Treatment of Diabetes and Long-term Survival Following Insulin and Glucokinase Gene Therapy

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ABSTRACT

Diabetes is associated with severe secondary complications, caused largely by poor glycemic control. Treatment with exogenous insulin fails to prevent these complications completely, leading to significant morbidity and mortality. We previously demonstrated that it is possible to generate a “glucose sensor” in skeletal muscle through co-expression of glucokinase (Gck) and insulin (Ins), increasing glucose uptake and correcting hyperglycemia in diabetic mice. Here, we demonstrate long-term efficacy of this approach in a large animal model of diabetes. A one-time intramuscular administration of adeno-associated viral vectors of serotype 1 (AAV1) encoding for Gck and Ins in diabetic dogs resulted in normalization of fasting glycemia, accelerated disposal of glucose after oral challenge, and no episodes of hypoglycemia during exercise for >4 years after gene transfer. This was associated with recovery of body weight, reduced glycosylated plasma proteins levels, and long-term survival without secondary complications. Conversely, exogenous insulin or gene transfer for Ins or Gck alone failed to achieve complete correction of diabetes, indicating that the synergistic action of Ins and Gck are needed for full therapeutic effect. This study provides the first proof-of-concept in a large animal model for a gene transfer approach to treat diabetes.
INTRODUCTION

Diabetes is a chronic disease for which there is currently no cure. Type 1 diabetes (T1D) patients need insulin replacement therapy to survive, but glycemia is not always properly regulated. Chronic hyperglycemia leads to development of diabetes-associated microvascular, macrovascular and neurological complications, which can be delayed by intensive insulin therapy (1). However, this treatment is not suitable for all diabetic patients because of its high risk of hypoglycemia secondary to excessive insulin dosage (1). Thus, precise regulation of glucose homeostasis is a major challenge in diabetes management. Therapeutic benefit has been obtained with islet transplantation (2) but access to human islets and the necessary immunosuppressive therapy are important limitations. Alternative cell- and gene-based therapies, centered around the engineering of non-pancreatic tissues to produce insulin, or the generation of stem cell-derived β-cells, are under investigation (3,4); however, long-term safety and efficacy data in large animal models are lacking.

Genetic engineering of skeletal muscle to counteract hyperglycemia is an attractive strategy to correct diabetes. Skeletal muscle is responsible for the disposal of ~70% of circulating glucose after a meal. In muscle, glucose utilization is controlled by insulin-stimulated glucose transport through the glucose transporter type 4 (GLUT4) (5) and phosphorylation by hexokinase II (HKII), which has a low $K_m$ for glucose and is inhibited by glucose-6-phosphate, limiting glucose uptake (6). In diabetic muscle, due to the lack of insulin, GLUT4 translocation to the plasma membrane and HKII activity both decrease. In contrast to HKII, the liver enzyme glucokinase (Gck) has a high $K_m$ for glucose, is not inhibited by glucose-6-phosphate, and shows kinetic cooperativity with glucose (7). When expressed in skeletal muscle of transgenic mice, Gck facilitates glucose uptake only when
blood glucose is high (8). However, during diabetes, constant basal levels of insulin are required to ensure the presence of GLUT4 on the cell membrane (5). These observations led to the hypothesis that regulation of glycemia could be achieved by co-expression in skeletal muscle of Gck and low levels of Ins; in such a system, glucose influx is regulated by circulating glucose levels allowing glucose uptake only when hyperglycemia is present. Accordingly, intramuscular delivery of AAV vectors expressing Ins and Gck to diabetic mice resulted in disease correction (9).

AAV vectors are the vector of choice for in vivo gene therapy due to their excellent safety and efficacy profile. Pre-clinical studies have shown that AAV-mediated gene transfer results in long-term gene expression in small and large animal models (10). Recently, these preclinical data have been successfully translated into humans (11,12). In the most clear-cut examples of success, clinical studies of hemophilia B (12) and Leber’s congenital amaurosis (11) were preceded by convincing studies of efficacy in large animal models (13,14). However, for most proof-of-concept studies in mice, no successful scale-up and long-term efficacy has been reported. This goal has yet to be demonstrated in large animal models of diabetes with gene and cell therapy approaches. Here, we used dogs treated with β-cell cytotoxic drugs as model of experimental diabetes (15), since large animal models of autoimmune diabetes are not available. We demonstrate that after a single intramuscular injection of AAV1 vectors, Ins and Gck transgenes act synergistically to achieve tight control of glycemia. This represents the first proof-of-concept study of long-term correction of diabetes in a large animal model using gene transfer.
RESEARCH DESIGN AND METHODS

Animals. Male Beagle dogs were purchased from Isoquimen (Barcelona, Spain) and housed at Servei de Granges i Camps Experimentals of the Universitat Autònoma de Barcelona (UAB). Animals were fed individually once daily at 9:00 am with 30 g/kg body weight standard dry food (Nestle, Vevey, Switzerland) or twice daily at 9:00 am and 9:00 pm with 15 g/kg body weight with diabetic food (Prescription diets w/d, Hills, Topeka, KAN) when indicated. Dogs were monitored regularly at the UAB Veterinary Clinical Hospital. C56Bl6 male mice (Harlan Teklad, Barcelona, Spain) were fed ad libitum with a standard diet (Harlan Teklad) and maintained in the SPF-mouse facility at the Center of Animal Biotechnology and Gene Therapy under a 12-h light/dark cycle (lights on at 8:00 AM). The Ethics Committee on Animal and Human Experimentation approved all procedures.

Diabetes induction. Experimental diabetes was induced in 6-12 month-old dogs by a single intravenous injection of a mixture of streptozotocin (STZ) (35 mg/kg) and alloxan (40 mg/kg) (Sigma, St Louis, MO, USA) as described previously (15). When hyperglycemia developed, dogs were maintained without exogenous insulin treatment, unless indicated. Dogs receiving exogenous insulin were injected subcutaneously with Lantus™ (Sanofi Aventis, Paris, France). When indicated, exogenous insulin treatment was optimized individually, increasing gradually the insulin dose up to the maximum tolerated dose that did not cause hypoglycemia. To induce diabetes in mice, animals aged 8 weeks were given, on 5 consecutive days, an intraperitoneal injection of STZ (45 mg/kg bw) dissolved in 0.1 mol/l citrate buffer (pH 4.5) immediately before administration.
**AAV production and administration.** AAV vectors were produced by triple transfection of HEK293 cells and purified by a CsCl-based gradient method (16). Expression of an engineered human insulin gene containing an endoprotease furin cleavage signal, and of rat glucokinase, was driven by the CMV promoter in both vectors (9). For certain experiments as indicated, codon-optimized versions of human insulin (oIns) (with furin cleavage sites) and human glucokinase cDNAs (oGck) were used. Vectors were delivered to a total of 12-25 sites on the lateral aspect of the thigh (with a 5-prong needle syringe) and the craniolateral face of the leg (single point injections) of both hind limbs, with maximal vector dose per site of injection being <6x10^{11} vg (Supplementary Fig. 1). Dogs received a vector dose of 1x10^{12} vg/kg or 2x10^{12} vg/kg 2-4 weeks after diabetes induction (Supplementary Table 1). Mice were intramuscularly treated with 4x10^{12} vg/kg of AAV1-oGck two weeks after diabetes induction, distributed into tibialis cranialis, gastrocnemius and quadriceps muscles of both hindlimbs.

