite these positive findings, the long-term success of reprogramming strategies will rely on the ability of newly formed β cells to evade preexisting autoimmunity. This is particularly relevant given the abundance and diversity of previously identified autoantigens involved in the autoimmune destruction of native β cells [99]. It is expected that the more similar a surrogate β cell is to a native β cell, the more likely it will be targeted by the host's immune system and destroyed. For instance, while Ferber *et al.* found that hepatic expression of PDX1 was able to correct diabetic hyperglycemia, they also found that the mice developed hepatitis and were prone to autoimmunity against the newly formed β cells [18]. While other studies in

NOD mice, a mouse model of autoimmune diabetes provides hope that autoimmunity could be avoided through reprogramming strategies [27], these studies must be carried out for longer periods of time to assess true efficacy. Ultimately, it is likely that these strategies will either require lifelong immunosuppression or selective immunomodulation to ensure the survival of the surrogate β cells.

1.4. Insulin gene therapy

Given the autoimmune nature of T1DM, it may actually be unfavorable to produce surrogate cells that closely resemble native β cells. Since the primary function of β cells is to synthesize and secrete insulin, many groups have taken a humbler approach and simply aimed to restore insulin production in non- β cells, a field known as insulin gene therapy. Although insulin is also an autoantigen known to result in native β cell destruction [28], the theoretical risk of recurring autoimmunity is greatly reduced. Of course, it should be emphasized that β cells are extremely precise in their ability to secrete insulin upon demand, so simply expressing insulin in a non- β cell would likely not be an effective means of treating T1DM. For instance, an individual would not want to be producing high levels of insulin at all times because this would cause hypoglycemic episodes. Thus, to understand whether insulin gene therapy is indeed a viable option to treat T1DM, a clear understanding of the intricacies and aptitude of β cells must first be attained. In the following sections, we will outline what makes a β cell so adept at controlling glycemia and cover what criteria must be met for insulin gene therapy approaches to be successful. We will then present an overview of the field of insulin gene therapy to treat T1DM, with an emphasis on the unique challenges not found in other gene therapy applications. More specifically, we will go into detail about the choice of cell type to target *in vivo*, as most cell types are limited in their ability to adequately sense changing blood glucose levels. We will then discuss key features used within insulin expression cassettes to meet the specific needs of the field, with a particular emphasis on glucose-inducible response elements (GIREs), before discussing the advantages and disadvantages of various viral vectors as they pertain to the field of insulin gene therapy. Lastly, we will present future directions necessary to make insulin gene therapy a clinical reality.

2. Insulin gene therapy

2.1. Features of the β cell

In order to determine whether insulin gene therapy possesses the necessary elegance to be a viable treatment option, it is important to understand the key features of β cells that make them so adept at controlling glycemia and the minimum requirements that absolutely must be met to replace their function. Native β cells possess several important features that together allow them to precisely control blood glucose levels. Specifically, the β cell has the ability to regulate insulin production at the transcriptional, post-transcriptional, translational, and post-translational levels, as well as the ability to store and secrete insulin in a highly regulated fashion in response to glucose. While no individual feature is sufficient in itself to control

glycemia, nor specific to β cells, the combination of features makes it a remarkably competent cell for its task.

First and most importantly, β cells have the ability to sense and quickly respond to small changes in circulating glucose levels over a broad range of physiological concentrations (2–20 mM) through concentration-dependent entry and metabolism of glucose. They do so through the activity of glucose transporter-2 (GLUT2) and glucokinase. GLUT2 is a transmembrane protein that enables glucose transport across cell membranes, whereas glucokinase is an enzyme that phosphorylates glucose to initiate its intracellular metabolism. GLUT2 and glucokinase have been dubbed the “glucose sensors” of β cells because they enable β cells to sense glucose over a very broad range of concentrations. They are able to do so due to their high K_m for glucose (~17 and 8 mM, respectively), which allows their activity to vary substantially based on glucose availability [29].

β cells also have the ability to secrete insulin in a precisely regulated fashion in response to elevations in blood glucose levels. β cells do so through their capacity to (1) synthesize and store large quantities of insulin within secretory vesicles and (2) generate secondary stimulus-secretion coupling signals to activate nearly instantaneous insulin vesicle exocytosis. Remarkably, β cells can secrete substantial quantities of insulin within a minute after exposure to elevated glucose. This is a consequence of their metabolic capabilities; β cells possess a unique combination of metabolic enzymes that ultimately leads to the generation of signals capable of altering insulin secretion in response to glucose metabolism. Namely, β cells, but not other mammalian cell types, have negligible lactate dehydrogenase activity while displaying increased pyruvate carboxylase activity, which directs pyruvate almost entirely toward mitochondria for subsequent metabolism via the tricarboxylic acid cycle and oxidative phosphorylation [30]. In so doing, β cells increase their ratio of ATP/ADP and activate ATP-sensitive K^+ channels, a key stimulus leading to insulin vesicle exocytosis.

Furthermore, β cells have remarkable control of insulin biosynthesis at the transcriptional, post-transcriptional, translational, and post-translational levels, with each level of control being regulated by glucose availability. At the transcriptional level, increased glucose levels lead to upregulated insulin expression through the enhanced activity of the transcription factors, PDX1 and MafA [31, 32]. At the post-transcriptional and translational levels, β cells have glucose-responsive mechanisms to modulate insulin mRNA stability and the rate of translation, respectively. This glucose-dependent regulation is primarily governed by an RNA-binding protein known as polypyrimidine tract-binding protein (PTB). Association of PTB with a pyrimidine-rich stretch in the 3′ untranslated region (UTR) of the preproinsulin mRNA has been shown to be responsible for glucose-dependent changes in its stability and rate of translation [33, 34]. In fact, the half-life of preproinsulin mRNA has been shown to increase nearly 3-fold as a result of PTB association [35, 36]. At the post-translational level, β cells possess specific enzymes that allow them to process proinsulin—a precursor form—into fully active insulin. Proinsulin conversion involves removal of two basic pairs of amino acids—the C-peptide—and is mediated by the β cell endoproteases, PC1/3 and PC2, and the exopeptidase, carboxypeptidase-H [37, 38]. Altogether, β cells have a variety of glucose-dependent mechanisms to control insulin output.

β cells further refine insulin biosynthesis and secretion in response to other circulating metabolites. First, the gut produces the peptide hormones, glucose-dependent insulintropic polypeptide (GIP) and glucagon-like peptide-1, which bind specific receptors found predominantly on β cells to bolster insulin production [39]. Second, specific amino acids and free fatty acids can serve as insulin secretagogues. While some amino acids and free fatty acids can actively promote insulin secretion, most of them simply have a role in amplifying the stimulatory effects of glucose [40]. Third, metabolic stress can induce neuronal signals that influence insulin output [41]. Together, these inputs regulate insulin production and secretion from β cells to more precisely maintain glucose levels within a normal range.

