Incretin Based Gene Therapy Approaches for Diabetes

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Diabetes is a huge and growing problem, and the costs to society are high and escalating.
Global health expenditure due to diabetes (20-79 years)

Diabetes caused **5.1 million deaths** in 2013. Every six seconds a person dies from diabetes.
Developing a new medicine takes an average of 10-15 years; For every 5,000-10,000 compounds in the pipeline, only 1 is approved.

Drug Discovery and Development: A LONG, RISKY ROAD

- **DRUG DISCOVERY**
  - 5,000-10,000 Compounds
  - 3-6 Years
  - IND Submitted

- **PRECLINICAL**
  - 250
  - 6-7 Years

- **CLINICAL TRIALS**
  - Phase 1: 20-80 Volunteers
  - Phase 2: 100-300 Volunteers
  - Phase 3: 1,000-3,000 Volunteers
  - 6-7 Years

- **FDA REVIEW**
  - NDA Submitted
  - 0.5-2 Years

- **LG-SCALE MFG**
  - One FDA-Approved Drug

**PHASE 4: POST-MARKETING SURVEILLANCE**
Drug Discovery and Development

• The U.S. system of new drug approvals is perhaps the most rigorous in the world.

• It takes 10-15 years, on average, for an experimental drug to travel from lab to U.S. patients, according to the Tufts Center for the Study of Drug Development.

• Only five in 5,000 compounds that enter preclinical testing make it to human testing. And only one of those five is approved for sale.

• On average, it costs a company $1.2 billion, including the cost of failures, to get one new medicine from the laboratory to U.S. patients, according to a recent study by the Tufts Center for the Study of Drug Development.
MEDICINES IN DEVELOPMENT FOR DIABETES

BIOPHARMACEUTICAL RESEARCH COMPANIES ARE DEVELOPING

180 MEDICINES TO TREAT TYPE 1 & TYPE 2 DIABETES

INCLUDING 128 FOR DIABETES — AND — 52 FOR DIABETES-RELATED CONDITIONS

Source: PhRMA, 2014 Medicines in Development for Diabetes
Incretin based drugs

- The incretins are gastrointestinal hormones that work to increase insulin secretion in response to food ingestion. An incretin effect is defined as a biologic process where orally taken carbohydrates induce the release of intestinal hormones augmenting insulin secretion more than what could be achieved with intravenous glucose delivery.

- Since reduced incretin response to food ingestion is one of the primary defects associated with glucose intolerance and hyperglycemia in T2DM, incretin based treatment strategies recently gained a significant momentum as a novel class of medications with antidiabetic potential.

- Incretin based treatment agents represent a new class of medications used in the treatment of patients with diabetes.
Albiglutide is a dipeptidyl peptidase-4-resistant glucagon-like peptide-1 dimer fused to human albumin. In March 2014, GlaxoSmithKline PLC received approval from the European Commission to market albiglutide under the name 'Eperzan'. In April 2014, the FDA approved albiglutide under the name Tanzeum.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Incretin-Based Mechanism</th>
<th>FDA Approval Date</th>
<th>EMA Approval Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exenatide</td>
<td>GLP1 agonist</td>
<td>April 28, 2005</td>
<td>November 20, 2006</td>
</tr>
<tr>
<td>Sitagliptin</td>
<td>DPP4 inhibitor</td>
<td>October 16, 2006</td>
<td>March 21, 2007</td>
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<tr>
<td>Vildagliptin</td>
<td>DPP4 inhibitor</td>
<td>(Not approved by the FDA)</td>
<td>September 26, 2007</td>
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<tr>
<td>Saxagliptin</td>
<td>DPP4 inhibitor</td>
<td>July 31, 2009</td>
<td>October 1, 2009</td>
</tr>
<tr>
<td>Liraglutide</td>
<td>GLP1 agonist</td>
<td>January 25, 2010</td>
<td>June 30, 2009</td>
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<tr>
<td>Linagliptin</td>
<td>DPP4 inhibitor</td>
<td>May 2, 2011</td>
<td>August 24, 2011</td>
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<tr>
<td>Alogliptin</td>
<td>DPP4 inhibitor</td>
<td>January 25, 2013</td>
<td>September 19, 2013</td>
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<tr>
<td>Lixisenatide</td>
<td>GLP1 agonist</td>
<td>(Not approved by the FDA)</td>
<td>February 1, 2013</td>
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* GLP1 denotes glucagon-like peptide 1, an incretin; DPP4 denotes dipeptidyl peptidase 4, an exopeptidase that inactivates the incretins.
GLP-1-mediated gene therapy approaches for diabetes treatment
Major antidiabetic properties of GLP-1

• GLP-1 is one of the two essential gut-derived incretin hormones involved in the modulation of glucose homeostasis.

• GLP-1 is released from intestinal L cells located in the lower intestine (ileum). Target organs include, but not limited to, pancreas, liver, stomach, muscle, adipose tissue and brain. GLP-1 also suppresses glucagon secretion from alpha cells, and stimulates somatostatin secretion from pancreatic delta cells. In addition, GLP-1 reduces gastric acid secretion.