**Biodistribution.** Total DNA was isolated from Dog3 with MasterPureDNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA). Vector genome copy number was determined in 20 ng of genomic DNA by TaqMan qPCR with primers and probe specific for the CMV promoter: forward primer: 5'-CACCAATGGGCGTGGATAGC-3'; reverse primer: 5'GCAGTTGTTACGACATTTTGGAA-3'; probe: 5'-ATTTCAGTCTCCACC-3'

**RNA analysis.** Liver and Quadriceps total RNA was extracted (Qiagen, Valencia, CA, USA) from dog and mouse samples and analyzed by Northern blot with radiolabelled (GE Healthcare UK, Buckinghamshire, UK) insulin or glucokinase cDNA probes or by quantitative RT-PCR for detection of Glucokinase Regulatory Protein (GKRP), Forward
primer: CAAGCACCAAGCGGTATCA; Reverse primer: GTCAGTGGGTTGGACTTCTCT.

**Western blotting.** Nuclear and cytoplasmatic protein fractions were obtained from skeletal muscle and liver extracted from starved mice, or 15 min after intraperitoneal glucose injection (3g/kg bw) as described (17). Fractioned or total protein lysates were subjected to SDS-PAGE, electrotransferred on PVDF (Millipore) membranes, and probed with the following antibodies: Akt and pAkt, (Cell Signalling, Danvers, MA, USA); Gck (Sigma); Histone 3 and α-Tubulin (Abcam, Cambridge, UK) as previously described (18). All rabbit primary antibodies were immunodetected using HRP-conjugated polyclonal swine anti-rabbit immunoglobulins (Dako, Glostrup, Denmark). Loading was normalized by α-Tubulin.

**Morphological and immunohistochemical analysis.** Dog samples were fixed in 10% formalin, embedded in paraffin and sectioned. Double glucagon and insulin immunostaining was performed with mouse anti-glucagon (Sigma) and guinea pig anti-insulin (Sigma) antibodies. Biotinylated horse anti-mouse (Vector Laboratories, Burlingame, CA, USA) followed by streptavidin-conjugated Alexa488 (Molecular Probes, Leiden, The Netherlands) and Alexa568-conjugated goat anti-guinea pig (Molecular Probes) were used as secondary antibodies. β-cell area was measured on four sections of pancreas biopsy samples, or multiple areas from the whole pancreas in necropsy samples, stained with anti-insulin and horseradish peroxidase-conjugated rabbit anti-guinea pig immunoglobulin (Dako, Glostrup, Denmark). Pancreatic β-cell area was calculated by dividing the area of insulin-positive cells by total pancreatic area of each section. To analyze muscle integrity, cross-sections were stained with hematoxylin and eosin or rabbit
anti-laminin (Dako, Glostrup, Denmark). Periodic acid-Schiff (PAS) staining was used to evaluate muscle glycogen content (Sigma).

**Hormone and metabolite determinations.** Serum insulin was measured with human insulin radioimmunoassay (Millipore, Billerica, MA, USA). Serum human C-peptide was determined with C-Peptide radioimmunoassay (Millipore) that does not cross-react with insulin or with canine C-peptide. Serum glucagon was measured by radioimmunoassay (Millipore). Blood glucose levels were determined using a Glucometer EliteR analyser (Bayer, Leverkusen, Germany). Serum fructosamine concentration was measured by nitroblue tetrazolium reduction test. The concentration of glycogen in skeletal muscle were measured as previously described (9). Urine was analyzed by Multistix 10 SG Urinalysis Strips (Siemens, Munich, Germany).

**Oral glucose tolerance test.** Oral glucose tolerance tests (OGTT) were performed on either 12 or 24-hour fasted dogs. Briefly, animals were given an oral gavage of glucose 1.75 g/kg bw. Glycemia was determined at time 0, 15, 30 minutes and then every half hour up to 3 hours post glucose administration.

**Exercise test.** Fasted dogs (24 h) were subjected to 37-minute exercise under increasing speed and slope in a variable speed belt treadmill (Starker Hund S.A.S, Padua, Italy). The protocol was: 1) 5 min, 0 degree, 4 km/hour; 2) 5 min, 0 degree, 8 km/hour; 3) 5 min, 2.5 degree, 8 km/hour; 4) 5 min, 5 degree, 8 km/hour; 5) 5 min, 7.5 degree, 8 km/hour; 6) 5 min, 10 degree, 8 km/hour; 7) 5 min, 0 degree, 10 km/hour and 8) 2 min, 0 degree, 4 km/hour. Dogs were allowed a recovery time equal to the exercise period (Supplementary Video 1).
Glucokinase activity determination. To measure Gck activity in skeletal muscle, tissue biopsies were obtained from mice or from necropsy samples from Dog3. Frozen mouse gastrocnemius or dog quadriceps samples were homogenized in an ice-cold buffer (pH 7.4) containing 100 mM Gly-Gly, 200 mM KCl, 5 mM DTT and 65 mM Tris. Samples were then centrifuged to pellet insoluble material. The glucose phosphorylation capacity was assayed in the supernatants at 30°C in a buffer containing 50 mM Gly-Gly, 100 mM KCl, 2.5 mM DTT, glucose-6-phosphate dehydrogenase (1 U/ml), 0.5 mM NADP and 4.5 mM ATP-Mg. Glucokinase activity was calculated as the difference between the glucose phosphorylation capacity at 100 and 0.5 mM glucose. Protein content was measured by Bradford assay (Pointe Scientific, USA) and Gck activity was expressed as mU/mg of protein.

