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Urocortin 2 Gene Transfer Improves Glycemic Control and Reduces Retinopathy and Mortality in Murine Insulin Deficiency

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Type 1 diabetes affects 20 million patients worldwide. Insulin is the primary and commonly the sole therapy for type 1 diabetes. However, only a minority of patients attain the targeted glucose control and reduced adverse events. We tested *urocortin 2* gene transfer as single-agent therapy for insulin deficiency using two mouse models. *Urocortin 2* gene transfer reduced blood glucose for months after a single intravenous injection, through increased skeletal muscle insulin sensitivity, increased insulin release in response to glucose stimulation, and increased plasma insulin levels before and during euglycemic clamp. The combined increases in both insulin availability and sensitivity resulted in improved glycemic indices—events that were not anticipated in these insulin-deficient models. In addition, *urocortin 2* gene transfer reduced ocular manifestations of long-standing insulin deficiency such as vascular leak and improved retinal function. Finally, mortality was reduced by *urocortin 2* gene transfer. The mechanisms for these beneficial effects included increased activities of AMP-activated protein kinase and Akt (protein kinase B) in skeletal muscle, increased skeletal muscle glucose uptake, and increased insulin release. These data suggest that *urocortin 2* gene transfer may be a viable therapy for new onset type 1 diabetes and might reduce insulin needs in later stage disease.

INTRODUCTION

Type 1 diabetes (T1D) affects 1.3 million patients in the US with 40,000 new patients annually.[1] An estimated 20 million are affected by T1D worldwide.[2] Lifespan is shortened 11–13 years[3] due to kidney and heart disease. Tight glucose control reduces microvascular complications and adverse cardiovascular events.[4,5] Insulin therapy is essential for such patients, but has shortcomings. For example, only a minority of patients achieve targeted glucose control,[5] and aggressive insulin therapy increases serious hypoglycemic episodes, which shorten life.[6] In addition, the majority of T1D patients develop insulin resistance.[7–9] Cardiovascular risk is 2.5-fold higher in T1D patients with insulin resistance versus those with normal insulin sensitivity.[7] Despite these shortcomings, insulin remains the sole therapy currently recommended by the American Diabetes Association[10]—and one which has been used for nearly 100 years.[11] In the present study, we tested a new approach to address these shortcomings of T1D therapy, using rodent models of insulin deficiency.

Recent clinical trials have tested the efficacy and safety of a wide range of non-insulin agents, used in combination with insulin in T1D, to improve glycemic control, reduce insulin requirements and weight gain, and to lessen the frequency of serious hypoglycemic episodes. A recent review of these clinical trials reveals modest reductions in HbA1c and daily insulin requirements, but often at a cost of undesirable side-effects.[12] Only pramlintide has FDA approval as a co-treatment with insulin but it is not widely used. An ideal adjunct to insulin would reduce insulin needs, require infrequent administration, reduce weight gain, and favorably affect heart function. *Urocortin 2* (*Ucn2*) gene transfer fulfills these criteria in insulin resistant mice.[13] In the present study, we ask whether *Ucn2* gene transfer—in the absence of exogenous insulin—can normalize glycemic control in two murine models of insulin deficiency.

Gene transfer is justified because there is an unmet medical need for treating T1D more effectively and in a manner that promotes patient compliance, and because T1D is associated with high morbidity and mortality. We propose intravenous (i.v.) delivery of a vector encoding a transgene with beneficial paracrine and endocrine actions (Figure 1A). This strategy could enable patients to be treated during an office visit by a one-time injection of the vector, a feature that would increase compliance and reduce costs. The best vector to achieve these goals is adeno-associated virus type 8 (AAV8), encoding *Ucn2*. Ucn2, a recently discovered peptide, acts via corticotropin-releasing

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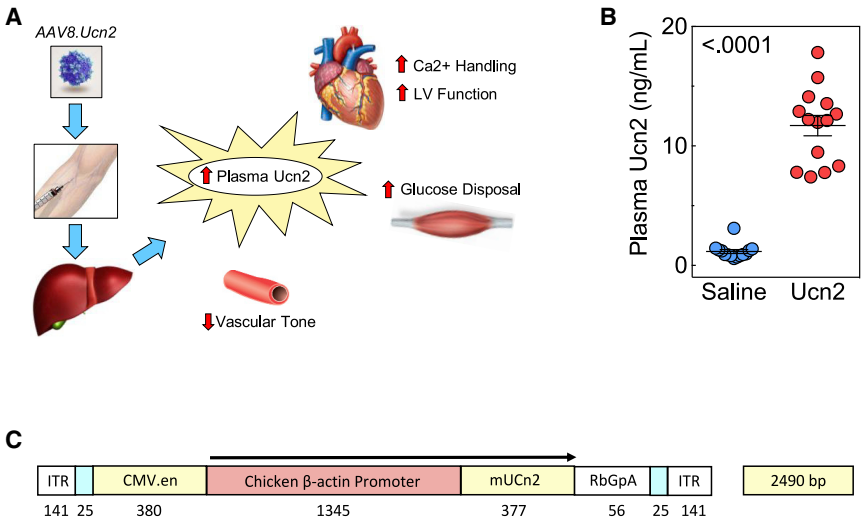

Figure 1. Gene Transfer of Peptides with Beneficial Paracrine and Endocrine Actions: *Ucn2* Gene Transfer