2.2. Requirements of insulin gene therapy

Combining all the remarkable features of the β cell, the end product is a cell with impressive glucose sensitivity, the ability to control insulin biosynthesis at several levels in a glucose-dependent fashion, and the ability to store, process, and secrete insulin almost instantaneously in response to glucose. The β cell is truly impressive. With this knowledge in mind, it would be easy to argue that insulin gene therapy lacks the sophistication necessary to adequately treat T1DM, especially given its relative simplicity. However, it is important to keep in mind that the most commonly used treatment for T1DM currently involves repeated injections of synthetic insulin. While this treatment option holds very little sophistication or biomimicry, it has still proven effective enough to remain a viable option since it was first employed as a medication in 1922. With that being said, any treatment that could provide better glycemic control while averting the cumbersome nature of synthetic insulin therapy would be a noteworthy improvement. So, beyond the ability to produce insulin alone (a feat that could likely be achieved in any cell type using a strong constitutive promoter), what other features of β cells are absolutely critical for the success of insulin gene therapy applications?

Given that glucose is the primary stimulus leading to the production of insulin and subsequent clearance of itself, the impressive glucose sensitivity conferred by GLUT2 and glucokinase is a necessary feature that must be present in an insulin-producing surrogate β cell. Without these proteins, a surrogate β cell would not be able to precisely sense glucose concentration and control insulin output over a broad range of circulating glucose concentrations. In addition to glucose sensitivity, it is also important that the insulin-producing surrogate cell has the ability to respond to changing blood glucose levels by modulating insulin output. In so doing, insulin output could be adjusted in a physiologically relevant manner to better control glycemia. It is likewise important for an insulin-producing cell to have the ability to process proinsulin into insulin, given that proinsulin has less than 10% of the biological activity of fully processed insulin [42]. Lastly, it would be ideal for an insulin-producing cell to have the ability to package, store, and secrete insulin almost instantaneously in response to elevated blood glucose levels. However, it is debatable whether this last feature is indeed critical to the success of insulin gene therapy. Diabetes is such a devastating disease because of the secondary complications that arise as a consequence of sustained hyperglycemia, not repeated episodes of transient hyperglycemia. Thus, as long as the insulin-producing surrogate cell has the ability to produce sufficient quantities of insulin within a reasonable time frame in a glucose-responsive fashion,

the difference between near instantaneous insulin secretion or secretion with a few minutes' delay may be less critical. The quantity of insulin secreted must simply be large enough to correct hyperglycemia, but not too large as to cause hypoglycemia.

Thus, a long-lasting treatment for T1DM using insulin gene therapy could be achieved, but several criteria must be considered. First, the appropriate cells must be targeted for insulin production. At a minimum, these cells would need to express the glucose sensors, GLUT2 and glucokinase. Second, insulin transgene expression must be responsive to fluctuating blood glucose levels, being upregulated during hyperglycemia and downregulated during euglycemia. There are a variety of mechanisms available to endow an insulin-producing surrogate β cell with this ability. Third, there must be some mechanism in place for the target cell to process proinsulin into mature insulin. Lastly, an appropriate gene correction tool must be utilized to safely and effectively drive long-term insulin expression.

While meeting these criteria provides several challenges, treating T1DM through insulin gene therapy presents additional challenges not associated with other gene therapy strategies. First, insulin has a relatively short half-life compared to many other proteins being used for gene therapy applications. For instance, the circulating half-life of coagulation factor IX, which is deficient in hemophilia B patients, is estimated to be around 18–25 hours [43], whereas the circulating half-life of insulin has been estimated to be around 4–6 minutes. To compensate for the short circulating half-life of insulin, it is necessary to produce large amounts of insulin either by developing a highly effective gene expression system or by transducing a larger number of cells than other gene therapy applications. This makes the choice of gene delivery system a critical one, as the delivery vehicle must be produced in great abundance and transduce cells efficiently *in vivo*. Second, basal insulin production must be kept low during fasting periods and upregulated only when blood glucose levels become elevated. Other gene therapy applications need only to deliver the therapeutic protein of interest constitutively at low levels to correct the clinical manifestation, owing to greater protein stability and the particular function of the protein. If insulin was expressed constitutively at low levels to satisfy basal metabolic activity, there would be long periods of postprandial hyperglycemia, and if the level of constitutive insulin production was increased to effectively control postprandial glucose levels, there would be a very high possibility of hypoglycemia during fasting periods. Thus, insulin expression cannot be driven by a strong constitutive promoter; it instead must be responsive to fluctuating blood glucose levels. This makes the design of the insulin expression cassette extremely important. It also creates a much narrower therapeutic window for dosing than other gene therapy applications. In the following sections, we will discuss these factors in depth.

2.3. Target cells for insulin gene therapy

At a minimum, the target cells chosen for insulin gene therapy would need to express GLUT2 and glucokinase and have innate mechanisms for glucose-responsiveness, thus giving them the ability to sense and respond to continually fluctuating blood glucose levels. Without them, insulin secretion from surrogate β cells would be far less precise. Besides β cells, the only cells that express both GLUT2 and glucokinase are hepatocytes and cells of the hypothalamus and

small intestine. Thus, these cells serve as a nice starting point for targeting of insulin transgene expression. It should be noted, however, that a variety of other cells have been targeted for insulin expression, including skeletal myocytes, fibroblasts, and mesenchymal stem cells. An interesting example is the targeting of skeletal myocytes. Skeletal myocytes do not express GLUT2 or glucokinase. Instead, they express GLUT4 and hexokinase, which each have a higher affinity (i.e. lower K_m) for glucose. To endow skeletal myocytes with enhanced glucose sensitivity, Callejas *et al.* co-expressed insulin with glucokinase. Whereas insulin alone was insufficient to adequately treat T1DM in dogs using skeletal myocytes as surrogate β cells, co-expression of glucokinase with insulin was able to normalize fasting hyperglycemia and accelerate glucose disposal after oral challenge [44]. These findings emphasize the importance of glucose sensitivity in treatment of T1DM.