• The action of GLP-1 related to food intake includes delay of gastric emptying, inhibition of gastric acid secretion and reduction of appetite.
Why GLP-1 but not GIP based drugs?

• Isoglycaemic glucose tolerance tests demonstrated that type 2 diabetes (T2DM) patients manifested a 50% reduction in the incretin effect, despite a 300% increase in glucose-induced insulin secretion of healthy controls.

• Thus, the loss of incretin response certainly results in glucose intolerance in patients with T2DM, since incretins are the main modulators of postprandial glucose excursions.

• Interestingly, meal-stimulated GLP-1 response, but not postprandial GIP secretion, was severely reduced in patients with T2DM. Moreover, GLP-1 retained its insulino-tropic effect in T2DM patients, while no incretin response was obtained with GIP administration.

• Because GLP-1 infusions restored down-regulated beta-cell response to glucose in T2DM patients, GLP-1 has been considered a therapeutic agent for the treatment of T2DM.
GLP-1 mediated gene delivery?

- Treatments such as insulin secretagogues or parenteral administration of insulin are associated with significant risks of hypoglycemia. Because GLP-1 increases insulin secretion only in the presence of elevated glucose, the risk of hypoglycemia is low. Thus, GLP-1 is a potent stimulator of glucose-induced insulin release without causing reactive hypoglycaemia.

- However, GLP-1 has a short biological half-life (2–3 min) due to rapid truncation by the ubiquitous serine protease dipeptidyl peptidase-4 (DPP-4), which limits its therapeutic use. While frequent injections or larger quantities are needed to compensate for the short biological half-life of GLP-1, viral or non-viral vector gene delivery technologies were developed to provide a constant bioactive GLP-1 production and secretion.

- Gene delivery is the only way to provide constant incretin synthesis by a single injection.
Designing of GLP-1 gene delivery

Utilisation of the preproglucagon transgene might lead to unpredictable production of glucagon, or other processed peptides with unknown function.
GLP-1-mediated gene therapy approaches for diabetes treatment
Designing of GLP-1 gene delivery vector

• GLP-1 is encoded by the preproglucagon gene. Differential post-translational processing of preproglucagon in the intestine and pancreas results in the secretion of GLP-1 and glucagon, respectively. This processing is due to the actions of distinct prohormone convertases that are differentially expressed in these two tissues. GLP-1 is generated in the L-cells of the gut by the action of the prohormone convertase PC3, while glucagon is generated in pancreatic alpha-cells by PC2. These proteases are restricted to a subset of endocrine and neuroendocrine cell types and thus are not practical for generating GLP-1 from preproglucagon expressed in non-endocrine tissues.

• Furthermore, use of a preproglucagon transgene could result in the unpredictable production of glucagon, its C-terminally extended forms, or other processed peptides with unclear function. Therefore a GLP-1 minigene encoding only the 31 amino acids present in GLP-1 (7–37) needs to be synthesized in vitro.

• Because the active form of GLP-1 (7–37 or 7–36 amide) is rapidly cleaved to an inactive form (9–37 or 9–36 amide) by DPP-IV, the alanine at position 8 can be mutated to glycine to render the peptide DPP-IV resistant and thus prolong its circulating half-life.

• To facilitate its production and secretion in ectopic, non-endocrine tissues, it is necessary to attach a signal peptide to GLP-1 that would target the peptide to the constitutive secretory pathway and allow post-translational processing of the hybrid peptide by signal peptidase, a ubiquitous enzyme.
Designing of GLP-1 gene delivery vector

1. Because utilisation of the preproglucagon transgene might lead to unpredictable production of glucagon, or other processed peptides with unknown function, gene transfer experiments involving GLP-1 encoding sequence normally is restricted to GLP-1 (7–37) transfer rather than the entire preproglucagon cDNA.

2. In addition, since the first two amino acids of GLP-1 are essential for its receptor binding, constructs encoding GLP-1 (7–37) from a methionine start codon need to be synthesized using a DNA synthesiser.

3. A furin recognition site (RGRR) is introduced into the GLP-1 cDNA following the start codon to facilitate removal of the preceding amino acids by furin endopeptidases to generate the active form of the peptide before secretion.

4. Lastly, a secretory signal peptide is needed to target GLP-1 to the constitutive secretory pathway (CSP) to allow post-translational processing by a signal peptidase facilitating its production and secretion in non-endocrine tissues.
Animal models of Type 2 Diabetes

- *o b / o b*
- *DIO C57BL/6*
- *d b / d b*
- *ZDF (Zucker Diabetic Fatty) Rat*
Animal models of Type 2 Diabetes

• Leptin (from Greek leptos, "thin"), the satiety hormone, is a hormone made by adipose cells that helps to regulate energy balance by inhibiting hunger. Mutation either in the leptin gene or the leptin receptor leads to excessive hunger and overeating resulting in obese phenotype.

• The ob/ob or obese mouse is a leptin gene mutant mouse that eats excessively and becomes profoundly obese.

• The db/db mice genetically lack the leptin receptor and have a severe form of progressive diabetes characterized by loss of beta-cells.

• Male Zucker diabetic fatty fa/fa (ZDF) rats carrying a defect in leptin receptor develop obesity and insulin resistance at a young age, and then with aging, progressively develop hyperglycemia.