(A) A one-time i.v. injection of an adeno-associated virus 8 encoding *urocortin 2* (AAV8.*Ucn2*) exploits paracrine and endocrine actions of Ucn2, which increase glucose disposal, insulin sensitivity, and cardiovascular function. (B) i.v. delivery of AAV8.m*Ucn2* (n = 14) or saline (n = 14) to Akita mice. Data obtained 2 months after delivery to 2-month-old mice shows a 9.8-fold increase in plasma Ucn2 ($p < 0.0001$, Student's t test, unpaired, two-tailed; error bars denote SE). Vector dose was 2×10^{13} genome copies/kg, i.v. in all studies. (C) AAV8.m*Ucn2* vector map. ITR, inverted terminal repeat; CMV.en, human cytomegalovirus enhancer; m*Ucn2*, murine *urocortin 2*; RbGpA, rabbit beta-globin poly(A). Data are presented as mean ± SE.

hormone type 2 receptors (CRHR2), which are expressed in skeletal muscle, heart, gastrointestinal tract, vasculature, and brain.[14] Studies in animals and patients with heart failure have shown favorable cardiovascular effects of brief Ucn2 *peptide* infusions.[15,16]

The goal of the present study was to test the efficacy of this new approach for the treatment of T1D. We used Akita mice, a monogenic nonobese model of insulin deficiency,[17] which is also useful to study the microcirculatory abnormalities of T1D.[18] Confirmatory studies were conducted in mice rendered insulin deficient by streptozotocin (STZ). T1D is a major risk factor for several prevalent life-altering and life-terminating diseases: peripheral vascular disease, stroke, myocardial infarction, and heart failure. The discovery and development of more effective therapies that reduce the prevalence of the cardiovascular and microvascular complications associated with T1D is needed. The goal of the present study was to test a new approach. Our hypothesis was that *Ucn2* gene transfer would, even in the absence of exogenous insulin, increase glycemic control, and reduce retinopathy and mortality.

RESULTS

Ucn2 Gene Transfer in Akita Mice

Vector dose (AAV8.*Ucn2* and AAV8.*Null*) was 2×10^{13} gc/kg, i.v., in all studies. Plasma Ucn2 was increased 9.8-fold ($p < 0.0001$) to a mean value of 11.7 ng/mL (from 1.2 ng/mL) 8 weeks after i.v. delivery of AAV8.*Ucn2* (Figures 1B and 1C). This was associated with pronounced mRNA expression in liver (a 33,000-fold increase) and in left ventricle (651-fold increase). Significant but smaller increases were seen in skeletal muscle, kidney, and pancreas (ranging from 10-fold to 59-fold) (Table S1).

Phenotypic and Metabolic Features: Effects of *Ucn2* Gene Transfer

Cumulative food and water consumption, assessed for 2 months after treatment, were lower in Akita mice that received *Ucn2* gene transfer ($p < 0.0001$ for both; Figures 2A and 2B), but had not returned to the levels seen in non-diabetic control mice. Body weight was increased 2 months after *Ucn2* gene transfer ($p < 0.0002$; Figure 2C), and urine volume was reduced ($p = 0.04$; Figure 2D). Urine albumin-to-creatinine ratio was reduced in Akita mice that received *Ucn2* gene transfer ($p = 0.01$; Figure 2E) but serum creatinine showed no group differences (Figure 2F). Akita mice showed increased plasma glucagon-like peptide (GLP-1) compared to normal mice ($p = 0.001$; Figure 2G). *Ucn2* gene transfer increased GLP-1 levels in normal mice ($p = 0.03$; Figure 2G), but not in Akita mice. Fasting (basal) plasma glucagon levels were higher in Akita mice than in control mice ($p = 0.001$), but glucagon levels were not altered by *Ucn2* gene transfer (Figure 2H). Plasma leptin concentration was unaffected by *Ucn2* gene transfer in either normal mice or in Akita mice (Figure 2I). Non-fasting plasma triglyceride concentrations were lower in Akita mice that had received *Ucn2* gene transfer ($p = 0.0005$; Figure 2J). Metabolic analysis (Table 1) indicated that Akita mice that received *Ucn2* gene transfer showed 10%–11% reductions in the rates of oxygen consumption ($p < 0.0001$) and CO_2 production ($p = 0.0004$) with no change in the respiratory exchange ratio. Activity also was reduced in Akita mice after *Ucn2* gene transfer ($p = 0.019$).

Glucose Disposal

Ucn2 gene transfer reduced fasting ($p < 0.0002$; Figure 3A) and random glucose levels ($p < 0.0003$; Figure 3B) and was associated with reduced HbA1c ($p < 0.005$; Figure 3C), indicating reduced hyperglycemia. There was an enduring beneficial effect of *Ucn2* gene transfer on fasting hyperglycemia in Akita mice, with no evidence of decline for 4 months ($p < 0.0001$; Figure 3D). In addition, glucose tolerance tests showed sustained benefits of *Ucn2* gene transfer on glucose disposal ($p = 0.0006$; Figure 3E) and HbA1c ($p = 0.047$; Figure 3F) 4 months after treatment.

