Advances and potential of gene therapy for type 2 diabetes mellitus

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ABSTRACT
Gene therapy of type 2 diabetes mellitus (T2DM) and its complications has attracted intensive interest in recent years. In this paper, we critically review literature reports on gene therapy of T2DM published over the last five years. By reviewing these advances, three questions were addressed. First, which genes and how do they exert anti-diabetic effects? Target genes including those regulating glucose homeostasis, improving insulin secretion or sensitivity, and ameliorating diabetic induced complications are summarized. Second, how to deliver and which route is advantageous? All main methods that have been used for delivery of target genes into diabetic subjects are outlined and discussed regarding their pros and cons. Last, what are the future directions and how do these advances promote the study of T2DM gene therapy? Testing of novel targets in a parallel way and especially, using combinational approaches may be the main directions. In conclusion, there are a large number of genes playing important roles during the incidence and development of T2DM, and many of them hold great promise as potential targets for gene therapy. Oral delivery of target genes by probiotics may be a nice route with priority to develop, due to its high efficiency and safety for future gene therapy of T2DM.

Introduction to T2DM and gene therapy
Type 2 diabetes mellitus (T2DM) is a chronic metabolic disease characterized by hyperglycaemia resulting from insufficient insulin production, as well as insulin resistance [1]. Data from the International Diabetes Federation (IDF) show that over 90% of patients among 425 million diabetic adults worldwide suffered from T2DM in 2017, and 352 million people had impaired glucose tolerance (IGT) [2]. In T2DM patients, there are not only elevated glucose levels, but also multiple potential complications, such as heart attack, stroke, blindness, kidney failure, and lower limb amputation [3]. Epidemiological studies have proved that T2DM is a multifactorial disease involving both genetic and environmental factors [4]. These risk factors and the genetic susceptibility make T2DM complicated to cure [5].

Currently, oral administration of hypoglycaemic agents and injection of insulin (like) agents are the main therapeutic strategies for T2DM. Although these agents play important roles in T2DM therapy, they still have various adverse effects [6–8]. Therefore, new alternative strategies for T2DM treatment, including gene therapy, have been studied [9]. Gene therapy is a strategy correcting or compensating the symptoms of diseases caused by defective or abnormal genes through introduction of exogenous normal genes. Gene therapy has the advantage that diseases can be potentially cured by a single treatment, and it is now bringing new treatment options to multiple fields of medicine [10]. Nowadays, the manipulation of genetic material is not restricted to gene addition but also to gene regulation and editing. Gene therapy for diabetes is not a new idea, and this topic was reviewed several years ago [11,12]. Therefore, in this paper we make an updated mini-review of the advances over the past five years focussing on T2DM gene therapy, from gene targets to delivery routes.

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Potential targets for T2DM gene therapy

Although gene therapy for diabetes mainly focuses on T1DM, several genes have been investigated for potential therapy for T2DM, as the disease has a strong genetic predisposition [13]. Genetic association studies have identified at least 75 independent genetic loci for T2DM and various new therapeutic targets have been determined [14]. For example, three novel mutations in gene KCNJ11 are associated with the development of autosomal dominant, early-onset T2DM [15]. Contrasting with their limited effects on disease incidence and development, genetic loci may have much stronger effects on drug response [16]. A large number of genetic polymorphisms affect the response to oral anti-diabetic drugs. Genes encoding SLC2A2 and organic cation transporters (OCT1, OCT2, and OCT3) are associated with glycemic response to metformin [17–19], while SLCO1B3 and KCNQ are associated with sulfonylurea response [20,21]. However, these genes have little applicability in therapy due to the different polymorphisms among different populations.

However, there are still several genetic loci with potential for T2DM gene therapy. One good example is nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3). Inhibition of gene NLRP3 attenuates inflammation, protects pancreatic β-cells from apoptosis, and prevents T2DM development in mice [22]. Theoretically, all genes involved in the onset, development, and deterioration of T2DM, have potential as targets. For simplicity, we will address the genes (Table 1) that regulate glucose homeostasis, improve insulin secretion or/and sensitivity, and ameliorate diabetic induced complications in the following sections.