Insulin sensitivity test in mice. Awake, fed AAV1-oGck or AAV1-null treated diabetic mice were intraperitoneally injected with 0.75 IU/kg body weight of insulin (Humulin regular, Eli Lilly). Glucose concentration was determined in blood samples obtained from tail vein before and at 0, 15, 30, 45 and 60 min after the insulin injection.

Statistical analysis. All values are expressed as means ± SEM. Differences between groups were compared by unpaired Student’s t test. A p value < 0.05 was considered statistically significant.
RESULTS

Glycemic control by insulin alone

Following experimental diabetes induction, 3 dogs (DogDb1-3) were subjected to glycemic control with subcutaneous exogenous insulin, according to hormone administration regimens and feeding protocols indicated for diabetic companion dogs. Despite therapy, fasting normoglycemia was not achieved in any of the animals (Fig. 1A-C); with one of the animals, Dog-Db3, experiencing severe fasting hyperglycemia. Exogenous insulin therapy prevented weight loss in all dogs (Fig. 1A-C). All these animals showed elevated fructosamine levels (Fig. 2A), a marker of glycosylated proteins in blood and an indicator of recent (3 weeks) glycemic control used in veterinary medicine (19-21). Finally, diabetic dogs showed a marked, sustained rise in blood glucose compared to healthy animals when subjected to oral glucose tolerance tests (OGTT) at the American Diabetes Association (ADA)-recommended standard dose of glucose (1.75g/kg) (22) (Fig. 2B).

In an effort to further improve glycemic control, a new treatment regime with exogenous insulin was established. In DogDb1 and 2, and in an additional diabetic dog (DogDb4), insulin dosing was increased as much as possible without causing severe hypoglycemia; this was done on an individual basis with twice daily administrations. In addition, dogs were fed with diabetic food distributed in two servings. Despite treatment optimization, no major changes were observed in fasted glycemia (Fig. 1A,B,D), while the improvement in glycemic control resulted in lower fructosamine levels, which fell below the upper limit of good glycemic control in veterinary medicine (450 µmol/L, (19-21)) (Fig. 2A), and improved OGTT (Fig. 2B).
When the insulin transgene (Ins) alone was expressed in skeletal muscle of a diabetic dog by administration of $1 \times 10^{12}$ vg/kg of AAV1-Ins vector (DogIns), partial correction of fasting glycemia was achieved (Fig. 3A). This animal recovered the body weight initially lost after diabetes induction and had fructosamine levels that ranged between 250 and 300 µmol/l following gene transfer (Fig. 3B and C). Despite achieving normal levels of fasting insulinemia (Fig. 3D), the ability of DogIns to dispose of glucose following OGTT was only moderately improved compared to diabetic dogs (Fig. 3E).

**Insulin and glucokinase gene transfer to skeletal muscle corrects diabetes in dogs**

Five diabetic dogs (Dog1-4 and DogDb3+Ins/Gck) were intramuscularly administered with AAV1-Ins and AAV1-Gck vectors. Administration of $1 \times 10^{12}$ vg/kg of each vector to Dog1 and 2 resulted in rapid return to fasting normoglycemia and normoinsulinemia, recovery of body weight (Fig. 4A and B and Supplementary Video 2), and long-term survival (>4 years, observation ongoing). The administration of a twice-high vector dose ($2 \times 10^{12}$ vg/kg) of each vector to Dog3 was also safe and resulted in correction of diabetes with no episodes of hypoglycemia (Fig. 4C and D). Thus, Ins+Gck gene transfer results in better control of diabetes than exogenous insulin therapy or gene transfer for Ins only. This result was further confirmed in DogDb3 (Fig. 1C) which was treated with AAV1-Ins and AAV1-Gck after 5 months of poorly controlled glycemia (now named DogDb3+Ins/Gck), resulting in normalization of glycemia without exogenous insulin administration. Codon-optimized versions of human transgenes were also tested to achieve better glycemic control at doses of $1 \times 10^{12}$ vg/kg, as use of codon-optimized genes increases the production of proteins (23). Delivery of AAV1-oIns and AAV1-oGck to Dog4, led to rapid recovery of
normoglycemia, normoinsulinemia, and body weight (Fig. 4E), with no signs of fasting hypoglycemia detected during the five-month follow up.

In all Ins+Gck-treated dogs, fructosamine levels remained within the range of 250-350 µmol/l (Fig. 5A). When OGTT was performed at a dose of 1.75g/kg, treated dogs showed only a small rise in glycemia after the load, followed by return to normoglycemia within two hours, a profile considered non-diabetic by ADA guidelines (2h plasma glucose <200mg/dl)(22), with the exception of DogDb3+Ins/Gck which was around 200mg/dl at 120 min (Fig. 5B). Furthermore, Ins+Gck-treated dogs showed improved glucose disposal even after high-dose (3 g/kg) glucose load; peak glycemia was lower than that of diabetic dogs, and 2h glycemia dropped below 200mg/dl (Supplementary Fig. 2). Importantly, Dogs1-4 and DogDb3+Ins/Gck showed good glycemic control under exercise, with no development of hypoglycemia (Fig. 6). In agreement with normalization of glycemia, ketone bodies were never detected in the urine of these dogs (data not shown). Finally, Ins+Gck-treated dogs did not show signs of secondary complications, while DogDb1-3 developed cataracts few months after hyperglycemia development (Supplementary Table 2).

Both transgenes were expressed in skeletal muscle (Fig.7A) and Gck was active in this tissue (Fig 7B). Circulating insulin in AAV-treated dogs derived from expression of Ins transgene in muscle, as documented by the lack of surviving β-cells in pancreas biopsies (Fig. 7A,C,D) and with the detection of human C-peptide in serum (Fig. 7E). In contrast to healthy dogs, and in agreement with the lack of pancreatic insulin-producing cells, the first phase peak of insulin release after a meal was not observed in AAV1-treated dogs (Fig. 7F). Together, these findings suggest that circulating insulin detected in AAV-
treated dogs derives from the expression of the Ins transgene in skeletal muscle and not from residual expression from surviving β-cells. All AAV1-Ins+Gck-treated diabetic dogs showed normal circulating glucagon levels (Fig. 7G), indicating preservation of α-cell function.

The production of insulin in skeletal muscle increased the phosphorylated AKT/total AKT ratio in muscle fibers, indicating that insulin activated its signaling in an autocrine/paracrine manner (Supplementary Fig. 3). Also, in agreement with the absence of Glucokinase regulatory protein (GKRP) in skeletal muscle (data not shown) (24), Gck was detected only in the cytosol of AAV1-treated muscle fibers, even after a glucose challenge (Supplementary Fig. 4).