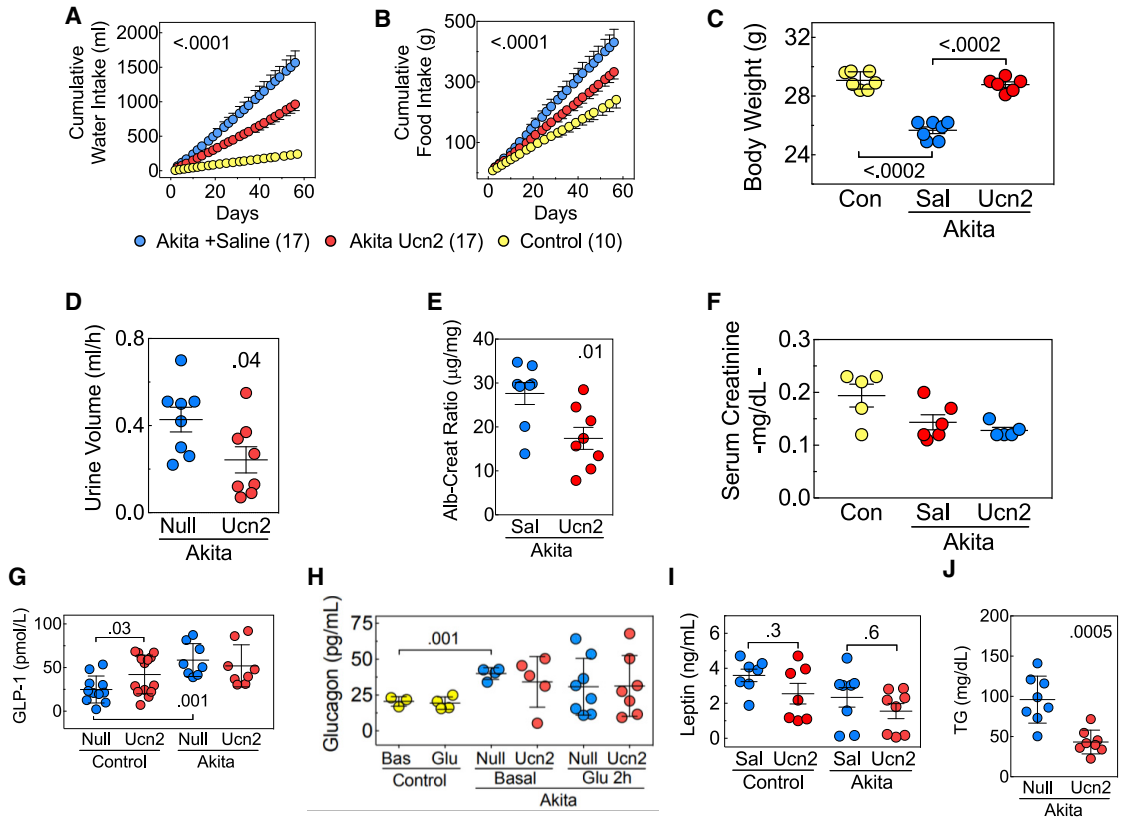

Figure 2. *Ucn2* Gene Transfer Reduced Polydipsia, Polyuria, Hyperphagia, and Hypertriglyceridemia and Normalized Body Weight in Akita Mice; GLP-1, Glucagon, and Leptin Were Unchanged after *Ucn2* Gene Transfer in Akita Mice

(A and B) Cumulative water and food intake were reduced in Akita mice that received *Ucn2* gene transfer. Controls are age-matched male C57BL/6J mice. (C) Body weight was higher after *Ucn2* gene transfer in Akita mice. (D) 3 h urine collection, 2 months after AAV8.Null or AAV8.*Ucn2* delivery to 2-month-old Akita mice, showing reduced urine volume. (E) There was reduced urine albumin-to-creatinine ratio after *Ucn2* gene transfer ($p = 0.01$; repeated twice). (F) Serum creatinine was similar in all three groups. (G) Plasma GLP-1 was increased following *Ucn2* gene transfer in normal control mice ($p = 0.03$), and Akita mice showed increased plasma GLP-1 versus normal mice ($p = 0.001$). However, *Ucn2* gene transfer in Akita mice had no effect on GLP-1 levels. (H) Plasma glucagon was measured before and 2 h after glucose stimulation (2.5 g/kg, oral). Fasting (basal) plasma glucagon was higher in Akita mice than in control mice ($p = 0.001$), but glucagon levels were not altered by *Ucn2* gene transfer in Akita mice. Experiments were repeated 3 times. (I) Plasma leptin was unaffected by *Ucn2* gene transfer in normal mice or in Akita mice. (J) Non-fasting plasma triglyceride (TG) was lower in Akita mice that had received *Ucn2* gene transfer ($p = 0.0005$). Akita mice received saline, AAV8.*Null* or AAV8.*Ucn2*. Mice were 2–4 months old at the time of vector or saline delivery except in (D) (6 months old) and data were collected 1–3 months later. (A and B) p value from two-way ANOVA, for gene effect; (C–J) p values from Student's t test (unpaired, two-tailed) with Bonferroni correction. Group sizes are indicated in graphs. Data are presented as mean ± SE.

Hyperinsulinemic Euglycemic Clamp

Blood glucose was matched from 80–120 min of the clamp (Figure 3G) through a 2.8-fold increase in glucose infusion rate ($p < 0.0001$; Figure 3H). Akita mice that received *Ucn2* gene transfer showed restoration of insulin-induced whole-body glucose clearance with reduced fasting total body glucose disappearance ($p < 0.004$; Figure 3I), increased insulin-induced change in rate of disappearance (Rd) ($p = 0.004$; Figure 3J), reduced hepatic glucose production before ($p = 0.008$) and during the clamp ($p = 0.02$; Figure 3K), and increased skeletal muscle glucose uptake (Figure 3L) in gastrocnemius ($p = 0.004$) and vastus lateralis ($p < 0.0002$).