• DIO C57BL6 mouse is predisposed to obesity when fed with high fat diet and hyperglycemia is induced following STZ injection.
Molecular targets in GLP-1 mediated gene transfer against diabetes

• Obesity,
• Hyperlipidemia,
• Non-alcoholic fatty liver disease (NAFLD),
• Insulin resistance,
• Hyperglucagonemia.

• Deficiency in insulin synthesis,
• Glucose intolerance,
• Beta cell loss,
• Hyperglycemia
• Type 1 or Type 2 diabetes.
Nonviral gene delivery approaches

- Nonviral gene delivery is more desirable than viral gene delivery because of its excellent safety profile (repeated administration) and ability to carry large amounts of DNA.
- However, the efficiency of nonviral gene delivery is not sufficient for therapeutic application.
- For a gene delivery carrier, polyethylenimine (PEI) has been widely used because its ability to form complexes with DNA within physiological pH range and to escape the endosome.
GLP-1 Gene Delivery for the Treatment of Type 2 Diabetes

<table>
<thead>
<tr>
<th>Gene Delivery Method</th>
<th>Route of Delivery</th>
<th>Target Tissue</th>
<th>Circulating GLP1 (Max)</th>
<th>Effects</th>
<th>The onset Duration</th>
<th>Animal Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEI-Plasmid</td>
<td>IV</td>
<td>Circulation, Liver</td>
<td>2x</td>
<td>Insulinotropic</td>
<td>1 wk</td>
<td>2 wks</td>
</tr>
</tbody>
</table>

Oh S et al, 2003 Mol Ther
Non-viral gene delivery approaches

Normally, the GLP-1 is produced in intestinal L cells and matures through the posttranslational process. In the case of nonviral gene delivery, most delivered DNA is taken up by hepatocytes.

A plasmid-based gene delivery method involving a modified GLP-1(7–37) cDNA with a furin cleavage site between the start codon and GLP-1 coding region was developed to evaluate the consequence of in vivo GLP-1 gene delivery in diabetic animals.

The chicken b-actin promoter instead of the CMV promoter was used to increase expression in the liver.

A single intravenous injection of polyethylenimine (PEI)/pGLP1 complex into Zucker diabetic fatty (ZDF) rats resulted in an increase in glucose-induced insulin secretion with a reduction in blood glucose level for 2 weeks.

Oh S et al, 2003 Mol Ther
### Glucagon-like Peptide-1 Plasmid Construction and Delivery for the Treatment of Type 2 Diabetes

Choi S et al, 2005 Mol Ther

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<th>The onset</th>
<th>Duration</th>
<th>Animal Model</th>
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</thead>
<tbody>
<tr>
<td>PEI-Plasmid</td>
<td>IV</td>
<td>Circulation, Liver</td>
<td>2.5x</td>
<td>Insulinotropic Anorectic</td>
<td>2 days</td>
<td>3 wks</td>
<td>DIO-C57BL/6J</td>
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</table>
Non-viral gene delivery approaches

• The SV40 early region promoter/enhancer has been used as a viral promoter and has shown very high levels of expression in muscle, liver, and pancreas.

• One way to enhance gene expression efficiency is to increase nuclear transport of plasmids into cells. The GLP-1 plasmid was further modified with nuclear factor kB (NFkB) binding sites to enhance nuclear import.

• To increase GLP-1 expression, an SV40 promoter with NF-κB- binding sites was incorporated into the plasmid carrying GLP1 (7–37) cDNA with furin cleavage site.

• A single systemic administration of PEI/pGLP1 complex into the diet-induced obese (DIO) mice resulted in increased insulin secretion and decreased blood glucose longer than 2 weeks.

Choi S et al, 2005 Mol Ther
Non-viral gene delivery approaches

- Food consumption decreased 2 days after injection and gradually returned to preinjection values after 14 days. Body weight also decreased after injection. Several reasons seem to be involved with reductions in body weight.

- First, GLP-1 decreases gastric emptying rates. It is possible that gastric distension activates GLP-1-containing neurons, so it can act as an inhibitor of food intake.

- However, the reduced sensation of appetite was reported not only in the postprandial state, but also in the fasting state and before meal ingestion in humans. This suggests that mechanisms other than decreased gastric emptying also contribute to body weight reduction.

- It has been shown that the central administration of GLP-1 inhibits food intake in rodents. Since circulating GLP-1 can access GLP-1 receptors in brain, it will participate in the regulation of appetite.

Choi S et al, 2005 Mol Ther
Gene therapy of diabetes using a novel GLP-1/IgG1- Fc fusion construct normalizes glucose levels in db/db mice

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<th>Duration</th>
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</tr>
</thead>
<tbody>
<tr>
<td>GLP-1/Fc Plasmid</td>
<td>IM</td>
<td>Circulation</td>
<td>3x</td>
<td>Insulinotropic</td>
<td>3 mos</td>
<td>NA</td>
<td>db/db mice</td>
</tr>
</tbody>
</table>

*Kumar M et al, 2007 Gene Ther*
Non-viral gene delivery approaches

- Because GLP-1 must be delivered through a parenteral route and has a short lifespan, a fusion protein consisting of an active human GLP-1 and mouse IgG1 heavy chain constant regions (GLP-1/Fc) was generated to prolong and enhance the therapeutic potency of GLP-1.