Intestinal K cells provide a particularly promising cell type for insulin gene therapy applications because they not only express GLUT2 and glucokinase, but they also possess the proinsulin processing enzymes and have the machinery for regulated insulin secretion. Cheung and colleagues exploited these unique advantages to generate insulin-producing surrogate β cells from K cells. To do so, they generated transgenic mice expressing human insulin under control of the GIP promoter, a K cell-specific promoter believed to be regulated by glucose [45]. They found that the GIP promoter was able to target insulin expression to K cells specifically, and the transgenic expression of insulin was effective at promoting normal fasting glucose levels and efficient glucose clearance in response to an oral glucose challenge for up to three months after mice were rendered diabetic with streptozotocin (STZ). However, while their findings hold promise, the translation of this strategy to the clinic will rely on their ability to address the following concerns: (1) The gut is one of the primary hubs for the immune system, and given that insulin itself is an autoantigen responsible for native β cell destruction [46], intestinal K cells may be particularly susceptible to recurring autoimmune attack; long-term protection from autoimmunity must be demonstrated. (2) More importantly, the GIP promoter is not only regulated by glucose intake but also by other sources—most notably fats. Thus, patients receiving this treatment would, at the very least, need to maintain a very strict diet to avoid potentially fatal consequences, like hypoglycemia. Further, another study found that glucose alone does not even regulate insulin secretion when controlled by the GIP promoter [47]. Regulation of the GIP promoter must be more thoroughly examined before moving toward human clinical trials.

The most commonly chosen target cells of insulin gene therapy applications, and the one we have chosen, are hepatocytes. Although hepatocytes do not have the machinery to store insulin within secretory vesicles and secrete it in a regulated fashion, they express GLUT2 and glucokinase and possess a robust capacity to synthesize and secrete proteins constitutively. In addition, hepatocytes are attractive targets for insulin expression because they (1) are closely related to β cells developmentally, (2) play a very important role in glucose homeostasis, and (3) are relatively easy to target. As a result, it has been the most commonly targeted organ for *in vivo* production of insulin-producing surrogate β cells and will be the focus for the remainder of this chapter.

2.4. Expression cassette design

After choosing an appropriate cell type for insulin production, there are several considerations that must be taken into account when designing the insulin expression cassette. Perhaps the first decision that must be made is which promoter to use to drive insulin expression. One of the most commonly used promoters within the field of gene therapy is the cytomegalovirus (CMV) promoter. The CMV promoter is a mammalian promoter from the human cytomegalovirus that drives strong, constitutive transgene expression. While the CMV promoter has been used quite frequently to drive insulin expression for treatment of T1DM, there is one fundamental reason why these studies could never be translated to the clinic: Insulin expression must be responsive to glucose, being upregulated when blood glucose levels rise and downregulated to low levels during fasting periods. The CMV promoter would drive consistent, high level expression of insulin even during fasting periods, which would ultimately cause blood glucose levels to fall dangerously low. Furthermore, even if regulatory elements were added to the expression cassette to endow glucose-responsiveness to insulin expression, the CMV promoter is so strong that it would override these elements. Thus, a weaker promoter must be used to maintain low levels of insulin production during fasting periods if insulin gene therapy is to be successful in treating T1DM.

Weaker tissue-specific promoters have been employed for hepatic insulin gene therapy applications to not only reduce the potential for hypoglycemia but also to improve targeting to the tissue of choice. For instance, several groups have used liver-specific promoters that activate insulin transgene expression in hepatocytes but remain inactive in other cell types. Interestingly, some liver-specific promoters are inherently glucose-responsive, making them a great choice for insulin gene therapy applications. For instance, Chen *et al.* used the glucose-6-phosphatase (G6Pase) promoter to drive insulin expression and found that elevated glucose concentrations enhanced promoter activity [48]. They also found that insulin strongly inhibited G6Pase promoter activity under low glucose conditions, creating a system with feedback inhibition [49]. The group then delivered the insulin gene to the liver of STZ-induced diabetic rats under the control of the G6Pase promoter and found that *ad libitum* hyperglycemia was significantly reduced, glucose utilization was accelerated after glucose challenge, and fasting glucose levels were within a normal range without hypoglycemia. Similarly, Burkhardt *et al.* used the liver-specific GLUT2 promoter to drive insulin gene expression in a glucose-inducible but insulin-repressive fashion and found an improvement in diabetic hyperglycemia [50]. However, it is worth noting that the activity of the wild-type insulin promoter used by native β cells is actually enhanced by insulin, creating a feed-forward system to amplify insulin expression.

To generate a more physiologically mimetic system driving insulin expression, Hsu and coworkers used the rat insulin-1 promoter, creating a system that is activated by both glucose and insulin, similar to native β cells [51]. In so doing, they were successful at driving insulin secretion from Huh7 hepatoma cells *in vitro* in response to glucose. They were also able to augment insulin expression *in vivo* in response to glucose and theophylline—a pharmacological activator of cAMP—and ameliorate hyperglycemia in STZ-induced diabetic mice. However, they did not test whether insulin activated transgene expression using this promoter,

and it is unclear how this promoter was active in hepatocytes, which do not ordinarily express the β cell-specific transcription factors necessary to upregulate insulin expression. Regardless, hepatocytes do not possess enough of the β cell-specific regulatory mechanisms to safely express insulin in a feed-forward manner with respect to insulin. Thus, for the sake of hepatic insulin gene therapy, it would be simpler to create a system that was unresponsive to insulin altogether.

Even if a liver-specific promoter does not possess glucose responsiveness, it can be endowed with sensitivity to glucose through incorporation of GIREs. GIREs are glucose-responsive DNA sequences found in the promoter region of several genes encoding lipogenic enzymes, like L-pyruvate kinase (L-PK), S_{14} , fatty acid synthase, and acetyl-CoA carboxylase [52]. GIREs are composed of two 6-bp motifs known as E boxes, with a consensus sequence of CACGTGnnnnnCACGTG (**Figure 1**). E boxes are generally recognized by transcription factors harboring basic helix-loop-helix/leucine zipper DNA-binding domains [53]. A specific transcription factor, dubbed carbohydrate response element-binding protein (ChREBP), has been found in great abundance in the liver, as well as the small intestine and adipose tissue, the most active sites of *de novo* lipogenesis [54].