Insulin Release

We next assessed glucose-stimulated insulin release in 5-month-old Akita mice, 2 months after vector delivery. Mice that received AAV8.Null had fasting plasma insulin levels 87% lower (0.12 ng/mL) than normal (C57BL/6J) mice and were similar to reduced insulin levels previously reported in Akita mice.[19,20] Importantly, Akita mice that received AAV8.Null exhibited a flat insulin response to oral glucose stimulation (Figure 4A). Akita mice that received AAV8.*Ucn2*, in contrast, showed increased plasma insulin ($p < 0.0001$), reaching 3-fold higher levels than those seen in mice that received AAV8.Null ($p = 0.008$) 120 min after glucose administration. Peak insulin concentration was lower than what is seen in normal mice but was sufficient to reduce fasting and random glucose, reduce HbA1c, and increase glucose tolerance (Figures 3A–3F). In addition, Akita mice that had received *Ucn2* gene transfer had higher plasma insulin levels before and 100–120 min after initiation of hyperinsulinemic euglycemic clamps (Figure 4B). Despite higher levels of plasma

Table 1. Metabolic Parameters

	Control				Akita			
	Saline (6)	*Ucn2* (6)	% Δ	p Value	Saline (6)	*Ucn2* (6)	% Δ	p Value
Energy, Kcal/h	0.51 ± 0.02	0.58 ± 0.02	13	<0.0001	0.54 ± 0.02	0.53 ± 0.02	−1.7	0.2
VO_2, ml/kg/h	3,450 ± 101	3,520 ± 94	2	ns	4,646 ± 143	4,129 ± 117	−11	<0.0001
VCO_2, ml/kg/h	3,243 ± 119	3,347 ± 118	3	ns	4,363 ± 183	3,936 ± 135	−10	0.0004
RER	0.94 ± 0.01	0.95 ± 0.01	1	ns	0.96 ± 0.01	0.95 ± 0.01	−0.14	ns
Activity	117 ± 14	136 ± 15	16	0.005	178 ± 18	151 ± 20	−15	0.019

Metabolic effects of *Ucn2* gene transfer in Akita mice. Control mice (C57BL/6J) and Akita mice (2 months old, male) received saline or *Ucn2* gene transfer, and metabolic data collected 2 months later for 6.5 days. Energy expenditure is the heat derived using the formula: $3.815 + 1.23 \times RER \times VO_2$. VO_2, rate of oxygen consumption. VCO_2, rate of carbon dioxide production. RER, respiratory exchange ratio. Activity represents horizontal ambulatory motor activity. Data are mean ± SE. p values are from Student t test (unpaired, two-tailed).

insulin during the glucose clamp studies, insulin levels were increased proportionately in both groups (Saline-Akita, 5.2 ± 0.9-fold pre-infusion insulin; *Ucn2*-Akita, 4.2 ± 0.5-fold pre-infusion insulin; p = 0.41). We also examined the effects of *Ucn2* gene transfer in insulin tolerance tests. *Ucn2* gene transfer was associated with increased glucose disposal in response to insulin (p < 0.001; Figure 4C). Thus, *Ucn2* gene transfer increases insulin release and insulin effectiveness *in vivo*. Finally, to determine whether Ucn2 had a direct effect on islet insulin secretion, we performed studies in isolated islets. Normal mice received i.v. saline versus AAV8.*Ucn2* with subsequent islet isolation, and evaluation of glucose-stimulated insulin release. The incubation of isolated islets with Ucn2 peptide (200 nM) increased insulin secretion from islets, whether isolated from mice that had received *Ucn2* gene transfer or saline (Figure 4D). This confirmed that Ucn2 peptide directly increases glucose-stimulated islet insulin release. These data provide a mechanism for increased glucose-stimulated insulin release in Akita mice that received *Ucn2* gene transfer (Figures 4A and 4B). There were no group differences in basal or Ucn2 peptide-stimulated islet somatostatin release at low or high glucose levels.

Diabetic Retinopathy

Next, we asked whether *Ucn2* gene transfer attenuates retinopathy in Akita mice. Akita mice (4 months old) received saline (n = 5) or AAV8.*Ucn2* (n = 4) and underwent fluorescein angiography 3 months later. Retinal vascular leak was lower in Akita mice that had received *Ucn2* gene transfer (19% ± 5% versus 43% ± 1%; a 56% relative reduction; p = 0.001; Figures 5A and 5B). Normal 6-month-old C57BL/6J mice show retinal vascular leak of 16% ± 3%.[21] A functional evaluation by electroretinography, which measures the electrical response of the retina to flashes of light, confirmed improved retinal function following *Ucn2* gene transfer. Scotopic b-wave response amplitude, corresponding to light-induced activity of the primary rod system, improved >80% following treatment, recovering to levels comparable to normal mice (p = 0.02; Figure 5C).

Mortality

34 Akita mice (2.5 months old) were randomized to receive i.v. saline (n = 17) or *Ucn2* gene transfer (n = 17). Mice that received *Ucn2* gene transfer showed decreased mortality—3 of 17 (18%) of these mice had died 7 months after gene transfer as compared to 16 of 17 (94%) in saline-treated Akita mice (p < 0.0001; Figure 5D). Previous studies have attributed early mortality to extreme hyperglycemia.[19]

Streptozotocin Model of Insulin Deficiency

We then asked whether *Ucn2* gene transfer would be effective in a second model of T1D, using STZ to induce an insulin-deficient state.[22] We found that 5 consecutive daily doses of STZ provided a stable model of insulin deficiency 3 weeks later. *Ucn2* gene transfer was associated with persistent reductions in fasting glucose (p < 0.005; Figure 5E), reduced AUC in glucose tolerance tests (p = 0.005; Figure 5F), and reduced HbA1c 4 weeks after treatment (p < 0.003; Figure 5G). These data indicate that the beneficial effects of *Ucn2* gene transfer on insulin deficiency are not model-limited. We saw a reduction in glucose-stimulated plasma insulin levels 7 weeks after STZ administration (p < 0.0001; Figure 5H), confirming that STZ in the doses and frequency used was associated with reduced plasma insulin levels.