Genes regulating glucose homeostasis

Glucose transporters (GLUTs) and sodium-glucose co-transporters (SGLTs)

Glucose transporters (GLUTs) play a fundamental role in the muscle and liver glucose fluxes. GLUT4 (Slic2a4 gene) and GLUT2 (Slic2a2 gene) are potential targets for T2DM gene therapy, as restoring their expression in muscle and liver improves glycemic control [23]. Sodium-glucose co-transporters (SGLTs) are responsible for the tubular reabsorption of filtered glucose from the kidney into the bloodstream. As found in human kidney biopsy specimens, the expression of SGLT1 is markedly increased in patients with T2DM, and SGLT1 mRNA is highly and significantly correlated with fasting and postprandial plasma glucose and HbA1c. In contrast, the levels of SGLT2 and GLUT2 mRNA are down-regulated in diabetic patients [24]. Therefore, inhibition of SGLT1 and/or enhancement of SGLT2 may be an effective strategy to alleviate hyperglycaemia in patients with T2DM.

Fibroblast growth factors (FGFs)

Fibroblast growth factors are a class of potential targets studied in relation to gene therapy of T2DM. Several FGFs, such as FGF1, FGF19, and FGF21, play significant roles in glucose homeostasis [25]. FGF1 knockout mice showed diabetic phenotype with increased blood glucose and insulin resistance. Non-ventricular injection of recombinant murine FGF1

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**Table 1. Potential targets can be used for T2DM gene therapy.**

| Class                          | Gene(s)          | Main function                                                                 | Reference |
|-------------------------------|------------------|-------------------------------------------------------------------------------|-----------|
| Genes regulating glucose homeostasis | GLUTs            | Re-absorption of filtered glucose from the kidney into the bloodstream        | [23]      |
|                               | SGLTs            | Play a fundamental role in the muscle and liver glucose fluxes                | [24]      |
|                               | FGFs             | Play significant roles in glucose homeostasis                                 | [25]      |
|                               | SIRT6            | Associated with increased glycolysis and GLUTs expression                     | [33]      |
| Genes improving insulin secretion and/or sensitivity | GLP-1 and its analogs/agonists | Increase beta-cell survival, stimulate insulin gene expression, and secretion | [35]      |
|                               | GPGRs and their agonists | Stimulate insulin and GLP-1 secretion                                         | [36]      |
|                               | CTB-APSL         | Promotes insulin secretion and insulin resistance                             | [40]      |
|                               | IRK c, TBK1      | Associated with weight reduction, insulin resistance, fatty liver, and inflammation | [38]      |
| Genes ameliorating diabetic induced complications | IL-1β            | Associated with inflammation and β-cell failure                              | [39]      |
|                               | ADPN             | Ameliorates diabetic nephropathy                                              | [45]      |
|                               | TGF-α            | Plays a role in DKD associated with nephron reduction                          | [41]      |
|                               | NLRP3            | Ameliorates diabetic cardiomyopathy                                           | [47]      |
|                               | CDKN2A/2B        | Associated with T-cell phenotype modulation and chronic inflammation          | [43]      |
|                               | HSP70            | Associated with mitochondrial bioenergetics and diabetic sensory neuropathy    | [44]      |
|                               | MicroRNAs        | Involved in regulating the diabetic microvasculature                           | [46]      |