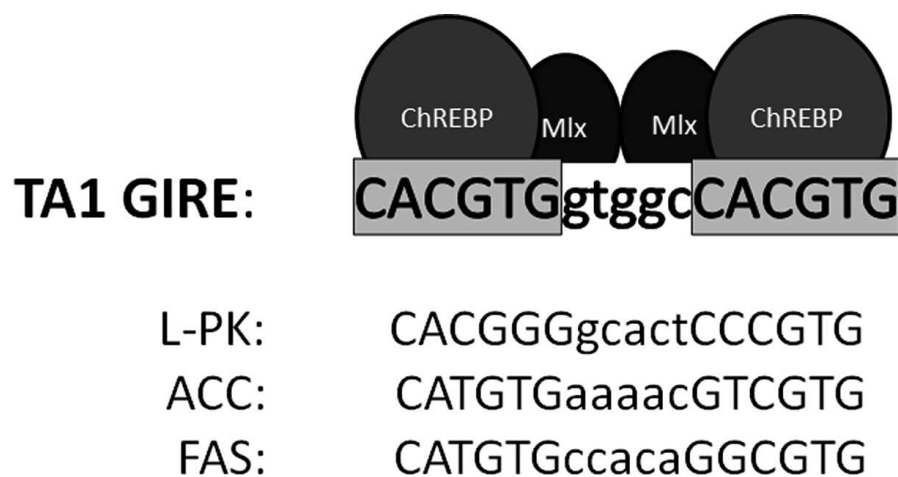


Figure 1. Glucose-inducible response elements (GIREs) and their transcriptional activators.

Incorporation of GIREs enables glucose-responsive control of gene transcription. GIREs are composed of two 6-bp motifs with a consensus sequence of CACGTG separated by 5 bp. A tetramer of ChREBP-Mlx binds each GIRE to amplify gene transcription in response to elevated glucose levels. GIREs have been identified in liver-specific genes like L-pyruvate kinase (L-PK), acetyl-CoA carboxylase (ACC), and fatty acid synthase (FAS).

Thule and colleagues leveraged GIREs to endow glucose-responsiveness to the liver-specific insulin-like growth factor binding protein-1 (IGFBP1) promoter. The IGFBP1 promoter is repressed by insulin, creating a feedback inhibition loop on insulin expression, but it is not inherently influenced by changes in glucose concentrations. To generate glucose-responsive insulin expression, they incorporated GIREs from the L-PK gene directly upstream of the IGFBP1 promoter and found that, depending on the number of GIREs incorporated, a 1.6- to

6.4-fold increase in promoter activity could be produced in response to elevated glucose concentrations in primary hepatocytes *in vitro* [55]. In addition, Thule and Liu used a recombinant adenovirus to deliver their glucose-responsive, insulin-repressive insulin construct into STZ-induced diabetic rats and found that it was able to produce near-normal glycemia and weight gain without inducing lethal hypoglycemia [56].

In our lab, we used the liver-specific albumin promoter—which is neither glucose- nor insulin-responsive—and inserted the GIREs from the *S*₁₄ gene upstream of the albumin promoter to create a system that is unresponsive to insulin but activated by elevated glucose levels (Figure 2) [57]. To test the effect that the *S*₁₄ GIREs have on glucose-responsive insulin output from the albumin promoter, we first generated insulin expression cassettes containing one to five GIREs. Interestingly, we found that the degree of glucose-induction on insulin output increased as the number of GIREs was increased up to three, after which there was only a marginal enhancement in insulin output. We observed a 9-fold increase in insulin output from primary hepatocytes between low and high glucose conditions when three GIREs were incorporated upstream of the albumin promoter (Figure 3). When we delivered this insulin expression cassette into the livers of STZ-induced diabetic rats through direct injection, we found that fasting blood glucose levels were reduced to normal, blood glucose levels of diabetic rats fed *ad libitum* were significantly reduced, and glucose clearance was significantly accelerated during an intraperitoneal glucose tolerance test. However, these effects only lasted for roughly a month, owing to the use of adenoviral vectors to deliver our insulin expression cassette.

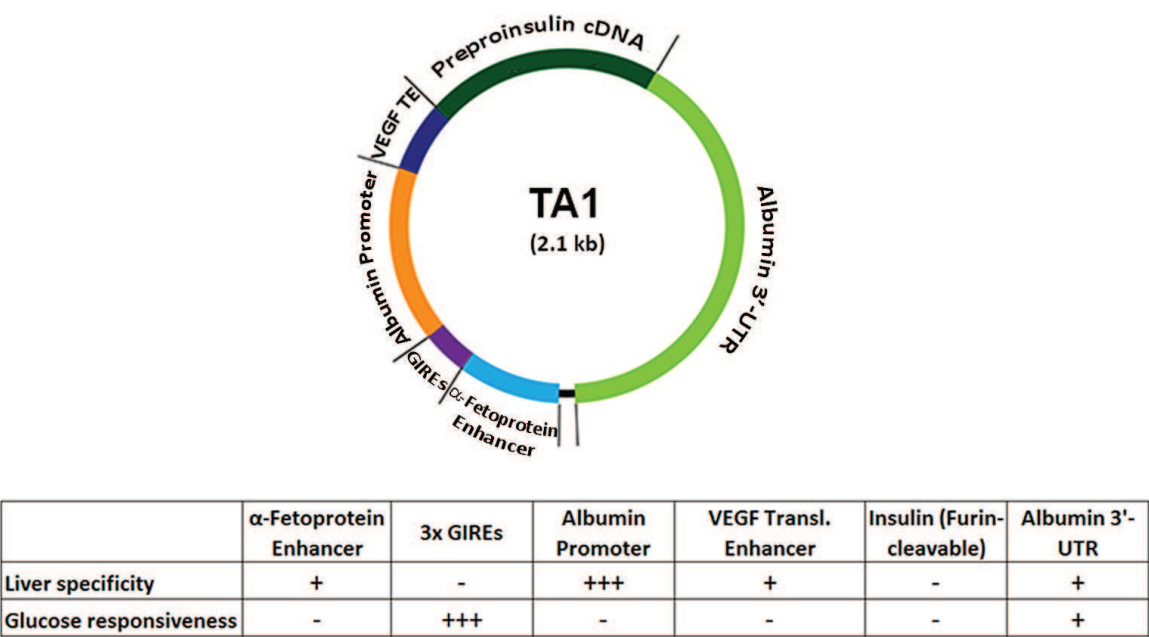


Figure 2. Elements of the insulin expression cassette—TA1.

TA1 is a 2.1-kb insulin expression cassette containing elements that drive insulin expression in both a liver-specific and glucose-responsive fashion. The albumin promoter is largely

responsible for restricting insulin expression to hepatocytes, while the α -fetoprotein transcriptional enhancer, vascular endothelial growth factor (VEGF) translational enhancer, and albumin 3'-UTR also promote liver specificity. Glucose responsiveness is primarily driven by three copies of GIREs, although the albumin 3'-UTR also promotes glucose-responsive insulin biosynthesis.