Dog3 was euthanized at 2.2 years after gene transfer and vector genome biodistribution analysis confirmed that most of the detectable vector was present in injected muscles (Supplementary Table 3). Normal muscle morphology without glycogenosis was documented in AAV-injected animals (Supplementary Fig. 5 and Supplementary Table 4).

**Role of the glucokinase transgene in glycemic control**

Two diabetic dogs were treated with AAV1-oGck vectors alone at 2x10^{12} vg/kg (DogGck1 and DogGck2). After vector delivery, both dogs remained hyperglycemic and required administration of exogenous insulin to reduce hyperglycemia and stabilize weight loss (Fig. 8A and B), demonstrating that Gck expression alone is not sufficient to counteract hyperglycemia. Consistently, fructosamine was elevated in both dogs (Fig. 8C) and developed cataracts few months after hyperglycemia development (Supplementary Table
2). Dogs expressing Gck alone showed impaired OGTT (1.75 g/kg), with glycemia curves similar to those of diabetic non-treated animals (Fig. 8D).

Similar to what it was done with diabetic control dogs, exogenous insulin treatment was optimized on an individual basis in DogGck1 and 2 with twice daily administrations. Although no drop in fasted glycemia or fructosamine levels was observed (Fig. 8A-C), an improvement in OGTT was documented (Fig. 8D).

The higher sensitivity to exogenous insulin of DogsGck was however evidenced when OGTT was performed with simultaneous subcutaneous injection of insulin. Both DogGck had a faster glucose disposal than diabetic control dogs, with glycemia dropping below 200 mg/dl in both animals (Fig. 8E). Increased sensitivity to exogenous insulin was also observed in diabetic mice treated with AAV1-oGck (Supplementary Fig. 6).
DISCUSSION

Since the 1922 breakthrough discovery of Banting and Best, who corrected hyperglycemia in dogs using pancreatic extracts, exogenous insulin administration has been the mainstay of diabetes therapy. Alternative therapies have been studied, but thus far only a handful of approaches, mainly involving allo- or xeno-transplantation of pancreatic islets, have reached clinical application (2). In the clinical translation of bench results, the scale up to a large animal model represents perhaps one of the most critical steps. This was nicely demonstrated by the work done in gene transfer for hemophilia B (12,25-27) and Leber’s Congenital Amaurosis (13,28), where results in dogs were fully predictive of the outcome in humans.

Our novel approach to control hyperglycemia, through genetic engineering of a “glucose sensor” in skeletal muscle using AAV vectors, has permitted long-term, clinically meaningful regulation of glycemia in a large animal model of diabetes. Currently, the goal of normalization of glycemia is pursued through intensive insulin therapy, which can delay the onset and slow the progression of secondary complications of diabetes (1). However, this treatment is not suitable for all diabetic patients because of its high risk of hypoglycemia secondary to excessive insulin dosage (1). Additionally, cell therapies, including cadaveric and stem cell-derived islet transplantation, require life-long immunosuppression (2,29). Our approach circumvents a number of the challenges of diabetes therapy. In contrast to allotransplantation where supplies of human pancreatic islets are limiting, AAV vector manufacturing is robust and unlimited. Moreover, long-term (>10 years) transgene expression has been documented in humans following AAV vector administration to skeletal muscle (30).
The intramuscular delivery of AAV vectors to engineer the skeletal muscle results in expression of both transgenes in the target tissue, and not in the liver, the other tissue where vector genomes are detected, probably due to the silencing of the CMV promoter (31). Our results support a model in which the production of insulin in skeletal muscle activates insulin signaling in an autocrine/paracrine manner, and the constant insulin production deriving from AAV-Ins transduced fibers is crucial for the system to work as a glucose sensor. Low, basal levels of insulin are required to keep muscle capable of uptaking glucose by ensuring continuous GLUT4 translocation to the plasmatic membrane (9). Thus, glucose transport does not become a rate-limiting step to the system in front of a glucose challenge. On the other hand, due to the absence of GKRP in muscle, the Gck produced in this tissue through gene transfer remains active in the cytosol (24). As a result, in a situation of hyperglycemia, Gck phosphorylates glucose efficiently, driving the uptake of large amounts of glucose through GLUT4 into the engineered muscle fibers. Then, AAV1-Ins alone is able to maintain a good control of fasted glycemia, but AAV1-Gck is required to cope with a glucose overload, as shown by the OGTTs. The advantage of expressing both genes in the same muscle cell would be that, by acting in an autocrine/paracrine manner, the levels of insulin required to achieve sufficient GLUT4 expression in the muscle fiber are low, and therefore safe. Regarding the systemic action of insulin derived from the skeletal muscle, we have previously demonstrated that transgenic mice expressing Ins+Gck under the control of a muscle-specific promoter or diabetic mice treated with AAV1-Ins+GcK gene transfer have restored Gck and markedly reduced PEPCK expression in the liver (9). PEPCK is the most important enzyme in the control of gluconeogenesis, and is regulated mainly at transcriptional level, being its expression upregulated in starvation or diabetes (32). This evidence suggests that hepatic
gluconeogenesis is reduced in Ins+Gck-treated diabetic animals when compared to untreated diabetic controls, which would contribute to a better glycemic control.

Insulin alone, either provided as exogenous therapy or expressed in muscle by an AAV vector, did induce glucose uptake, however it did not guarantee a tight control of glycemia, especially after a glucose challenge. Expression of Gck alone did not correct hyperglycemia either. Diabetic dogs treated with high doses of AAV1-Gck were hyperglycemic in fasting conditions. However, expression of Gck in muscle results in greater sensitivity to insulin, either when insulin is exogenously administered or when it is expressed at low, safe levels from an AAV vector. Furthermore, even if an intensive exogenous insulin treatment is adjusted on an individual basis for each diabetic control or AAV-Gck-treated dog, the glycemic control achieved by Ins+Gck gene therapy is superior, as evidenced by the lower fasted glycemia and fructosamine levels, and improved glucose disposal in AAV-Ins+Gck-treated animals. Thus, only the synergistic action of Ins and Gck allows for tight control of diabetic hyperglycemia.