**Note:** SGLTs, sodium-glucose co-transporters; GLUTs, glucose transporters; FGFs, fibroblast growth factors; SIRT6, Sirtuin 6; GLP-1, glycogen like peptide 1; GPGRs, G protein–coupled receptors; CTB-APSL, cholera toxin B subunit and active peptide from shark liver; ADPN, adiponectin; TGF-α, transforming growth factor-alpha; NLRP3, nucleotide-binding oligomerization domain-like receptor protein 3; DKD, diabetic kidney disease; HSP70, heat shock protein 70.
(rFGF1) in diabetic mice resulted in a normal glycaemia for more than two days and increased insulin sensitivity [26]. Moreover, single intracerebroventricular (ICV) injection of FGF1 brought glucose levels back to normal for at least 18 weeks without side effects such as hypoglycaemia and weight gain in diabetic mice [27]. In contrast, FGFR9 has a critical role in glycogen metabolism, and the anti-diabetic effect of FGFR9 has been confirmed to be related to the central nervous system. Injection (ICV) of FGFR9 ameliorates glycemic status and increases insulin sensitivity through suppression of hypothalamic AGRP/NPY neuron activity and the hypothalamic-pituitary-adrenal axis [28,29]. In addition, FGFR21 is involved in controlling glucose and lipid homeostasis. FGFR21 is elevated in patients with impaired glucose tolerance and progressively increases in patients with overt T2DM [30]. Mimetics targeting FGFR21 signalling have a beneficial effect on glycemic control [31].

Sirtuin 6 (SIRT6)
Recent studies revealed the critical role of SIRT6 in the pathophysiology of metabolic diseases like T2DM. These roles include promoting pancreatic insulin secretion, inhibiting hepatic gluconeogenesis and triglyceride synthesis, and suppressing adiposity [32]. SIRT6 has been investigated as a target for treating T2DM. Improvements in glucose tolerance, glycolysis, and the expression of glucose transporter GLUT1 and GLUT4 in skeletal muscle were achieved after inhibition of SIRT6 in mice [33]. However, muscle-specific SIRT6 knockout displayed impaired glucose homeostasis and insulin sensitivity. Mechanistically, deletion of SIRT6 in muscle decreased expression of genes involved in glucose and lipid uptake, fatty acid oxidation, and mitochondrial oxidative phosphorylation in muscle cells because of the reduced AMP-activated protein kinase (AMPK) activity. In contrast, over-expression of SIRT6 in C2C12 myotubes activates AMPK thereby regulating metabolic homeostasis [34].

Genes improving insulin secretion or sensitivity
Several genes are associated with insulin secretion and sensitivity. Glucagon-like peptide 1 (GLP1) and its analogues are incretins with such roles, for example, stimulation of glucose-dependent insulin secretion, increase in insulin gene expression and ß-cell survival [35]. G protein–coupled receptors (GPCRs) have also received attention in T2DM therapeutics, mainly the incretin receptors and the bile acid receptor GPBAR1 [36]. In fact, there are more than 30 GPCRs and many of them are directly involved in insulin resistance, ß-cell dysfunction, and the aetiology of inflammation that lead to obesity-induced T2DM [37]. Furthermore, inhibition of the inflammatory kinases IKKc and TBK1 in mice reduces weight, insulin resistance, fatty liver and inflammation, and improves insulin sensitivity and hepatic steatosis in T2DM patients [38]. A little differently, targeted immunization of the proinflammatory cytokine IL-1ß improves glucose tolerance and insulin sensitivity [39]. Oral administration of an exogenous fusion protein cholera toxin B subunit and an active peptide from shark liver (CTB-APSL) for five weeks promotes insulin secretion and insulin resistance in T2DM mice, suggesting that CTB-APSL is also a promising target for gene therapy [40].

Genes ameliorating diabetes-induced complications
T2DM has various complications that heavily affect the quality of life of patients. Diabetic kidney disease (DKD) is a common dysfunction in patients with T2DM and transforming growth factor alpha (TGF-α) is increased in serum of DKD subjects. Blockade of TGF-α attenuates nephron reduction in db/db mice thereby delaying kidney disease progression [41]. Diabetic cardiomyopathy (DCM) is triggered by metabolic disorder and cell death that is associated with NLRP3 inflammation. Silencing of gene NLRP3 ameliorates cardiac inflammation, pyroptosis, fibrosis, and cardiac function [42]. Progression of T2DM and T2DM-coronary artery disease (CAD) is accompanied by T-cell imbalance and chronic inflammation. CDKN2A/2B genes are associated with T-cell phenotype modulation, so these genes may restore immune cell homeostasis and delay disease progression [43]. Modulation of Hsp70 offers an effective approach to correcting sensory neuron bioenergetic deficits and diabetic peripheral neuropathy (DPN) in both T1DM and T2DM [44]. Delivery of the exogenous globular adiponectin (ADPN) gene ameliorates the progression of diabetic nephropathy (DN) [45]. In addition, microRNAs involved in a range of diabetes-associated complications such as retinopathy, nephropathy, wound healing, and myocardial injury, are potential targets for therapeutic gene intervention for T2DM and its complications [46].