LV Function

Akita mice randomized to i.v. AAV8.*Null* or AAV8.*Ucn2* at 6 weeks of age (hyperglycemia and insulin-deficient present) underwent echocardiography 10 weeks later. Echocardiography showed increased ejection fraction and velocity of circumferential fiber shortening, reductions in end-systolic and end-diastolic dimension (Table 2). Similar changes were seen in control mice after *Ucn2* gene transfer.[23]

Skeletal Muscle Insulin Signaling

The mechanisms for beneficial effects of *Ucn2* gene transfer include both increased islet insulin release and increased insulin sensitivity in skeletal muscle. We therefore examined relevant signaling pathways, including activation of adenosine monophosphate activated protein kinase (AMPK) and Akt (protein kinase B) in skeletal muscle from Akita mice. *Ucn2* gene transfer was associated with increased phosphorylation of Akt at Ser308 (p = 0.005; Figure 6A) and at Ser473 (p = 0.005; Figure 6A). In addition, Akita mice that had received *Ucn2* gene transfer showed higher levels of skeletal muscle

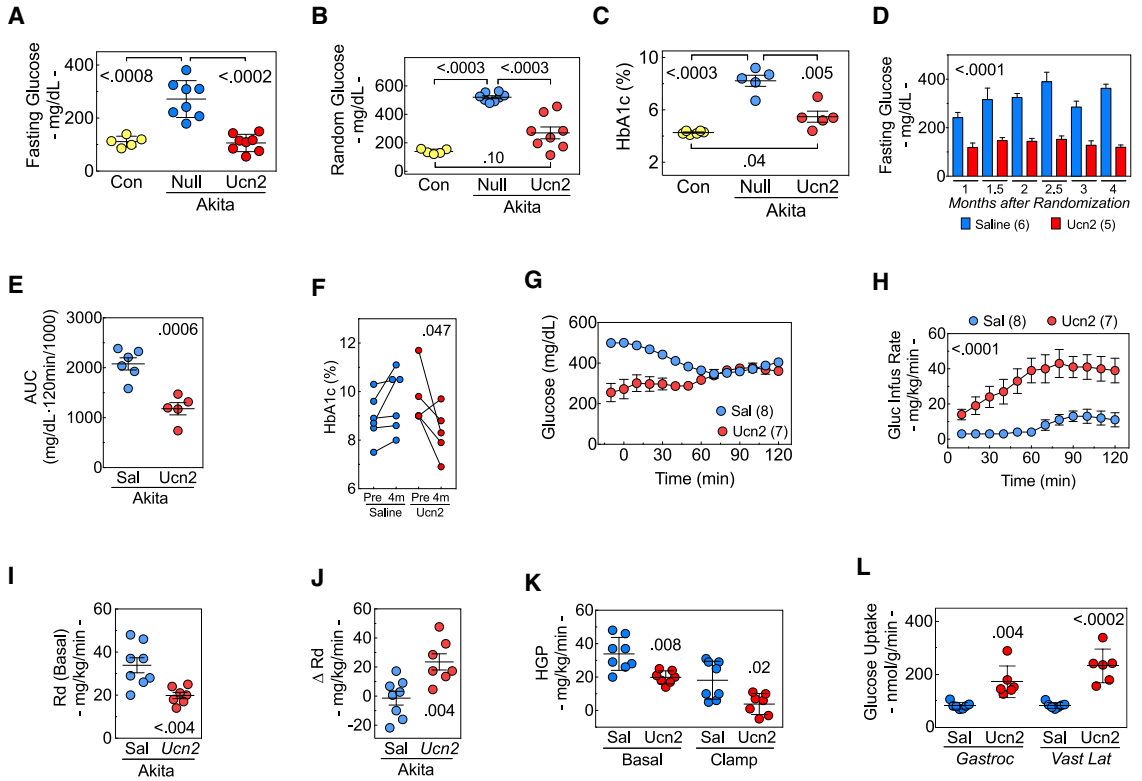

Figure 3. *Ucn2* Gene Transfer Provided Sustained Increases in Glucose Disposal and Skeletal Muscle Insulin Sensitivity

(A–C) Glucose disposal. Akita mice received AAV8.*Ucn2* or AAV8.*Null* (C57BL/6J; Con). (A and B) 1 month later, 12 h fasting glucose (A; $p < 0.0002$) and random glucose (B; $p < 0.0003$) were lower after *Ucn2* gene transfer. (C) HbA1c, measured 2.5 months after treatment, was reduced after *Ucn2* gene transfer ($p < 0.005$). (D–F) Duration of effects. (D) Beneficial effects of *Ucn2* gene transfer on fasting glucose 1–4 months after treatment ($p < 0.0001$, two-way ANOVA). (E) Increased glucose disposal 4 months after treatment ($p = 0.0006$; AUC analysis from glucose tolerance test 0–120 min after glucose, 2 g/kg, i.p). (F) HbA1c was reduced 4 months after *Ucn2* gene transfer ($p = 0.047$; two-way ANOVA, Ucn2 effect). (G–L) Hyperinsulinemic-euglycemic clamp. Akita mice received i.v. saline or i.v. AAV8.*Ucn2* and underwent clamp 4 months later. (G) Blood glucose was matched during the clamp (80–120 m). (H) The glucose infusion rate required to maintain similar glucose concentrations was higher (2.8-fold; $p < 0.0001$, two-way ANOVA) in Akita mice that received *Ucn2* gene transfer, indicating increased insulin sensitivity. (I) 5 h fasting glucose flux (rate of disappearance, Rd) was lower in Akita mice that had received *Ucn2* gene transfer ($p < 0.004$). (J) The change in glucose disposal (clamp minus basal) was increased in Akita mice that had received *Ucn2* gene transfer ($p = 0.004$). (K) *Ucn2* gene transfer reduced basal ($p = 0.008$) and insulin-stimulated hepatic glucose production (HGP; $p = 0.02$). (L) *Ucn2* gene transfer increased glucose uptake in gastrocnemius (Gastroc; $p = 0.004$) and vastus lateralis (Vast Lat; $p < 0.0002$). Mice were 2–2.5 months old at the time of vector or saline delivery, and data were collected 1–4 months later. (A–C, E, I–L) Student's t test (unpaired, two-tailed) with Bonferroni correction in (A)–(C), (K), and (L); (D and H): ANOVA. Group sizes are indicated in graphs. Data are presented as mean ± SE.