- IgG–Fc homodimerisation would result in the formation of bivalent GLP-1 peptide ligands with longer half-life compared to native GLP-1, since the formation of large molecular weight homodimers slows renal clearance and reduces degradation of the conjugated peptide.

- The anti-diabetic effects of the GLP-1/Fc plasmid injection took time to develop, as delivery of GLP-1/Fc fusion protein normalised fasting blood glucose levels three months after the first injection in db/db mice resulting in augmented glucose-induced insulin secretion and reduced glucagon release.

- However, the GLP-1/Fc fusion protein could not penetrate through the blood–brain barrier, so body weight and peripheral insulin sensitivities were not affected by this treatment.

Kumar M et al, 2007 Gene Ther
Effective and safe gene-based delivery of GLP-1 using chitosan/plasmid-DNA therapeutic nanocomplexes in an animal model of type 2 diabetes

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<th>The onset</th>
<th>Duration</th>
<th>Animal Model</th>
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<tr>
<td>Chitosan/plasmid-DNA</td>
<td>SC, IM</td>
<td>Circulation</td>
<td>5x</td>
<td>Insulinotropic Anorectic</td>
<td>NA</td>
<td>24 days</td>
<td>ZDF rats</td>
</tr>
</tbody>
</table>

Jean M et al, 2011 Gene Ther
Non-viral gene delivery approaches

As the only natural polysaccharide with positive charge, chitosan has several unique properties as a carrier for gene therapy since chitosan:

• is safe, non-toxic and biodegradable;
• provides tunable electrostatic binding to negatively charged DNA;
• is mucoadhesive to permit interaction between the delivered macromolecule and membrane epithelia; and
• can open intercellular tight junctions to facilitate transport into cells.

Compared with other cationic polymers such as polylysine and polyethleneimine, chitosan is less toxic and has better tolerability.
Non-viral gene delivery approaches

• A chitosan-based gene delivery system was constructed by taking advantage of the natural ability of cationic polymers to condense plasmid DNA through electrostatic interaction to protect it from a nuclease attack. In addition, nanoparticles made of chitosan are small enough to pass through intercellular tight junctions to gain entry into cells to deliver GLP-1-encoding plasmid DNA.

• The therapeutic efficacy of chitosan-based nanocomplexes containing GLP-1-encoding plasmid DNA with a furin recognition site and cytomegalovirus (CMV) promoter was assessed in 12-week-old ZDF rats with overt diabetes mellitus.

• A significant increase in the amount of plasma GLP-1 was detected at day 49 after five injections of chitosan–GLP-1 nanoparticles. In spite of the improvement in glucose tolerance and reduced weight gain in the treated rats, the increase in circulating insulin was transient and only lasted 14 days following the last injection.

• Intriguingly, subcutaneous (s.c.) injection of the nanocomplexes was more efficient than intramuscular (i.m.) gene delivery presumably due to an inflammatory reaction at the injection site that interfered with vector distribution.

Jean M et al, 2011 Gene Ther
Lessons learned from non-viral GLP-1 mediated gene transfer

- All non-viral gene delivery methods involved the use of ubiquitous promoters to express GLP-1 transgene. This is mainly due to the need for high levels of transgene expression to manifest anorectic effects of GLP-1 since there was no concern about reactive hypoglycemia upon GLP-1 gene expression.

- Although, chitosan-mediated gene delivery systems yielded transient GLP-1 gene expression requiring repeated administration of the chitosan-DNA complex to retain insulinotropic activity, such agents were necessary to obtain high levels of transfection necessary to obtain and prolong the therapeutic outcome of gene delivery.

- Discovery of novel transfection agents and the use of alternative routes of gene delivery systems should be employed to increase gene transfer efficacy of non-viral gene delivery methods.
Virus mediated gene delivery approaches

- Previously published studies on plasmid-mediated GLP-1 gene delivery used a form of GLP-1 that was not engineered for secretion and achieved only minimal elevations in circulating GLP-1 with modest and transient effects on blood glucose in a rat model of type II diabetes or mouse model of diet-induced obesity.

- On the other hand, viral expression vectors carrying GLP-1 gene can generate significantly elevated circulating peptide levels in vivo.

- Ectopic expression of GLP-1 in vivo represents an alternate strategy for peptide delivery. Cells transduced with a GLP-1 expression vector could serve as a depot for continuous production of GLP-1 and because the insulinotropic actions of GLP-1 are glucose dependent, constitutively elevated GLP-1 should not cause hypoglycemia.
Adenoviruses (members of the family Adenoviridae) are medium-sized (90–100 nm), nonenveloped (without an outer lipid bilayer) viruses with an icosahedral nucleocapsid containing a double stranded DNA genome.
Adenovirus vectors for gene delivery

• Among the viral vectors tested, adenoviral vectors are very efficient in transducing a wide range of tissues with an ability to infect both dividing and non-dividing cells, to produce high titre yield and accommodate large transgenes.

• However, adenovirus-transduced cells are quickly cleared by the immune system due to antigenicity to adenovirus encoded viral peptides severely limiting the longevity of transgene expression.