Skeletal muscle has a number of key advantages as a target tissue for AAV-mediated gene transfer. Muscle is easily accessible by non-invasive procedures, and vector delivery leads to minimal systemic biodistribution (Supplementary Table 2). Moreover, gene delivery to muscle is not limited by the presence of pre-existing neutralizing antibodies against AAV (33), a key aspect given the relatively high prevalence of anti-AAV antibodies in the general population (34). In addition, muscle has a metabolism highly based on glucose consumption and can efficiently secrete proteins into the bloodstream. Insulin can be produced, processed and secreted as a mature protein from skeletal muscle, provided the gene has a genetic modification allowing the cleavage by the
endoprotease furin (9). We have been unable to detect insulin within muscle fibers of transgenic or AAV-treated mice, suggesting that the insulin produced in muscle fibers is not accumulated in vesicles and that its secretion is unlikely to be regulated by vesicle-mediated exocytosis (as in pancreatic beta cells). In contrast, secretion from the skeletal muscle seems to be constitutive.

Long-term follow-up of treated dogs suggests that muscle expression of Ins and Gck is well tolerated over a prolonged period. Studies confirmed the safety of the approach even under marked physical exertion, when high levels of glucose consumption increase the risk of hypoglycemic episodes. The use of a large animal model with a long lifespan allowed us to follow animals for early indicators of secondary complications. The absence of clinical findings such as cataracts or urinary tract infection, and the reduction of biomarkers such as glycosylated proteins (fructosamine), suggest that Ins and Gck gene transfer may prevent diabetes complications. Hence, normalization of glycemia with a one-time intervention could result in a substantial improvement in patients’ quality of life, particularly in populations with difficulties in diabetes management, such as brittle diabetes (35).

One possible limitation of the results presented here is that the dog model of diabetes used in this study does not fully mimic the immunological state of type 1 diabetic patients. However, while future studies in autoimmune models of diabetes are warranted, studies in mice (36), dogs (37), and humans (38) would suggest that targeting muscle with AAV vectors may at least partially escape immune recognition. This may be the result of lower levels of MHC class I presentation in this tissue, or the result of the induction of apoptosis of reactive T cells (36,38).
In summary, our data represent the first demonstration of long-term correction of diabetes in a large animal model using gene transfer. Future safety and efficacy studies will help to determine the range of Ins and Gck vector doses that are therapeutic, as well as the Gck/Ins expression ratio that is optimal for a tight control of glycemia. These studies will provide the bases for the initiation of a clinical veterinary study in companion animals with diabetes, a strategy also proposed for the clinical development of cancer therapeutics (39). The proposed clinical trial in diabetic pet dogs will greatly help defining the safety and efficacy profile of our approach in humans. One added advantage of this strategy is also related to the fact that large experimental animal models of autoimmune diabetes do not exist, thus companion animals with naturally occurring diabetes constitute an extremely valuable and stringent model. In conclusion, this study lays the foundation for the clinical translation of this approach to veterinary medicine, and possibly to humans.
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Conflict-of-interest statement:

F.B., D.C. and E.A. are inventors in a patent application regarding the use of insulin and glucokinase treatment of diabetes.
F.M. and K.A.H. are inventors in patent application on the AAV vector technology.

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FIGURE LEGENDS

FIG. 1. Glycemic control by exogenous insulin. (A-D) Follow-up of fasting glycemia and body weight of diabetic control dogs (Dog-Db1-4) daily treated with exogenous insulin (dosage (IU) and timing (24h vs. 12h) is shown). Db, dog treatment with STZ+aloxan. A, indicates the period of adjustment of the insulin dosing regime. Grey bar: fasting normoglycemia range in dogs (20).

FIG. 2. Fructosamine levels and glucose disposal after a load in dogs treated with exogenous insulin. (A) Monitoring of serum fructosamine in DogDb1-4 receiving exogenous insulin every 24 or 12 h. The fructosamine upper limit for good diabetes control in dogs is indicated with dashed lines. According to (20), fructosamine concentrations between 350-400µmol/L indicate an excellent glycemic control, 400-450µmol/L indicate a good glycemic control, 450-500µmol/L indicate a fair glycemic control and >500µmol/L indicate a poor glycemic control. Results are shown as mean±SEM of 2-4 determinations. (B) OGTT were performed at 1.75g/kg glucose before (Healthy) and after diabetes induction in DogDb1-3 treated with exogenous insulin every 24 h (left panel) or in DogDb1, 2 and 4 receiving insulin every 12 h (right panel). Mean±SEM, n=3.

FIG. 3. Glycemic control by AAV-Ins alone. (A and B) Diabetic dog treated with AAV1-Ins (1x10^{12} vg/kg) (DogIns) showed partial normalization of fasting blood glucose levels, (A) and recovery of body weight loss (B). (C) Fructosamine monitoring in DogIns. Results are shown as mean±SEM of >4 measurements/year. (D) Fasting insulinemia of DogIns. Dashed lines: average maximum and minimum fasting insulinemia values obtained by random measurements in 6 healthy dogs. (E) No major improvement was observed in the
ability of DogIns to dispose of glucose during an OGTT at 1.75g/kg performed 4 years after gene transfer when compared to diabetic dogs under optimized exogenous insulin treatment. Db, dog treatment with STZ+alloxan. Grey bar: fasting normoglycemia range in dogs (20).

**FIG. 4.** Treatment with AAV1-Ins and AAV1-Gck corrects diabetes in dogs. (A-D) Follow up of glycemia, body weight and insulinemia. Five diabetic dogs (Dog1-4 and DogDb3+Ins/Gck) were treated with AAV1-Ins and AAV1-Gck vectors at $1 \times 10^{12}$ vg/kg each, Dog1 and 2 (A and B) and at $2 \times 10^{12}$ vg/kg each, Dog3 and DogDb3+Ins/Gck (C and D), or with AAV1-oIns and AAV1-oGck vectors at $1 \times 10^{12}$ vg/kg each, Dog4 (E). Dogs had serum insulin levels that remained within the range of fasted healthy animals (dashed lines). Db, dog treatment with STZ+alloxan. Grey bars: fasting normoglycemia range in dogs (20).

**FIG. 5.** AAV1-Ins and AAV1-Gck treatment normalizes serum fructosamine and recovers glucose disposal after a load. (A) Dog1-4 and DogDb3+Ins/Gck had levels of serum fructosamine that ranged from 200 to 350 $\mu$mol/l. (B) OGTT at a dose of glucose of 1.75g/kg. Ins+Gck-treated dogs showed a glucose profile similar to healthy animals and below the range for diabetes diagnosis according to ADA guidelines (2h plasma glucose <200 mg/dl). Data are represented as mean ± SEM of 2-3 OGTT performed every year or during the study period, in dogs with shorter follow-up. OGTT for diabetic control dogs (red line) were performed during the period of intensive exogenous insulin treatment.