AMPK activation ($p = 0.036$; Figure 6B). *Ucn2* gene transfer did not alter skeletal muscle Akt phosphorylation at Ser473 or AMPK activity in normal mice (Figure 6C).

Skeletal Muscle, Liver, and Pancreas mRNA Expression

There were only 2 instances in which *Ucn2* gene transfer was associated with >3-fold changes in gene expression in skeletal muscle, liver, or pancreas that also were statistically significant: liver fibroblast growth factor 21 (*FGF21*) (5.1-fold increase, $p < 0.01$) and liver *Ucn2* (>30,000-fold increase; $p = 0.003$) (Tables S2–S5). The increase in *Ucn2* expression was expected, but increased *FGF21* expression was not. We measured plasma FGF21 concentrations in 4-month-old male control mice (C57BL/6J) and Akita mice, 2 months after *Ucn2* gene transfer, and found increases in plasma FGF21 in Akita mice (3.9-fold increase; $p = 0.026$) (Figure 6D) and confirmed increased liver FGF21 expression ($p < 0.013$; Figure 6D).

Necropsy and Histology

Necropsy (Table S6) was performed on Akita mice and C57BL/6J mice with and without *Ucn2* gene transfer. Group differences were observed only in body weight, which was increased in Akita mice that had received *Ucn2* gene transfer ($p = 0.0003$). Histological inspection of liver and LV showed no group differences in fibrosis, and fatty infiltration in the liver was minimal and similar between groups (Figure S1). No inflammatory infiltrates were seen. Kidney histology did not show increased mesangial expansion in Akita mice compared to control mice, and no group differences were seen in other histological features. Pancreata showed similar degrees of fibrosis (Figure S1A). A 3.3-fold increase in islet number was seen

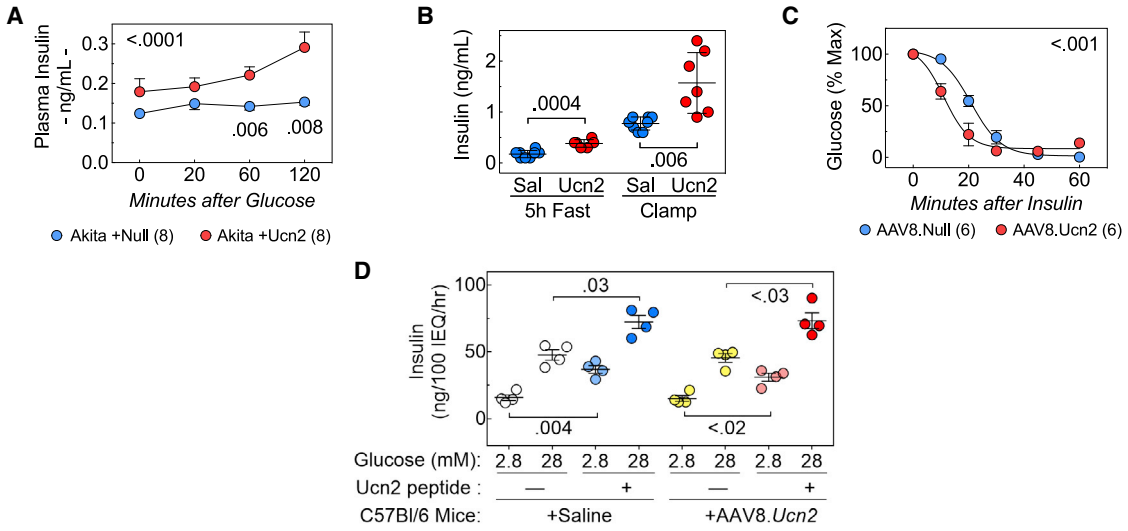

Figure 4. Effects of *Ucn2* Gene Transfer on Insulin Release and Insulin Tolerance

(A) *In vivo* insulin release. Akita mice received AAV8.*Null* or AAV8.*Ucn2* (n = 8 both group); 2 months later, after 12 h fast, glucose (2.5 g/kg p.o.) was given and plasma insulin measured. *Ucn2* gene transfer increased insulin release ($p < 0.0001$, gene effect, two-way ANOVA); post hoc p values ($p = 0.006$ at 60 min; $p = 0.008$ at 120 min) from Student's t test with Bonferroni correction. (B) Plasma insulin levels during glucose clamp. Akita mice that had received *Ucn2* gene transfer showed increased plasma insulin levels before ($p = 0.0004$) and 100–120 min after initiation of the clamp ($p = 0.0006$; n = 6–8 per group); Student's t test with Bonferroni correction. (C) Insulin tolerance test. Akita mice received AAV8.*Null* or AAV8.*Ucn2* (n = 6 both groups); 2 months later, nonfasted mice received insulin (0.75 U/kg, i.p.) and blood glucose was assessed. *Ucn2* gene transfer was associated with increased insulin-induced glucose disposal ($p < 0.001$). Group sizes are indicated in graphs. (D) Glucose-stimulated insulin secretion, normal mice. C57BL/6J male mice (2 months old) received i.v. saline (n = 8) or i.v. AAV8.Ucn2 (n = 8), and islets were isolated 1.5 months later. Each data point represents pooled islets from each of 2 mice within the group. In all instances, high glucose concentration (28 mM) stimulated more insulin release than low glucose concentration (2.8 mM), as expected. When islets were incubated with Ucn2 peptide (200 nM, 1 h), insulin release was greater than when incubated without peptide, at low and high glucose concentrations, and whether or not mice had received *Ucn2* gene transfer. p values from Student's t test (unpaired, two-tailed) with Bonferroni correction. Data are presented as mean ± SE.