• Furthermore, systemic delivery of adenovirus vectors at high doses might result in severe adverse effects, and repeated administration of the vector is not feasible due to the presence of neutralising antibodies.

• Contrary to first-generation adenovirus vectors, helper-dependent (gutless) adenoviral (HDAd) vectors encode no viral proteins due to deletion of almost all viral genes except ITRs, resulting in negligible toxicity and sustained (even lifelong) transgene expression.
To facilitate its production and secretion in ectopic, non-endocrine tissues, it was necessary to attach a signal peptide to GLP-1 that would target the peptide to the constitutive secretory pathway and allow post-translational processing of the hybrid peptide by signal peptidase, a ubiquitous enzyme. The signal peptide from secreted alkaline phosphatase (SEAP) was linked to the GLP-1-coding region (Figure 1a). We also tested another means of processing the hybrid peptide by fusing GLP-1 to a furin protease recognition sequence that was then linked to the leader sequence from EX4 (Figure 1a). Using this strategy, a panel of GLP-1 expression vectors containing a variety of leader sequences was generated, including signal peptides from SEAP and clusterin, and leaders from EX4, glucose-dependent insulinotropic peptide (GIP), and helodermin (Hel).
Expression vectors containing these hybrid mini-genes were transfected into 293 cells and the cell supernatants were assayed for the presence of GLP-1 3 days following transfection. The use of radioimmunoassays specific for the N- and C-termini of GLP-1 confirmed the presence of significant amounts of processed GLP-1 in the cell supernatants. The data from a representative experiment using the N-terminal radioimmunoassay (RIA) is shown in Figure 1b and demonstrates that constructs containing a furin-cleavable leader sequence (solid bars) produce significantly more GLP-1 than constructs containing just a signal peptide (striped bars).
In order to confirm that the constructs are capable of producing elevated circulating GLP-1 levels in vivo, plasmid DNA was administered to BALB/c mice via high-volume tail vein injection. The next day following vector administration, high levels of GLP-1 could be detected in the plasma of injected mice (Figure 1c). The RIA is specific for the C-terminus of GLP-1 (7–37) and thus measures GLP-1 (total), the combined amount of active- and DPP-IV-cleaved GLP-1. As endogenous GLP-1 is predominantly in the 7–36 amidated form, there was no detectable GLP-1 in the control vehicle-injected mice. Circulating GLP-1 levels in the mice injected with a GLP-1 expression vector were several orders of magnitude greater than the reported endogenous levels of 10–20 pM. Consistent with the transient transfection experiments, constructs containing a furin-cleavable leader yielded significantly higher GLP-1 levels than a construct employing just a signal peptide.
Effect of Ad2/GLP-1Gly8 in db/db mice

We next wished to determine whether GLP-1 expressed in vivo would be effective in improving glycemic control in a model of type II diabetes. Diabetic db/db mice are deficient in leptin signaling and are obese and hyperphagic. They are also severely insulin resistant and start developing hyperglycemia by 4 weeks of age.

GLP-1 linked to the EX4 leader sequence was cloned into an expression vector harboring the CMV enhancer linked to the ubiquitin promoter and intron (CUbi). The CUbi vector is capable of directing expression for more than 1 month in the liver.

Third-generation adenoviral vectors provide a simple and efficient tool for achieving sustained, high levels of gene expression. Therefore, an adenoviral vector containing the CUbi-EX4GLP1Gly8 transcription cassette was constructed for use in experiments in diabetic animal models. The vector backbone contains deletions in the E1, E3B, and E4 regions but retains E4 ORF6.

The vector was administered to the tail vein of db/db mice at a dose of 1 x10e11 viral particles per mouse. Control mice received adenovirus devoid of a transgene (empty vector (EV)).
The blood glucose of Ad2/GLP-1-treated db/db mice declined to a level equivalent to that of lean mice within 1 day following vector administration (Figure 2a). Glucose levels rose out of the euglycemic range over the next week but even at 6 weeks post-vector administration, glucose levels were still significantly below the level of the Ad2/EV-treated mice. GLP-1 vector administration to non-diabetic lean mice resulted in a small but statistically significant decline in blood glucose without evidence of hypoglycemia.

GLP-1 levels were measured using an RIA specific for the N-terminus of GLP-1 (7–37) and thus detected active GLP-1 and not the inactive DPP-IV cleavage product. Plasma levels of active GLP-1-Gly8 (7–37) in db/db mice were larger than 1 nM 6 weeks after vector administration (Figure 2b).
A glucose tolerance test performed at 2 weeks post-vector administration also showed that the GLP-1-treated db/db mice had improved glucose homeostasis (Figure 2c).

Although the two groups had similar peaks of blood glucose excursion, the GLP-1-treated animals had significantly lower glucose levels at 120 and 180 min time points.