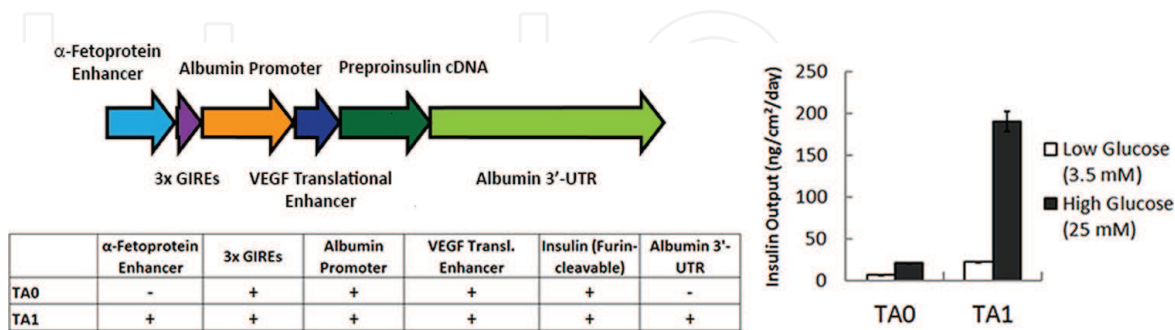


Figure 3. Glucose induction of insulin expression and the effect of hepatocyte-specific enhancer elements on overall insulin output.

The insulin expression cassettes, TA0 and TA1, differ in the presence of α -fetoprotein transcriptional enhancer and the albumin 3'-UTR. Inclusion of these elements greatly increases overall insulin output, while both constructs display similar glucose responsiveness.

To improve our insulin expression cassette, we investigated whether inclusion of various liver-specific enhancer elements could further enhance insulin production. Specifically, we incorporated a transcriptional enhancer from the human α -fetoprotein gene, an intron from the human growth hormone gene previously shown to improve mRNA processing efficiency, a translational enhancer from the vascular endothelial growth factor (VEGF) gene that functions as an internal ribosomal entry site, and the 3'-UTR from the human albumin gene that also contains an intron to improve mRNA processing (**Figure 3**). The ability of each element to enhance glucose-inducible insulin expression was first examined by transducing primary rat hepatocytes *in vitro* using an adenovirus. After testing different constructs containing a variety of different combinations of these elements, we ultimately found that insulin expression cassettes incorporating the α -fetoprotein transcriptional enhancer, VEGF translational enhancer, and albumin 3'-UTR led to increased insulin production *ex vivo*. Specifically, the VEGF translational enhancer led to a 4- to 6-fold increase in insulin output alone at both low and high glucose concentrations. Incorporation of the transcriptional enhancer and 3'-UTR led to another 5- to 8-fold increase in insulin output (**Figure 3**). Together, these modifications to the insulin expression cassette resulted in a 20- to 50-fold increase in insulin output *in vitro* compared to the original constructs, thus allowing us to more efficiently drive insulin expression, a particularly important factor when less efficient delivery vehicles must be used for gene therapy.

We confirmed the utility of this improved insulin construct *in vivo* by delivering our insulin expression cassette in the form of minicircle DNA, which can be readily produced in large quantities. Upon intravenous injection of this minicircle DNA into STZ-induced diabetic rats,

we observed a DNA dose-dependent correction in hyperglycemia in both fasted rats and rats fed *ad libitum*. In addition, we observed a full restoration in the rate of weight gain in STZ-induced diabetic rats comparable to that of healthy, non-diabetic rats, and intraperitoneal glucose tolerance tests demonstrated glucose-inducible increases in insulin production capable of correcting hyperglycemia within 45 minutes. A single injection of minicircle DNA led to normalization of serum levels of albumin, triglycerides, cholesterol, aspartate transaminase, alanine aminotransferase, and alkaline phosphatase, thus demonstrating restoration of healthy liver function. Further, there were no signs of hepatic inflammation, underscoring the safety of hepatic insulin gene therapy. Together, we were able to create a treatment for T1DM possessing glucose-responsive insulin production (due to the natural expression of GLUT2 and glucokinase from hepatocytes, and the presence of GIREs in the insulin expression cassette) that is capable of fully correcting diabetic hyperglycemia.

Another novel feature of our insulin expression cassette is the presence of the albumin 3'-UTR. As mentioned previously, the albumin 3'-UTR contains an intron that improves mRNA processing. However, in addition to that, it also contains two pyrimidine-rich stretches known to bind PTB [58]. PTB is a ubiquitously expressed mRNA binding protein that serves as a common mediator of mRNA stability. As mentioned previously, PTB binding sequences are also found in the 3'-UTR of the preproinsulin gene. This is a particularly important feature for hepatic insulin gene therapy applications, as the half-life of preproinsulin mRNA has been reported to be less than 6 hours in hepatocytes. That is much less than the 29–77 hours found in β cells. Perhaps even more importantly, the presence of PTB binding sites would also confer glucose-responsive control of preproinsulin mRNA translation in hepatocytes [34]. Thus, the presence of the albumin 3'-UTR endows hepatocyte-derived surrogate β cells with improved mRNA processing and stability, as well as glucose-responsive control of translation.

One final consideration when designing an expression cassette for insulin gene therapy is the preproinsulin sequence used. A mature insulin molecule is composed of two polypeptide chains—the A and B chains—linked together by two disulfide bonds. However, the insulin gene produces a single preproinsulin polypeptide that contains two basic pairs of amino acids separating the A and B chains, known as the C-peptide, as well as a 24-residue signal peptide. The signal peptide is removed as preproinsulin is translocated into the rough endoplasmic reticulum, forming proinsulin. Proinsulin undergoes further maturation within secretory granules through the action of prohormone convertases PC1/3 and PC2, as well as carboxypeptidase-H. These enzymes are co-packaged with proinsulin in secretory granules and together act to remove C-peptide and produce mature insulin. However, prohormone convertases PC1/3 and PC2 are only found in β cells and other cells with the regulated secretory pathway, like pituitary cells and intestinal K cells. Thus, for insulin gene therapy applications to be successful, it is important to maintain proinsulin processing, even if researchers choose to target cell types that do not have the regulated secretory pathway, like hepatocytes. In these instances, modifications can be made to the preproinsulin sequence to bypass the necessity of PC1/3 and PC2. The most commonly used modification is incorporation of furin cleavage sites [59, 60]. Furin is a ubiquitously expressed endoprotease that can efficiently cleave proteins at

paired basic amino acid sites. Through incorporation of furin cleavage sites, any cell of the body can produce fully functional insulin.

Further modifications can be made to the preproinsulin sequence to enhance bioactivity or production for insulin gene therapy applications. First, the preproinsulin sequence can be mutated to alter the stability of the resulting insulin molecules. The most prevalently used mutation is the B10 mutation—a naturally occurring mutation where the histidine residue at position 10 on the B chain is replaced by aspartic acid [59, 61]. This mutation results in enhanced stability and the accumulation of 10- to 100-fold more mature insulin than wild-type insulin. Other mutations have been found to result in highly potent insulin analogues, including HisA8, ArgA8, and GluB10 [61].