in Akita mice that had received Ucn2 gene transfer versus saline (Figure S1B; $p = 0.04$); mean islet size was similar between groups. Compared with normal C57BL/6J mice, reductions in islet insulin intensity were seen, as expected in Akita mice (data not shown), with no group difference between saline and *Ucn2* gene transfer (Figure S1C; $p < 0.12$).

DISCUSSION

The present study shows that *Ucn2* gene transfer improves glycemic control and reduces retinopathy and mortality in insulin deficiency (Figures 3, 4, and 5)—in the absence of exogenous insulin therapy. *Ucn2* gene transfer increases insulin-stimulated glucose uptake in skeletal muscle and improves insulin-induced suppression of hepatic glucose production (Figure 3) yet does not cause hypoglycemia. In the Akita model of insulin deficiency, despite an 87% reduction in plasma insulin compared to normal C57BL/6J mice, *Ucn2* gene transfer improved glycemic control, often to normal levels, in the absence of insulin treatment (Figure 3) by increasing both insulin release and insulin sensitivity (Figures 3 and 4). We also found that *Ucn2* gene transfer was effective in improving disordered glucose disposal in the STZ model of insulin deficiency (Figure 5), indicating that the therapy is not model-dependent. *Ucn2* gene transfer improves glucose metabolism to an extent that it renders glucose fluxes (Rd, HGP) in insulin-deficient Akita mice indistinguishable from glucose fluxes observed in normal C57BL/6J mice in glucose clamp studies.[24] In addition to benefits on insulin sensitivity, *Ucn2* gene transfer increased insulin release. For example, we saw increases in plasma insulin in responses to glucose stimulation (Figure 4A), and increased plasma insulin levels before and 100–120 min after initiation of the clamp (Figure 4B). We showed that Ucn2 directly stimulates islet insulin release (Figure 4D). The combined effects of increased insulin availability and increased insulin sensitivity result in normalization of glycemic indices, substantial reduction of retinopathy and increased survival. We found that *Ucn2* gene transfer had enduring effects on glycemic control 4 months after a single administration and reduced cumulative mortality 7 months after therapy. *Ucn2* gene transfer was not associated with differences in GLP-1 or glucagon in Akita mice, suggesting that incretin signaling did not play a major role in its beneficial effects on glucose disposal.

Our findings were unexpected. We previously reported resolution of disordered glucose homeostasis by *Ucn2* gene transfer in type 2 diabetes,[13] where endogenous insulin is abundant and the mechanism of Ucn2 action was increased skeletal muscle insulin sensitivity. However, the primary problem in T1D is insulin deficiency—a problem unlikely to be resolved by anything but exogenous insulin. Indeed, previous efforts to achieve superior glucose control in T1D with insulin sensitizing drugs have been relatively ineffective.[12] That *Ucn2* gene