At the last time point, the glucose levels of the treated db/db mice were not significantly different from the lean controls.
Diabetic db/db mice are severely insulin resistant and thus have greatly elevated circulating insulin levels. On the C57BLKS/J (black Kaliss) background, db/db mice experience a progressive beta-cell failure that eventually results in a large reduction in circulating insulin levels and a dramatic rise in blood glucose.

db/db mice treated with Ad2/GLP-1 experienced only a small, statistically insignificant, reduction in plasma insulin levels, whereas the Ad2/EV-treated db/db mice showed a greater than 60% decline in circulating insulin (Table 1). Thus, GLP-1 treatment resulted in a delay of the appearance of insulin deficiency in this strain.
Insulin immunostaining (brown) of pancreatic sections. (a) Ad2/EV-treated lean, (b) Ad2/EV-treated db/db, and (c) Ad2/GLP-1-treated db/db. (d) Quantitation of insulin staining as a percentage of total tissue area. Magnification 10-fold. Data are mean values +/- s.d. from three slides per db/db mouse. Each group contains four animals. Data for the lean group is derived from three animals, seven slides total.

db/db mice were injected with Ad2/CUBiEX4GLP-1Gly8 or Ad2/EV. At 28 days after vector administration, pancreatic sections were immunostained for insulin. Representative images are shown in Figure 3a–c. Lean mice showed small, densely staining, islets (Figure 3a), whereas db/db mice had larger lightly staining islets (Figure 3b and c). GLP-1- treated db/db mice tended to have larger islets (Figure 3c) than control db/db mice. This increased islet size resulted in a significant increase in the overall fraction of the pancreas staining positive for insulin (Figure 3d).

Parsons GB et al, 2007 Gene Ther
Effect of Ad2/GLP-1Gly8 in db/db mice

• Furthermore, GLP-1 has been reported to have positive effects on beta-cell mass through stimulation of beta-cell replication, beta-cell neogenesis, and inhibition of apoptosis. In addition to these direct actions of GLP-1, correction of hyperglycemia is predicted to diminish beta-cell death owing to glucotoxicity.

• Consistent with other published reports, greater than twofold increase in the beta-cell mass of GLP-1-treated db/db mice was observed compared to control empty vector-treated db/db mice. Multiple kinetic studies will be required to determine whether this increase is owing to changes in rates of beta-cell replication, neogenesis, or inhibition of apoptosis.

• Alternatively, increases in insulin transcription or translation may facilitate greater detection of beta-cells.
Type 1 diabetes results from insulin deficiency caused by destruction of pancreatic beta cells. Glucagon-like peptide (GLP)-1 stimulates beta cell growth and differentiation. To determine whether continuous expression of GLP-1 in vivo can regenerate beta cells and remit type 1 diabetes in mice for a prolonged time, an adenoviral vector was constructed containing the cytomegalovirus promoter/enhancer and albumin leader sequence followed by GLP-1 cDNA (rAd-GLP-1).
Remission of diabetes in rAd-GLP-1-treated STZ-induced diabetic mice

To determine whether treatment of diabetic NOD/SCID mice with rAd-GLP-1 remits diabetes, STZ-induced diabetic NOD/SCID mice were injected with rAd-GLP-1 and measured blood glucose levels at various times after treatment.

It was found that blood glucose levels gradually decreased and became normal within 10 days after rAd-GLP-1 injection, and normoglycemia remained for 20 days thereafter, when this part of the experiment was terminated.

When STZ was injected into these normoglycemic mice 30 days after virus injection, the mice reverted to hyperglycemia. In contrast, rAd-bGAL-treated mice remained hyperglycemic.
Figure 4 Histological and immunohistochemical analysis of the pancreas in rAd-GLP-1-treated STZ-induced diabetic mice. STZ-induced diabetic NOD/SCID mice were treated with rAd-GLP-1 (STZ + rAd-GLP-1) or rAd-βGAL (STZ + rAd-βGAL). (a) 30 days after viral injection, serial pancreatic sections were stained with hematoxylin and eosin (HE) or double-stained with anti-insulin (red) and anti-glucagon (green) antibodies. (b) Mice were injected daily with BrdU (100 mg/kg body weight, i.p.) for 5 days beginning on the 8th day after virus injection. Pancreatic sections were prepared on the last day of BrdU injection and double-stained with anti-pdx-1 and anti-BrdU antibodies. Arrows indicate double-positive cells. Images were analyzed with a laser scanning confocal microscope. Representative islets are shown (original magnification × 400; bar = 40 μm). (c) The insulin-positive area was measured after anti-insulin antibody staining at 30 days after virus injection and expressed as a percentage of the area found in normal mice. **p < 0.001 compared with STZ + rAd-βGAL-treated group.
Regeneration of insulin-producing cells in the pancreas of rAd-GLP-1-treated diabetic mice

• To determine whether the restoration of normoglycemia in rAd-GLP-1-treated STZ-induced diabetic mice is a result of regeneration of insulin-producing cells, pancreatic sections were double stained with anti-insulin and anti-glucagon antibodies at 30 days after rAd-GLP-1 or rAd-bGAL treatment. In rAd-bGAL-treated mice, insulin-positive cells were rarely seen in pancreatic islets and most of the stained cells were glucagon-positive.

• In contrast, insulin-positive cells were clearly increased in pancreatic islets from rAd-GLP-1-treated mice as compared with rAd-bGAL-treated mice (Figure 4a). When pancreatic sections were double stained with anti-pdx-1 and anti-BrdU antibodies, double-positive cells were found in rAd-GLP-1-treated mice but not in rAd-bGAL-treated mice, indicating that insulin-producing cells were newly generated in rAd-GLP-1-treated mice (Figure 4b).