Another way the preproinsulin sequence can be modified is through codon optimization. A codon is a series of three nucleotides that encode a specific amino acid. There are 64 different codons but only 20 different amino acids, which means that many amino acids are encoded by multiple codons. It is generally acknowledged that different organisms have codon preferences as a result of the composition of their respective tRNA pool. In other words, specific codons are preferred by specific organisms because they have that specific tRNA in greater abundance. It is thought that cDNA sequences with optimized codons will achieve faster rates of translation and accuracy, thus improving translational efficiency and production of the transgene product. For gene therapy applications, this has been shown to improve the potency of treatment. For instance, Cantore *et al.* observed a 2- to 3-fold increase in potency of their factor IX treatment for hemophilia B in dogs upon codon optimization [62]. Codon-optimized versions of human insulin have also been shown to achieve better glycemic control in diabetic dogs due to increases in insulin production [44].

In summary, there is some flexibility in the design of the expression cassette for insulin gene therapy applications; the relatively small size of the preproinsulin gene is advantageous for the design of an expression cassette and its subsequent delivery to target cells. Regulatory elements capable of improving cell type specificity, overall insulin output, and glucose responsiveness can be employed to yield insulin expression with greater precision. In addition, the preproinsulin sequence itself can be modified to improve production and functionality. Once a sufficient level of control has been attained over insulin expression, it then becomes a matter of delivering the expression cassette to target cells in a safe and efficient manner.

2.5. Delivery vehicles for insulin gene therapy

When choosing a delivery vehicle for insulin gene therapy applications, two important considerations must be taken into account. First, the delivery vehicle must be able to promote long-term insulin expression. This is important because an antibody response from the initial treatment will reduce the efficacy of subsequent treatments. Thus, repeated administration of most delivery vehicles is largely ineffective, especially if it occurs more than two weeks after the initial treatment. Second, it must be possible to affordably and reliably produce the gene delivery vehicle in the large quantities needed for gene therapy. This is particularly important in the field of insulin gene therapy because the insulin molecule has a relatively short circu-

lating half-life, estimated at around 4–6 minutes [63]. As a result, a greater number of cells must be targeted for insulin expression than other gene therapy applications.

Many gene delivery vehicles exist and can be broadly grouped into two categories: viral and non-viral. Non-viral methods have the advantages of being safer and inducing less of an immune response. However, non-viral methods typically only drive transgene expression transiently, as most are incapable of supporting chromosomal integration. In addition, they tend to deliver genes inefficiently *in vivo*. Regardless, we explored the potential of delivering the insulin gene in the form of minicircle DNA to validate the *in vivo* efficacy of our expression cassette. We chose to use minicircle DNA because they can be produced in large quantities and contain no bacterial or viral elements, improving their likelihood of evading the immune system. We found that delivering our insulin expression cassette as a minicircle was able to correct diabetic hyperglycemia in a dose-dependent fashion in STZ-induced diabetic rats (Figure 4). While the effects of this treatment only persisted for about a month, the reduced immunogenicity of minicircle DNA allowed for repeated administration, although the second injection was not nearly as effective as the first (Figure 5) [64].

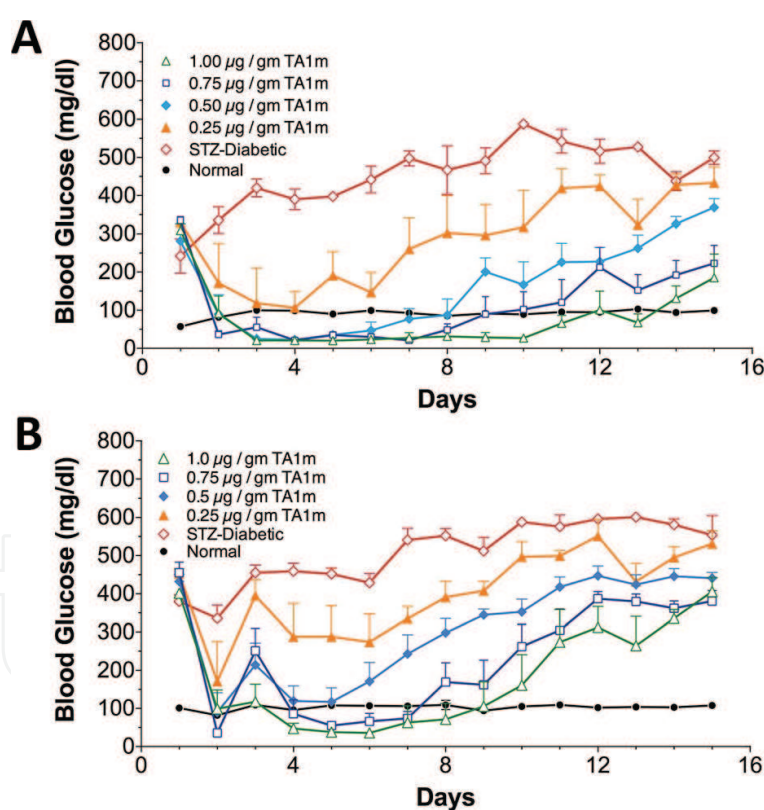


Figure 4. Dose-dependent effect of insulin minicircle DNA treatment on hyperglycemia in rats.

STZ-induced diabetic rats were intravenously injected with the indicated dose of TA1m minicircle DNA and measured for both fasting (A) and *ad libitum* (B) blood glucose levels. There was a dose-dependent correction of diabetic hyperglycemia that persisted for at least 10 days.

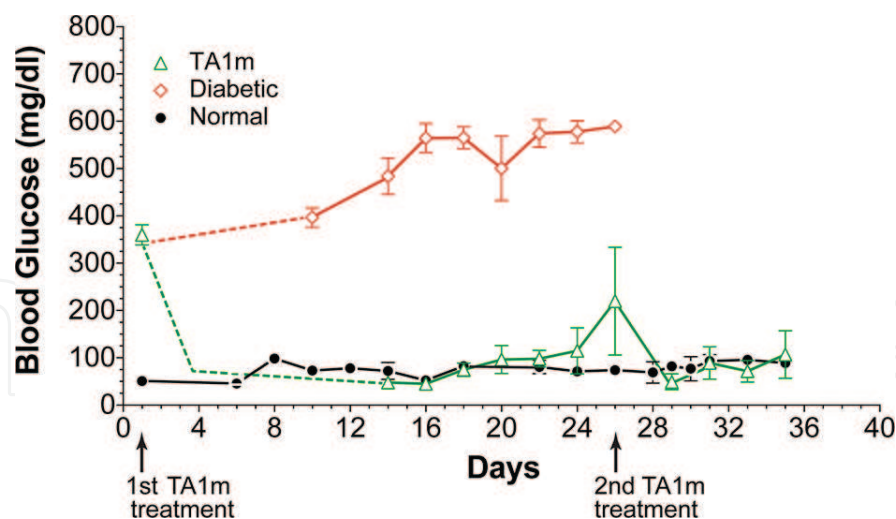


Figure 5. Effect of repeated administration of insulin minicircle DNA on fasting hyperglycemia in diabetic rats.