Methods of gene delivery
The success of gene therapy requires suitable gene delivery systems or vectors to provide the therapeutic
Table 2. Gene therapy of T2DM in the last five years.

| Gene target | Delivery route/method | Treatment period | Main results | Reference |
|-------------|-----------------------|------------------|--------------|-----------|
| GLP-1       | OA/recombinant         | 11 hours         | Significant decrease ($p < 0.05$) in blood glucose levels during 2–11 hours post dosing and a significant increase in insulin levels | [60] |
| 5 × GLP-1   | OA/recombinant         | 16 days          | Has no effect on glucose levels | [61] |
| 10 × GLP-1  | OA/recombinant         | 16 days          | Ameliorates weight loss, decreases blood glucose levels | [62] |
| GLP-1       | OA/plasmid DNA encoding GLP-1 | 32 days   | Decreases diabetic glucose levels to the normoglycemic range with significant weight reduction | [65] |
| GLP-1       | IP/HIV based lentiviral vector encoding GLP-1 | 24 days   | Reduces blood glucose levels, improves insulin sensitivity and glucose tolerance, normalizes glycemia, and plasma triglyceride levels | [63] |
| Exendin-4   | Submandibular gland injection/dsAAV | 8 weeks   | Increases insulin levels, decreases blood glucose levels | [64] |
| NLRP3       | Jugular-vein injection/ NLRP3-miRNA | 8 weeks   | Gene silencing of NLRP3 ameliorates diabetic cardiomyopathy | [42] |
| ADPN        | IP/recombinant plasmid mediated by lipofectamine | 12 weeks | Ameliorates diabetic nephropathy, attenuates urine albumin excretion, reduces the generation of ROS, and prevents interstitial fibrosis | [45] |
| mGCK        | SC/adenovirus-based vector encoding mGCK | 8 weeks | Decreases blood glucose levels over a period of 12 and 70 days without inducing hypoglycemia | [66] |
| VIP         | IP/HIV based lentiviral vector encoding VIP | 4 weeks | Improves insulin sensitivity, glucose tolerance and reduces serum triglyceride/cholesterol levels | [67] |
| ANGPTL8     | UTMD delivery of human ANGPTL8 gene plasmids | 4 weeks | Promotes the proliferation of beta cells, improves glucose tolerance, and the fasting blood insulin level | [68] |
| FGF21       | IV/liver specific AAV encoding a murine optimized FGF21 | 69 weeks | Reduces body weight, adipose tissue hypertrophy and inflammation, hepatic steatosis, inflammation and fibrosis, and insulin resistance | [69] |

Note: ADPN, adiponectin; OA, oral administration; IP, intraperitoneal injection; SC, subcutaneous injection; IV, intravenous injection; dsAAV, double-stranded adeno-associated virus; FGF21, fibroblast growth factor 21; GLP-1, glucagon like peptide 1; mGCK, mutant glucokinase; NLRP3, nucleotide-binding oligomerization domain-like receptor protein 3; UTMD, ultrasound-targeted microbubble destruction; VIP, vasoactive intestinal peptide.

effect where needed [47]. Therefore, determination of delivery methods is as important as identification of gene targets. However, it is well-recognized that gene delivery technology is a major obstacle to the success of gene therapy at present [48]. Based on the vectors used for loading/delivering genes, these methods can be classified into viral gene delivery and non-viral gene delivery. Virus packaged genes can be either injected or orally administered exploiting the natural ability of viruses to enter cells and to transfer their genetic material to the nucleus and express proteins [49]. Previously, delivery systems based on non-viral vectors only included the direct administration of naked nucleic acids and the uses of different materials to ferry the genetic material into the target cell, such as liposomes or cationic polymers [50]. In recent years, delivery systems based on bacteria (mainly probiotics) have been quickly developed and orally applied, and provide more efficient alternatives to traditional non-viral delivery systems [51].