**FIG. 6.** Effect of exercise on glycemia. Blood glucose was measured in 24-hour fasted dogs before, immediately after exercise and during post-exercise recovery. None of the
dogs was hypoglycemic over the periods analyzed. Dog1-4 and DogDb3+Ins/Gck showed good glycemic control under exercise, comparable to that of healthy dogs. A drop in blood glucose was observed after exercise in dogs treated with AAV1-Ins or with AAV1-oGck vectors only; these dogs had baseline glucose levels much higher than all other animals treated with AAV1-Ins and AAV1-Gck vectors.

**FIG. 7.** Skeletal muscle is the source of insulin after AAV1-Ins and AAV1-Gck treatment. (A) Transgene expression in skeletal muscle of AAV-treated dogs. Northern blot analysis showing expression of insulin (Ins) and glucokinase (Gck) in skeletal muscle biopsy specimens of DogIns and Dog1, and necropsy samples of Dog3, obtained 9 months and 2.2 years post treatment, respectively. Uninjected muscle was used as a control (Con). (B) Measurement of Gck activity in skeletal muscle of healthy (H) dog and two different necropsy samples of Dog3. (C) β-cell area was quantified in necropsy samples (whole pancreas) from a healthy (H) dog, a diabetic dog (Db) and Dog3 (2.2 years post-treatment), and in pancreas biopsies from Dog1, Dog2 and DogIns obtained 9 months after AAV administration. Diabetes induction led to a >90% reduction in β-cell mass in all dogs. (D) Representative images of pancreatic sections stained with insulin (red) and glucagon (green) illustrating the marked reduction of insulin-producing cells in Dog3 by 2.2 years after diabetes induction. Original magnification 200X. (E) Human C-peptide was detectable in serum of all AAV1-Ins treated dogs, but not in healthy (H) or untreated diabetic (Db) dogs, indicating that proinsulin was produced and processed in skeletal muscle. ND, non detected. Results are shown as mean ± SEM (n=23-56 measurements per dog). (F) Time-course of insulin secretion after a meal 4 years after AAV treatment of Dog1, Dog2 and DogIns. Basal levels correspond to a 24-hour fasting period, after which animals were fed a 30 g/kg serving of standard diet. Only the healthy dogs showed the first
peak of insulin release, which corresponds to pancreatic secretion of the insulin stored in secretory granules. (G) Normal circulating glucagon levels were observed in AAV1-Ins and AAV1-Gck treated dogs indicating preservation of α-cell function. Results are shown as mean ± SEM (n=5-6 measurements per dog).

**FIG. 8.** Diabetic dogs treated with AAV1-oGck alone. (A and B) Follow up of glycemia and body weight of diabetic dogs treated with AAV1-oGck vectors (2x10^{12} vg/kg), DogGck1 (A) and DogGck2 (B). DogGck remained hyperglycemic during fast and required daily administration of exogenous insulin (dosage (IU) and timing (24h vs. 12h) is shown). (C) Fructosamine levels were altered in both DogGck receiving exogenous insulin every 24 or 12 h. Results are shown as mean±SEM 2-4 determinations. (D) Both DogGck showed similar OGTT profile at 1.75g/kg to diabetic untreated dogs when administered with exogenous insulin once daily. Upon implementation of the optimized treatment, OGTT improved. (E) The synergistic effect of Gck and insulin. DogGck1 and DogGck2 and diabetic control dogs were given a glucose load (1.75g/kg) together with a subcutaneous injection of insulin (6 IU). Data are represented as mean ± SEM of 3 OGTT. Db, dog treatment with STZ+alloxan. Grey bars: fasting normoglycemia range in dogs (20).
Figure 1

Diabetes

A

Insulin (IU/h)

6/24
A
3+2/12

DogDb1

Glucose (mg/dl)

0 50 100 150 200 250

Time (days)

Db

100

Weight (kg)

6 7 8 9 10 11 12 13 14

6/24
A
3+2/12

B

Insulin (IU/h)

6/24
A
8+6/12

DogDb2

Glucose (mg/dl)

0 50 100 150 200 250

Time (days)

Db

100

Weight (kg)

6 7 8 9 10 11 12 13 14

C

Insulin (IU/h)

3/12
4/12
5/12
6/24
5/24
A4/12

DogDb3

Glucose (mg/dl)

0 20 40 60 80 100 120 140

Time (days)

Db

100

Weight (kg)

6 7 8 9 10 11 12 13 14

D

Insulin (IU/h)

A
5+4/12

DogDb4

Glucose (mg/dl)

0 10 20 30 40 50 60 70 80

Time (days)

Db

100

Weight (kg)

6 7 8 9 10 11 12 13 14
**Figure 2**

A. Fructosamine (µmol/l) levels for different groups over time:

- Healthy
- DogDb1
- DogDb2
- DogDb3
- DogDb4

B. Glucose (mg/dl) levels over time for different insulin conditions:

- Insulin/24h
- Insulin/12h

Graphs show the variation in glucose and fructosamine levels across different time points and conditions.
**Dog1**
(AAV1-Ins+AAV1-Gck 1x10^{12} vg/kg)

**Dog2**
(AAV1-Ins+AAV1-Gck 1x10^{12} vg/kg)

**Dog3**
(AAV1-Ins+AAV1-Gck 2x10^{12} vg/kg)

**DogDb3+Ins/Gck**
(AAV1-Ins+AAV1-Gck 2x10^{12} vg/kg)

**Dog4**
(AAV1-oIns+AAV1-oGck 1x10^{12} vg/kg)
Figure 5

### A

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<th>Healthy</th>
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<th>Dog2</th>
<th>Dog3</th>
<th>DogDb3+Ins/Gck</th>
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**Time (years)**

### B

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</table>

- **Healthy**
- **Diabetic**
- **Dog1**
- **Dog2**
- **Dog3**
- **DogDb3+Ins/Gck**
- **Dog4**
Diabetes

Figure 6

Glucose (mg/dl)

- Resting
- Post exercise
- Recovery

H, Dog1, Dog2, Dog3, DogDb3+Ins/Gck, Dog4, DogIns, DogGck1, DogGck2
**Figure 7**

Diabetes

Panel A: Autoradiographic analysis of Gck activity in different genotypes. 

Panel B: Gck activity (mU/mg) comparison between H and Dog3 genotypes.