STZ-induced diabetic rats were intravenously injected with 0.8 $\mu\text{g/gm}$ body weight of TA1m via tail vein, and blood glucose measurements were made after a 4-hour fast. TA1m was able to correct diabetic hyperglycemia for nearly a month before the effects began to diminish. A second TA1m injection (0.8 $\mu\text{g/gm}$ body weight) was made on the 26th day and was able to re-correct fasting blood glucose levels, thus demonstrating that a substantial humoral response had not been mounted against the minicircle DNA.

We have also explored the use of viral delivery vehicles, as viruses are highly evolved and proficient at delivering genetic information into target cells. Of course, viral vectors will inevitably elicit an immune response, with some viruses invoking a greater immune response than others. Several viral vectors have been used for insulin gene therapy, with each possessing their own unique features. Refer to **Table 1** for an overview of the features inherent to various viral vectors.

| Viral vector | Packaging capacity | Length of expression | Relative viral titer | Transduction efficiency | Infect both dividing and non-dividing cells | Immunogenicity |
|------------------------|--------------------|----------------------|----------------------|-------------------------|---|----------------|
| Adenovirus | 7.5 kb | Transient | +++ | +++ | Yes | High |
| Adeno-associated virus | 4.5 kb | Transient and Stable | ++ | ++ | Yes | Low |
| Oncoretrovirus | 8 kb | Stable | + | + | No | Moderate |
| Lentivirus | 8 kb | Stable | + | ++ | Yes | Low |

Table 1. Features of various viral vectors for gene therapy applications.

Adenovirus, adeno-associated virus, oncoretrovirus, and lentivirus are the most commonly used delivery vehicles for gene therapy applications. Each viral vector possesses their own unique features that affects their suitability for insulin gene therapy applications.

Adenoviruses were among the first viral vectors used in the field of insulin gene therapy due to their ability to be produced in very high titers and transduce non-dividing cells with high efficiency. These features allow researchers to transduce a large number of cells *in vivo* and overcome the lack of insulin protein stability. However, adenoviral vectors are unable to integrate their genetic cargo into the host's genome and thus provide only transient gene expression [65, 66]. As a proof-of-principle, we initially used adenoviral vectors to establish the efficacy of our insulin expression cassette *in vivo*. Indeed, we were successful at correcting fasting blood glucose levels and improving *ad libitum* glucose levels in diabetic rats (**Figure 6**). As expected, however, the observed reduction in blood glucose levels only persisted for about a month. This observation is in agreement with other studies using adenoviral vectors to deliver the insulin gene into diabetic animals, which likewise noted an improvement of hyperglycemia for only a month [67]. Unfortunately, unlike minicircle DNA, a humoral response is elicited by the first treatment, precluding repeated administration of adenovirus-based treatments. Thus, while adenoviral vectors are extremely efficient gene delivery tools, they are not well suited as a long-term therapeutic tool for treatment of T1DM, at least in their initial form.

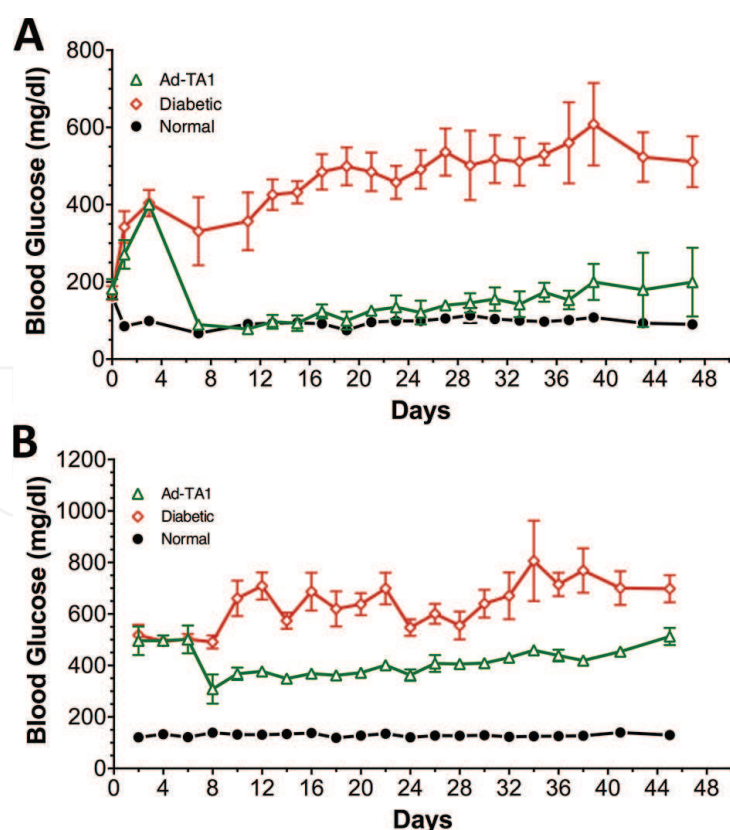


Figure 6. Effect of TA1 on hyperglycemia in diabetic rats when delivered via adenovirus.

STZ-induced diabetic rats were injected with 2×10^{10} adenoviral pfu/rat, and both fasting (A) and *ad libitum* (B) blood glucose measurements were made. Treatment with adenoviral vectors carrying the TA1 insulin expression cassette were able to fully correct diabetic hyperglycemia after an overnight fast and partially correct *ad libitum* blood glucose levels for around one month.