• The insulin-positive area was significantly increased in rAd-GLP-1-treated mice as compared with rAd-bGAL-treated mice and reached about 80% that of normal mice (Figure 4c).
Viral gene delivery approaches

Adeno Associated Virus

Sanlioglu S et al, 2000 JV
AAV based gene therapy approaches

• Although both plasmid-based and adenoviral systems have been used to express GLP-1R agonists recently, these systems yield relatively short-term expression. AAV has been considered another attractive vector.

• AAV vectors are derived from the AAV type 2 virus, a nonpathogenic, replication-defective parvovirus, that can integrate into the host genome at a specific locus.

• AAV vectors are able to effectively transduce both dividing and non-dividing cells in vitro and in vivo, allowing stable gene transfer by either integration into the host chromosomes or persistence as an episome.

• In addition, the lack of cytotoxicity and minimal cellular immune responses after AAV-mediated in vivo gene transfer also contribute to the success of long-term gene delivery using this vector in a variety of tissues.
AAV based gene therapy approaches

- Unlike other DNA-based viral and nonviral vectors, such as adenoviral vectors and plasmids, AAVs package and deliver a single-stranded DNA genome that is transcriptionally inactive until it is converted into a double-stranded template.

- The rate-limiting steps for the conversion of ssDNA to dsDNA are the de novo synthesis of the second strand of DNA and the annealing of the plus and minus strands from two separate viral particles co-infecting the same cell. Therefore, there is an extended lag period before transgene expression actually occurs although AAV is a safe vector for gene therapy.

- Wang et al. discovered that if the D-sequence with the terminal resolution site is deleted from one of the ITRs, cleavage by Rep cannot occur and, consequently, the dimers are not resolved into monomers, allowing the double-stranded genomes to be packaged as large hairpin DNA molecules. McCarty et al. used a double-stranded form of the AAV single-stranded genome to reduce the lag period for transgene expression.
Advantages of AAV based gene therapy approaches

• Although the packaging capacity of AAV vectors is limited, it has not been associated with any disease or pathology. It is infectious to both diving and non-dividing cells. Furthermore, unlike adenovirus, there is a lack of a significant immune response directed against the AAV vector, contributing to the ability of the vectors to persist in vivo.

• Despite neutralizing antibodies may limit repeated administration of AAV vectors, AAV8 appears not to be neutralized by antisera directed against other AAV serotypes. Only low levels of neutralizing antibodies against AAV8 have been detected in normal human subjects, suggesting that immune responses that have been observed following AAV2-mediated gene therapy may be minimized following AAV8 administration.

• Pancreatic beta-cells proliferate at a slow rate and long-term episomal maintenance of transgenes delivered through AAV vectors has been demonstrated in slowly dividing cells. Thus, long-term, stable expression of GLP-1 could be expected in human beta-cells following AAV administration.
Figure 1 Construction and characterization of dsAAV GLP-1 vector. (a) A schematic representation of the GLP-1 expression vector containing a GLP-1 molecule (7–37). The schematic diagram of the GLP-1 expression cassette. CB, CMV enhancer/chicken β-actin promoter; Igk, Ig κ-chain leader sequence; HA, hemagglutinin A (HA) epitope; furin cleavage site, furin proprotein convertase recognition sequence: GLP-1 7–37 amino acids, Ala8 → Gly8. (b) Characterization of the genome from dsAAV and ssAAV. On the neutral gel (0.8%), dsAAV DNA (arrowheads) migrated to the 2.1 kb size, whereas ssAAV DNA (arrowheads) run aberrantly next to the 3 kb. On the alkaline denaturing gel (0.8%), dsAAV DNA was denatured into 4.2 kb whereas ssAAV DNA remained at 4.7 kb.
Long-term effects of dsAAV GLP-1 vector transduction in vivo: Diabetic db/db mice (7- to 8-week old) were used to characterize the effects of dsAAV GLP-1 in vivo. To transduce liver, 150 ul (total 1x10e12 viral particles per mouse) of either dsAAV GFP or dsAAV GLP-1 was injected into portal vein. Blood glucose levels were evaluated every week after injection of viral vectors. The fasting blood glucose levels of dsAAV GLP-1-treated mice were significantly lower than control mice, and this effect lasted for approximately 4 months (Figure 4a). In addition, a glucose tolerance test performed at 12 weeks after administration of vector showed that dsAAV GLP-1- treated db/db mice had improved glucose homeostasis (Figure 4b). Although blood glucose excursion was similar between the two groups, the GLP-1-treated animals showed rapid serum clearance of glucose at the 90- and 120-min time points (Figure 4b).
**Long-term effects of dsAAV GLP-1 vector transduction in vivo:** To investigate whether the maintenance of circulating insulin levels was reflected in the preservation of beta-cell mass, pancreatic sections were immunostained for insulin at 14 weeks after vector administration. Lean C57BL/6 J mice showed small, densely staining islets, whereas db/db mice had larger, lightly staining islets. The dsAAV GLP-1-treated db/db mice tended to have larger islets than did the control db/db mice (Figure 4c). This analysis demonstrated a 1.4-fold increase in b-cell mass in dsAAV GLP-1-treated mice compared with dsAAV GFP-treated mice.