All the above-mentioned delivery methods have been tested in diabetic animals, and most of those developed before 2014 were based on viral vectors, including adenovirus and adeno-associated virus (AAV) [35]. The viral DNA integrates into the host cell genome, thus conferring stable therapeutic gene expression. However, viruses have wild tropism to transduce multiple cell types, and genetic manipulation of viruses is needed before the virus genome with the gene target is packaged [52]. Direct injection of naked DNA is of low immunity and low cost and is convenient for preparation, but the main barrier for its wide use is poor delivery efficiency and transient gene expression [53]. Administration of (nano-scale) DNA-material conjugates significantly improved delivery efficiency and stability but its expression and localization are still uncontrollable [54,55]. In contrast to the above delivery methods, gene delivery by bacteria is much more controllable and can reach high-level gene expression as host bacteria can be manipulated as live microbial cell factories for long-term production of targets [56].

Current advances in T2DM gene therapy

As mentioned in the gene targets and delivery methods section, there have been great advances in T2DM gene therapy over the last five years. Currently, studies on T2DM gene therapy mainly concern ameliorating insulin resistance in peripheral tissues, enhancing insulin production or increasing the functionality of β-cells. Here, we summarize some advances in gene therapy for T2DM in Table 2 and compare their designs and their main results.
Gene therapy with GLP-1 and its analogs

The majority of studies on gene therapy for T2DM are focussed on GLP-1 and its derivatives. GLP-1 is a short incretin hormone that secretes mainly from the proximal small intestine endocrine L cells. It is now used as a leading therapeutic drug for T2DM which modulates insulin secretion in a glucose-dependent manner, enhancing insulin sensitivity, suppressing glucagon secretion, and promoting pancreatic β-cell proliferation. However, the active forms of GLP-1, GLP-1(7–36) amide, and GLP-1(7–37), have a very short physiological half-life of less than 2 minutes. Different forms of human GLP-1 are expressed in different hosts but are still used as peptides and applied after protein purification [57–59].

Many groups have already investigated GLP-1 gene therapy for T2DM. Using probiotic Lactococcus lactis, GLP-1 gene was orally delivered into ZDF rats [60]. The therapy resulted in a significant decrease (p < 0.05) in blood glucose levels 2–11 hours after dosing and a significant increase in insulin (p < 0.01) compared with the free solution of GLP-1 peptide. A similar study, using Lactobacillus paracasei as host, orally delivered pentameric GLP-1 into diabetic Goto-Kakizaki (GK) rats. However, delivery of 5 × GLP-1 for 16 days had no added anti-diabetic effect, though L. paracasei itself significantly lowered the blood glucose level [61]. This result might be because of either the low level expression or incorrect enzymatic degradation of 5 × GLP-1 protein in vivo. In contrast, oral administration of recombinant Saccharomyces cerevisiae expressing 10 × GLP-1 for 16 days has shown less weight loss and lower glucose levels in T2DM rats [62].

Injection (IP) of lentiviral vector encoding GLP-1 not only reduces blood glucose and plasma triglyceride, but also improves insulin sensitivity and glucose tolerance for diabetes [63]. Apart from GLP-1, its derivatives, like Exendin-4, have been loaded into double-stranded AAV (dsAAV) and injected into the submandibular gland of diabetic rats. After 8 weeks of intervention, the levels of blood glucose were lower and insulin concentration was higher in the Exendin-4/ dsAAV group than in the control group [64]. Different from the above studies, oral administration of the plasmid DNA encoding GLP-1 decreased diabetic glucose levels to the normoglycemic range with significant weight loss [65]. To facilitate oral delivery, the plasmid was conjugated with a nano-sized complex coated with heparin-taurocholic acid (HTCA). This novel oral system delivers GLP-1 gene through the enterohepatic recycling pathways of bile acids.