Panel C: β-cell area/pancreas area (% of healthy).

Panel D: Fluorescence images of healthy and Dog3 pancreas.

Panel E: Human C-peptide (ng/ml) levels in different genotypes.

Panel F: Insulin (ng/ml) levels over time (min) for healthy and Dog3 genotypes.

Panel G: Glucagon (pg/ml) levels for different genotypes.
SUPPLEMENTARY FIGURE LEGENDS

**Supplementary Fig. 1.** Efficient transduction of canine skeletal muscle with AAV1 vectors. (A) A 5-prong needle syringe was used as vector administration device. (B) Intramuscular delivery of the AAV1 vectors with this device results in efficient transduction of a large number of muscle fibers. AAV1-CMV-GFP vectors were injected at a dose of 5x10^{11}vg/injection site; representative images of GFP immunostaining are shown. Original magnification 200X.

**Supplementary Fig. 2.** OGTT at a dose of glucose of 3g/kg in Ins+Gck-treated dogs showed improved glucose disposal, lower peak glycemia and 2h glycemia dropped below 200 mg/dl. Data are represented as mean ± SEM of 2-3 OGTT performed every year during the study period. * glucose levels >600mg/dl.

**Supplementary Fig. 3.** Insulin signaling in skeletal muscle of diabetic mice treated with AAV-Ins and AAV-Ins+Gck. Two weeks after diabetes induction mice were injected i.m. with 2x10^{12}vg/kg of AAV1-null, AAV1-Ins or AAV1-Ins+GcK vectors. Fourteen weeks after vector administration the content of phospho-Akt (p-Akt) and total Akt was measured by Western blot in injected muscle samples. α-Tubulin was used as load control. Representative images of the Western blots are shown in the left panel. The right panel shows the ratio of p-Akt/Akt obtained from densitometric analysis of the western blots (n=4 per group). * p<0.05 vs AAV1-null

**Supplementary Fig. 4.** Subcellular localization of Gck. Healthy mice were injected i.m. with 4x10^{12} vg/kg of AAV1-null or AAV1-oGck. Three weeks after vector delivery One
group of AAV1-oGck-treated mice was injected with 3g/kg of glucose and sacrificed 15 min later (shown as + glucose). The rest of the animals were sacrificed in starved conditions. Skeletal muscles (A) and liver (B) were fractioned in nuclear and cytoplasmic portions as indicated in Research Design and Methods. Left panels show representative images of Western blots for glucokinase (Gck), α-tubulin (a cytoplasmic protein) and histone3 (nuclear protein). Left panels show the ratio of Gck protein that was detected in the cytoplasmic fraction (identified as the fraction enriched in α-tubulin) vs. Gck that was detected in the nucleic fraction (identified as the fraction enriched in Histone 3. n=2, per group. ND, not detected. N, nucleic fraction. C, cytoplasmic fraction.

Supplementary Fig. 5. Integrity of muscle structure after AAV1-Ins and AAV1-Gck gene transfer. Morphologic analysis performed on skeletal muscle biopsies of DogIns obtained 9 months after AAV1 vector administration and on necropsy samples from Dog3 (2.2 years after treatment). No signs of muscle pathology or inflammation were observed by hematoxylin and eosin (H&E) staining or by laminin immunodetection. Periodic acid Schiff (PAS)-staining showed normal levels of glycogen storage. Original magnification 200X.

Supplementary Fig. 6. Expression of Glucokinase in skeletal muscle increases insulin sensitivity. STZ-treated mice were intramuscularly administered with either AAV1- oGck or AAV1-null (non-coding) vectors (4x1012 vg/kg). (A) Glucokinase activity was detected in the skeletal muscle of mice injected with AAV1-oGck 3-months after vector administration. (B) Blood glucose profile of diabetic mice treated with AAV-oGck or AAV-null vectors. Glycemia was determined in fed conditions in the absence of exogenous insulin treatment. (C) Insulin sensitivity was determined in diabetic mice 3-
months after treatment with AAV-oGck or AAV-null vectors. Fed mice were given an intraperitoneal injection of insulin (0.75IU/kg, Humulin regular, Eli Lilly) and glycemia was followed for 180 min. In contrast to diabetic null mice that remained highly hyperglycemic, Gck-expressing diabetic mice showed marker reduction of glycemia. Results are expressed as the mean ± S.E.M. of n=12 mice per group. *, indicates significant difference with p<0.05.

SUPPLEMENTARY TABLE LEGENDS

Supplementary Table 1. Summary of the experimental groups and the time of follow-up of each dog. N.A., not applicable.

Supplementary Table 2. Evaluation of secondary complications in AAV-treated and untreated diabetic dogs. Dogs were periodically monitored at the Veterinary Clinical Hospital at UAB. This Table summarizes the secondary complications described in companion diabetic dogs and the evaluation of these complications in AAV-treated and untreated diabetic dogs throughout the study over a period of 4 years. *Dog3 showed moderate azotemia immediately after STZ and Alloxan administration, compatible with a toxic effect of these drugs. No proteinuria or urinary tract infections were detected in Dog3 for the duration of the study.

Supplementary Table 3. AAV1 vector genome biodistribution in Dog3. Systemic biodistribution of vector genomes in Dog3 2.2 years after the administration of 2.0x10^{12} vg/kg of AAV1-Ins and AAV1-Gck vectors. Most of the detectable vector was found in injected muscles. Low vector gene copy numbers were detected in most peripheral tissues.
Expression of insulin in the liver was undetectable (data not shown) despite the detection of vector genomes. This is likely due to silencing of the CMV promoter in liver.

**Supplementary Table 4. Glycogen content in skeletal muscle biopsies.** Glycogen content in skeletal muscle was determined in necropsy samples from an uninjected control (Con) dog and from Dog3 (2.2 years post-treatment), and in muscle biopsies from DogIns and from Dog1 obtained 9 months after AAV1 administration. Results are mean±SEM of 3 samples for dog Con and Dog3 samples obtained from different muscle areas. Results for DogIns and Dog1 correspond to a single biopsy sample.

**SUPPLEMENTARY VIDEO LEGENDS**

**Supplementary Video 1.** Dog exercise test. Fasted dogs (24 h) were subjected to 37-minute exercise under increasing speed and slope in a variable speed belt treadmill.