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Figure 5  Effect of dsAAV GLP-1 on blood GLP-1 levels, insulin levels and β-cell staining in db/db mice. (a) Plasma was collected before injection and 2, 14, 18 and 28 weeks after injection. GLP-1 levels were determined using a C-terminal-specific GLP-1 RIA kit. (b) Blood insulin levels were measured using ELISA at 14 weeks after a 15h fast. (c) Mouse pancreata collected 14 weeks after viral transduction were immunostained with anti-insulin and anti-glucagon antibodies and visualized using FITC- and rhodamine-conjugated second antibodies, respectively (left). Morphometric analyses of 30 islets per pancreas from 3–4 mice for each group were carried out to measure the size of the insulin-positive areas (right). The positive area in a dsAAV GFP-transduced db/db mouse is shown by the open column, whereas that for a dsAAV GLP-1-transduced db/db mouse is shown by the gray column. Each column represents a mean ± s.d. Bar=100 μm. *P<0.01, **P<0.001.
Prolonged GLP-1 expression and increased insulin levels in dsAAV GLP-1-infected mice

Plasma GLP-1 levels were more elevated in mice injected with dsAAV GLP-1 than the control (Figure 5a) at 2 weeks after viral injection. Fasting blood glucose levels rose again 4 months after injection, correlating with low levels of GLP-1 in the bloodstream (Figure 5a). Furthermore, nonfasting insulin levels were significantly higher in the dsAAV GLP-1-injected mice than in the control mice (Figure 5b).

To determine the influence of dsAAV GLP-1 on the size of the area containing insulin-positive cells, double immunological staining of pancreatic sections was performed. GLP-1 viral vector treatment resulted in an increase in the size of insulin-positive areas (Figure 5c). Quantitative morphological analysis suggested that the insulin-positive areas in individual islets were significantly increased by dsAAV GLP-1 treatment more than 2.5-fold to control, whereas dsAAV GFP-treated islets only had a few insulin-producing beta-cells and spatial derangement of the islet endocrine cells (Figure 5c).
RNA levels in tissues after transduction with dsAAV vectors. The db/db mice were killed 14 weeks after transduction, and RNA was isolated from the liver and pancreas.

(a) Liver samples from dsAAV GLP-1-transduced and dsAAV-GFP-transduced mice were confirmed by RT–PCR specific for GLP-1 and GFP each (upper). RNA from liver was also amplified without prior reverse transcription (No RT) (lower) to show that the signal was produced from RNA and not from contaminating DNA.

(b) Pancreatic insulin (INS-1) and CHOP mRNA levels assessed by RT–PCR in control virus and dsAAV GLP-1-treated mice (upper). The normalized levels of CHOP and INS-1 to b-actin determined by densitometry are illustrated in the graphs (lower).

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Increased gene expression in the liver and pancreas of dsAAV vector-treated mice

To confirm whether the elevated expression of GLP-1 was due to production of exogenous GLP-1, GLP-1 mRNA levels were measured in the livers of infected mice using reverse transcription (RT)–PCR with gene-specific primers. GLP-1 transcripts were detected in only dsAAV GLP-1-treated db/db mice, whereas GFP transcripts were detected only in only dsAAV GFP-treated db/db mice. No transcripts were detected in the total RNA extracted from liver tissues without reverse transcription (Figure 6a). These data show that RNA levels correlate well with plasma GLP-1 levels as shown in Figure 5a. A significant increase in insulin mRNA transcripts was detected in dsAAV GLP-1-treated mice (Figure 6b). In other words, GLP-1 transduction stimulated insulin biosynthesis and increased insulin exocytosis.

Diabetes is associated with the development of endoplasmic reticulum stress in beta-cells. The transcription factor CHOP has been shown to be a critical component of the endoplasmic reticulum stress response. Significantly lower levels of CHOP mRNA were found in dsAAV GLP-1-treated pancreas tissue than in dsAAV GFP-treated pancreas tissue (Figure 6b).
• GLP-1 gene delivery has produced favourable results in both pre-diabetic and fully diabetic animals, suggesting that a GLP-1 gene therapy approach may be a reasonable alternative to constant infusions or daily injections of GLP-1 peptide.

• Protocols using dsAAV vectors have produced some successful results, similar or enhanced results are expected using lentivirus vectors targeting pancreas with glucoregulatory function. This is especially true when the long-term beneficial neuroprotective and/or cardioprotective effects of GLP-1 are expected.

• Even though gene therapy appears to be a promising technique for achieving a long-term increase in GLP-1 synthesis and secretion, the most effective gene delivery method has yet to be identified.

• It is important to keep in mind, though, that many of these published results showing the benefits of GLP-1 gene therapy were conducted in small rodent models of T2DM, making it crucial to continue testing of this therapy in larger animal models (such as cats, dogs, pigs and even primates) to increase the clinical relevance of experimental findings and design future clinical trials.
GLP-1-mediated gene therapy approaches for diabetes treatment

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