Gene therapy with other novel targets

Other genes have also been delivered through different routes, and they were either over-expressed or inhibited in vivo to exert anticipated effects. For example, expression of exogenous ADPN by injection (IP) of recombinant plasmid mediated by lipofectamine ameliorated diabetic nephropathy [45]. Expression of a mutant glucokinase (mGCK), which is an enzyme critical for glucose metabolism, by injection (SC) of adenovirus-based vector resulted in steady decrease in blood glucose over a long period (12–70 days) without inducing hypoglycaemia [66]. Expression of vasoactive intestinal peptide (VIP) by injection (IP) of HIV-based lentiviral vector encoding VIP improved insulin sensitivity, glucose tolerance, and reduced serum triglyceride/cholesterol levels [67]. ANGPTL8 gene delivery by ultrasound-targeted micro-bubble destruction promoted β-cells proliferation, expanded the β-cell mass, improved glucose tolerance, and increased the fasting blood insulin level in a diabetic rat model [68]. Gene silencing of NLRP3 by jugular-vein injection of NLRP3-miRNA for 8 weeks ameliorated diabetic cardiomyopathy [42]. Moreover, a recent landmark study showed, excitingly, that FGF21 gene delivery by AAV resulted in reductions in body weight, adipose tissue hypertrophy and inflammation, hepatic steatosis, inflammation and fibrosis, and insulin resistance for more than 1 year after a single injection. Furthermore, this therapy prevented the increase in weight and insulin resistance in healthy animals, without side effects [69].

Future directions and potential of T2DM gene therapy

As summarized, there are many genes classified into different classes with potential for gene therapy (Table 1). However, current studies on gene therapy for T2DM are limited to a few targets and only in animals (Table 2). Therefore, the effects of other genes need to be investigated in the future. Crucially, parallel comparisons cannot be made due to the different designs of these reported gene therapies. Although GLP-1 is the most widely studied target for gene therapy for T2DM, contradictory results still exist [61,62]. Hence, we believe parallel studies should be carried out by more groups/centers as reproducibility is crucial for a therapy, for example, using the same gene target, the same delivery method, and the same animal models.

Gene therapy works through two different mechanisms: gain or loss of function achieved by over-expression or inhibition. Gene therapy by inhibition of targets
(e.g. microRNA) needs further study as it is little reported at present. The majority of studies obtained therapeutic effects by over expression of delivered gene targets. As known, heterogeneous over-expression is restrained by many factors, especially the expression vector. Therefore, more attention should be paid when designing vectors. Fusion is also important for many gene targets, as this may significantly improve their stability or accessibility in vivo [70,71].

Probiotics, especially many lactic acid bacteria, have many appealing features as delivery live vectors for biomedical purposes [72,73]. In fact, many animal studies have highlighted the great potential of probiotic intervention in the management of diabetes. Probiotics have many health-promoting properties including anti-diabetic properties, and there are different intervention approaches for T2DM using probiotics [74]. As noted, probiotic L. paracasei significantly lowers the blood glucose level [61]. Lactobacillus reuteri SDS865 significantly improves the secretion of GLPs, insulin and C-peptide [75]. Therefore, these strains may be of advantage when used as delivery hosts considering their beneficial effects on T2DM, especially during the early intervention stage. Also, other targets like FGF21 hold great promise for probiotic delivery.

Conclusions
Overall, there have been great advances in gene therapy for T2DM and the therapies hold great promise. Many and various genes affect T2DM and its complications. These target genes of therapeutic potential can be delivered through either viral or non-viral based methods. However, more studies have to be conducted in a comparable way to pave the way for gene therapy for T2DM. Efficiency, stability, specificity, safety, and convenience are key factors requiring careful consideration before clinical application of any gene delivery system. A combination of gene therapy and other interventions may be necessary to combat T2DM effectively.

Disclosure statement
The authors declare that there is no conflict of interest.

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