More recently, researchers have generated a newer version of “guttated” adenoviral vector that has been stripped of all viral coding sequences, greatly reducing their immunogenicity [68, 69]. This is an important advancement in the field of gene therapy because adenoviral vectors have proven so immunogenic in past human clinical trials that their administration led to the death of a patient in 1999, temporarily halting progress in the field of gene therapy [70, 71]. Immunogenicity is undoubtedly a very large concern with adenoviral vectors, so any improvement is useful. However, it seems unlikely that immunogenicity will ever be completely eliminated from adenoviral vectors, or any viral vector for that matter. Further advancements have now made it possible to improve upon the innate capabilities of adenoviral vectors by adding the potential to integrate their genetic cargo into a host's genome and drive long-term transgene expression. The advancement was a result of the merging of two technologies, where chromosomal integration is mediated by the Sleeping Beauty (SB) transposon system and efficient gene delivery accomplished by the gutted adenoviral vectors. DNA transposons translocate from one DNA site to another through a simple cutting-and-pasting process, enabling the integration of defined DNA sequences into mammalian genomes [72]. To achieve stable transgene expression using this system, two separate adenoviral vectors must be administered and co-transduce a single cell. The first vector represents the transposon donor vector, which contains a transposon encoding the transgene of interest. The second vector encodes the SB transposase, which mediates relocation. This system has been used successfully to enable persistent phenotypic correction of hemophilia B in dogs [72] and holds great potential for the treatment of other diseases that require persistent gene expression.

To combat issues related to immunogenicity and short-term expression, other groups have employed adeno-associated virus (AAVs). AAVs are able to transduce both dividing and non-dividing cells. In dividing cells, AAVs are able to integrate transgenes into the host's genome at a specific site on chromosome 19 [73]. Within non-dividing cells, the AAV genetic cargo remains largely episomal, as the chromatin is less accessible. AAVs are less immunogenic than adenoviral vectors and are reported to cause relatively long-term transgene expression in non-dividing cells. The primary disadvantage of using AAVs is that their packaging capacity is less than 5 kb, limiting the use of larger expression cassettes with greater complexity for regulated expression. However, the preproinsulin gene is quite small, so even when the gene is accompanied with multiple regulatory elements in a complex expression cassette, the maximum size limitation is unlikely to become an issue for insulin gene therapy applications. Indeed, AAVs have been used to successfully drive insulin expression within non- β cells. Park *et al.* used AAV to deliver insulin under control of the CMV promoter into STZ-induced diabetic rats and found improved glucose tolerance (at 2 g/kg) comparable to that of non-diabetic control rats [74]. Additionally, they observed a less pronounced immune response using AAV when compared to the same treatment using adenoviral vectors.

Retroviral vectors are another widely used gene delivery vehicle, owing to their ability to integrate their genetic cargo into a host's genome and attain sustained gene expression. However, retroviral vectors are greatly limited by their inability to integrate their cargo into the chromosomes of non-dividing cells, a problem that severely hinders their utility in insulin gene therapy applications. In cases where retroviral vectors have been used to deliver insulin, hepatocyte proliferation must first be stimulated [75]. This, of course, greatly limits the translation of retroviral vectors for treatment of T1DM. Retroviral vectors have also been shown to have a preference to integrate their genetic cargo in close proximity to the transcriptional regulatory sequences of proto-oncogenes, as observed in 1999 following the treatment of nine severe-combined immunodeficiency patients [76]. Insertional mutagenesis led to the development of leukemia in four of the nine patients, ultimately halting the field of clinical gene therapy temporarily. All in all, retroviral vectors do not possess favorable features for insulin gene therapy applications.

Lentiviral vectors are a type of retrovirus that provide two key advantages over other retroviruses: (1) they are able to integrate their genetic cargo into the genome of both dividing and non-dividing cells and (2) have less preference to integrate near regulatory sequences of proto-oncogenes, reducing the risk of insertional mutagenesis [77, 78]. Their ability to transduce non-dividing cells is critical for gene therapy strategies, as most cells of the body are either non-dividing or slowly dividing. An additional advantage of lentiviral vectors is that they do not elicit a strong immune response. Unfortunately, lentiviral vectors possess two major pitfalls limiting their widespread translation to human clinical trials: (1) Lentiviral vectors are difficult to produce in high titer [79] and (2) the efficiency of lentiviral transduction *in vivo* is significantly lower than other vectors, especially adenoviral vectors. Given the relative instability of insulin, the need to transduce a larger number of cells than other gene therapy applications, and the fact that—unlike other diseases—a partial correction of hyperglycemia is not sufficient to adequately treat T1DM, these pitfalls pose some limitations to the use of lentiviral vectors for insulin gene therapy applications. Nonetheless, lentiviral vectors offer long-term transgene expression with reduced immunogenicity and an improved biosafety profile and are thus a viable candidate as a therapeutic tool for treatment of T1DM.

To combat issues related to lentivirus infectivity, researchers have modified lentiviral vectors to improve their *in vivo* efficacy. For example, Naldini *et al.* has previously shown that inclusion of viral protein R—a viral protein present in native HIV-1 particles—within synthetic lentiviral particles is critical for hepatocyte transduction [80]. Conversely, Schaffer *et al.* has generated vesicular stomatitis virus envelope proteins that show improved serum resistance [81], which could improve lentiviral efficacy *in vivo*. A combination of modifications will hopefully yield a more potent lentiviral particle *in vivo*. Interestingly, Ren and colleagues used lentiviral vectors to deliver insulin to the livers of STZ-induced diabetic rats [82] and NOD mice [83] and observed long-term correction of diabetic hyperglycemia with no evidence of impaired liver function, intrahepatic inflammation, or recurring autoimmunity against the newly formed insulin-producing cells. While their work gives validity to the use of lentiviral vectors for insulin gene therapy applications, it should be noted that insulin expression was driven by the CMV promoter and displayed no responsiveness to circulating glucose levels.

3. Concluding remarks

Overall, insulin gene therapy provides a promising alternative to current treatments for T1DM. Although this treatment option will inevitably lack the full sophistication of native β cells, it would certainly improve upon current treatment options. Insulin gene therapy opens the possibility of having a one-time treatment option that can provide long-term correction of diabetic hyperglycemia through physiologically relevant mechanisms, like glucose-dependent alterations in insulin transcription and translation. Further, the simplicity of the treatment should yield reproducible results with excellent success rates and additionally help newly-formed insulin-producing surrogate β cells evade recurring autoimmunity. However, several hurdles must still be overcome.

In order for the treatment to be a viable option, long-term insulin expression must be driven to avoid repeated injection. However, the viral vectors currently used to drive long-term transgene expression, like lentivirus, are generally difficult to produce in high titer and limited by their transduction efficiency. As a result, most successful long-term efforts in the field have employed the CMV promoter, which can only drive strong constitutive expression of insulin. In order to take those research efforts to the next level, weaker tissue-specific promoters must be used that drive low basal levels of insulin expression during fasting periods and substantially upregulated insulin expression upon increases in glucose availability. To date, this has yet to be achieved. We are currently exploring several viral vectors for their capacity to deliver our insulin expression cassette—which has elements to drive liver-specific, glucose-responsive insulin expression—at a therapeutic level. In so doing, we hope to produce an affordable, long-term treatment option for patients with T1DM.

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