**Supplementary Video 2.** Dog2 before and after combined AAV1-Ins and AAV1-Gck treatment. One month after development of hyperglycemia and without exogenous insulin treatment, Dog2 showed apathetic behavior and significant cachexia. Seven months after treatment with AAV1-Ins and AAV1-Gck, Dog2 restored normoglycemia and recovered weight. Accordingly, the dog behaved more actively, like healthy control dogs.
Supplementary Figure 3

AAV1-null  AAV1-Ins  AAV1-Ins+Gck

p-Akt

Akt

α-Tubulin

pAKt/Akt levels (relative to healthy)

AAV1-null  AAV1-Ins  AAV1-Ins+Gck

*  *

For Peer Review Only
A

Skeletal muscle

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<td>N</td>
<td>C</td>
<td>N</td>
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<tr>
<td>Gck</td>
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<tr>
<td>α-Tubulin</td>
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Gck Citoplasm/nucleus (fold change)

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B

Liver

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Gck Citoplasm/nucleus (fold change)

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ND
Supplementary Figure 5

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Supplementary Table 1. Experimental design and follow-up time.

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<th>Dose of AAV vector</th>
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<td>DogDb2</td>
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<td>DogDb4</td>
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<td>2.6</td>
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<tr>
<td>DogIns</td>
<td>AAV1-Ins</td>
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<td>53.3</td>
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<tr>
<td>Dog1</td>
<td>AAV1-Ins + AAV1-Gck</td>
<td>1x10^{12} vg/kg (each vector)</td>
<td>53.3</td>
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<tr>
<td>Dog2</td>
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<td>53.3</td>
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<tr>
<td>Dog3</td>
<td>DogDb3+Ins/Gck</td>
<td>2x10^{12} vg/kg (each vector)</td>
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N.A., Not applicable.
Supplementary Table 2. Evaluation of secondary complications in AAV-treated and untreated diabetic dogs.

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<th>Complication (Frequency in diabetic companion dogs)</th>
<th>Dog</th>
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<td><strong>Ocular complications</strong></td>
<td>Detected in DogDb1-3, DogGck1 and DogGck2 soon after diabetes induction with worsening over follow up period.</td>
</tr>
<tr>
<td><strong>Cataract</strong> (Common)</td>
<td>Not detected in DogDb4, DogIns and Dog1-Dog4.</td>
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<tr>
<td><strong>Uveitis</strong> (Common, secondary to cataract)</td>
<td>Not detected</td>
</tr>
<tr>
<td><strong>Retinopathy</strong> (Uncommon)</td>
<td>Not detected</td>
</tr>
<tr>
<td><strong>Urinary tract infection</strong></td>
<td>Not detected</td>
</tr>
<tr>
<td>(Common)</td>
<td></td>
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<tr>
<td><strong>Nephropathy</strong></td>
<td>Not detected in DogDb1-4, DogIns, Dog1, Dog2, Dog4, DogDb3+Ins/Gck, DogGck1 and DogGck2. Drug-induced moderate azotemia in Dog3*.</td>
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<tr>
<td><strong>Azotemia</strong> (Uncommon)</td>
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<tr>
<td><strong>Proteinuria</strong> (Uncommon)</td>
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<tr>
<td><strong>Clinical Peripheral Neuropathy</strong></td>
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<tr>
<td>Weakness, knuckling, abnormal gait, muscle atrophy, depressed limb reflexes, deficits in postural reaction testing (Uncommon)</td>
<td></td>
</tr>
</tbody>
</table>

Dogs were periodically monitored at the Veterinary Clinical Hospital at UAB. This Table summarizes the secondary complications described in companion diabetic dogs and the evaluation of these complications in AAV-treated and untreated diabetic dogs throughout the study over a period of 4 years. *Dog3 showed moderate azotemia immediately after STZ and Alloxan administration, compatible with a toxic effect of these drugs. No proteinuria or urinary tract infections were detected in Dog3 for the duration of the study.
Supplementary Table 3. AAV1 vector genome biodistribution in Dog3.

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<th>Tissue</th>
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<tr>
<td>Kidney (medulla)</td>
<td>0.38</td>
</tr>
<tr>
<td>Lung (right middle lobe)</td>
<td>0.67</td>
</tr>
<tr>
<td>Lung (right caudal lobe)</td>
<td>0.61</td>
</tr>
<tr>
<td>Heart (left ventricle)</td>
<td>0.19</td>
</tr>
<tr>
<td>Heart (left atrium)</td>
<td>0.22</td>
</tr>
<tr>
<td>Gonads (testis)</td>
<td>0.04</td>
</tr>
<tr>
<td>Liver (left lateral lobe)</td>
<td>1.15</td>
</tr>
<tr>
<td>Liver (quadrate lobe)</td>
<td>1.12</td>
</tr>
<tr>
<td>Liver (right medial lobe)</td>
<td>0.40</td>
</tr>
<tr>
<td>Liver (right lateral lobe)</td>
<td>0.88</td>
</tr>
<tr>
<td>Liver (caudate process)</td>
<td>0.55</td>
</tr>
<tr>
<td>Liver (papillary process)</td>
<td>0.81</td>
</tr>
<tr>
<td>Pancreas (left lobe)</td>
<td>0.15</td>
</tr>
<tr>
<td>Pancreas (right lobe)</td>
<td>0.03</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.57</td>
</tr>
</tbody>
</table>

Systemic biodistribution of vector genomes in Dog3 2.2 years after the administration of 2.0x10^{12} vg/kg of AAV1-Ins and AAV1-Gck vectors. Most of the detectable vector was found in injected muscles. Low vector gene copy numbers were detected in most peripheral tissues. Expression of insulin in the liver was undetectable (data not shown) despite the detection of vector genomes. This is likely due to silencing of the CMV promoter in liver.
Supplementary Table 4. Glycogen content in skeletal muscle biopsies.

<table>
<thead>
<tr>
<th>Dog</th>
<th>Glycogen (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con</td>
<td>3.31 ± 0.55</td>
</tr>
<tr>
<td>DogIns</td>
<td>0.910</td>
</tr>
<tr>
<td>Dog1</td>
<td>2.711</td>
</tr>
<tr>
<td>Dog3</td>
<td>2.76 ± 0.47</td>
</tr>
</tbody>
</table>

Glycogen content in skeletal muscle was determined in necropsy samples from an uninjected control (Con) dog and from Dog3 (2.2 years post-treatment), and in muscle biopsies from DogIns and from Dog1 obtained 9 months after AAV1 administration. Results are mean±SEM of 3 samples for dog Con and Dog3 samples obtained from different muscle areas. Results for DogIns and Dog1 correspond to a single biopsy sample.