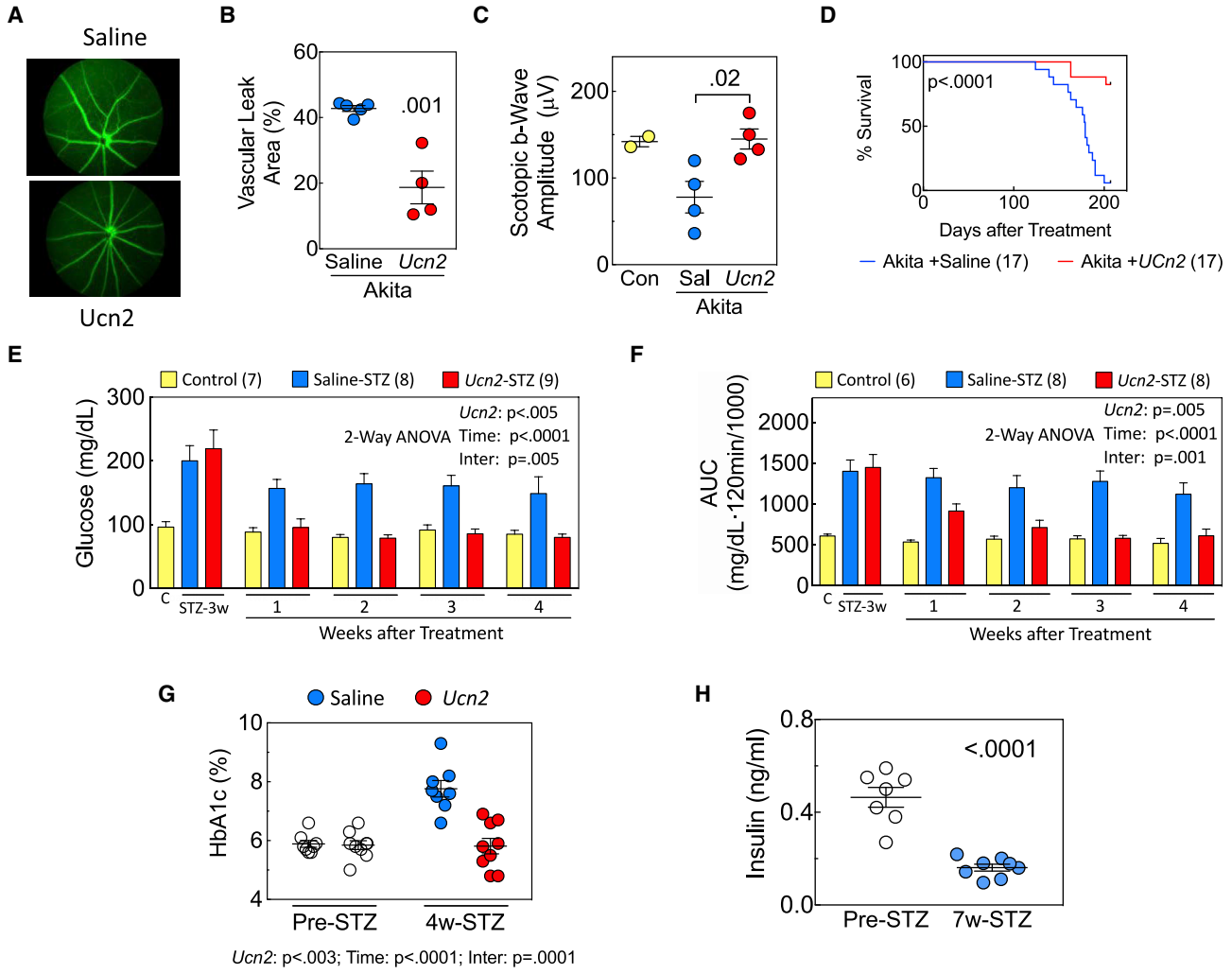

Figure 5. *Ucn2* Gene Transfer Reduced Diabetic Retinopathy and Mortality in Akita Mice, and Effects on Glucose Disposal Were Also Seen in a Second Model of Insulin Deficiency

(A–C) Retinopathy. Akita mice (4 months old) received saline (n = 5) or *Ucn2* gene transfer (n = 4) and underwent retinal imaging 3 months later. (A) Fluorescein angiography was performed to quantitate retinal vascular leak. (B) The graph shows the mean values of both eyes from each of 9 mice. *Ucn2* gene transfer reduced vascular leak ($p = 0.001$). (C) Electroretinography indicated improved retinal function ($p = 0.02$). (D) Mortality. 34 Akita mice (2.5 months old) received i.v. saline (n = 17) or AAV8.*Ucn2* (n = 17). *Ucn2* gene transfer increased survival ($p < 0.0001$). (E–G) Glucose disposal after *Ucn2* gene transfer in a second model of insulin deficiency: streptozotocin (STZ)-treated mice. (E) 2-month-old male control mice (C57BL/6J) received STZ and randomized 3 weeks later to receive saline or *Ucn2* gene transfer. Studies to assess glucose disposal were conducted weekly. Control mice (C) received no STZ or AAV8.*Ucn2*. 12 h fasting glucose levels showed persistent reduction after *Ucn2* gene transfer versus saline in STZ-induced insulin deficient mice (two-way ANOVA, $p < 0.005$, Ucn2 effect). Fasting glucose returned to levels similar to control mice. (F) Similar improvements in glucose tolerance (2 g/kg glucose, i.p.) were seen after *Ucn2* gene transfer in STZ-treated mice (two-way ANOVA, $p = 0.005$, Ucn2 effect). (G) We measured HbA1c 4 before STZ-treatment and again 4 weeks after Ucn2 gene transfer, which confirmed sustained glycemic control (two-way ANOVA, $p < 0.003$, Ucn2 effect). (H) Blood was obtained 2 h after glucose stimulation (2 g/kg, i.p.) before (pre) and 7 weeks after the final dose of STZ. Plasma insulin, as anticipated, was reduced after STZ administration ($p < 0.0001$); Student's t test (paired, two-tailed). p values in (B), (C), and (H) from Student's t test (unpaired, two-tailed); (D) Kaplan-Meier analysis; (E–G) from two-way ANOVA. Group sizes are indicated in graphs. Data are presented as mean ± SE.

transfer normalized glucose disposal despite marked reduction in insulin levels underscores its therapeutic potential in T1D.

The microvascular complications of T1D are tightly linked with degree of glucose control. Retinopathy is a major cause of diabetes-related blindness. Consistent with previous investigations of age-matched Akita mice,[18,21] eyes of untreated Akita mice showed signs of diabetic vascular damage such as vascular leakage and new vascular beds. The significant reduction in retinal vascular leak (Figures 5A and 5B) in Akita mice that had received *Ucn2* gene transfer is a promising finding vis-à-vis potential therapy for the prevention and treatment of early clinical diabetic retinopathy. Prolonged vascular

Table 2. LV Size and Function

	Control			Akita		
	AAV8.*Null* (6)	AAV8.*Ucn2* (6)	p Value	AAV8.*Null* (8)	AAV8.*Ucn2* (8)	p Value
EF, %	66 ± 2	79 ± 2	0.0001	65 ± 2	79 ± 2	<0.001
EDD, mm	3.8 ± 0.1	3.5 ± 0.1	0.03	3.8 ± 0.1	3.5 ± 0.1	<0.04
ESD, mm	2.5 ± 0.1	1.8 ± 0.1	0.0003	2.4 ± 0.1	1.9 ± 0.1	<0.001
VCFc, circ/s	23 ± 1	34 ± 2	0.0003	23 ± 1	34 ± 1	<0.0001
HR, bpm	566 ± 11	550 ± 8	0.25	549 ± 8	547 ± 7	0.9

Effects of *Ucn2* gene transfer on left ventricular size and function in Akita mice. Control mice (C57BL/6J) and Akita mice (2 months old, male) received AAV8.*Null* (as controls) or AAV8.*Ucn2* (2×10^{13} gc/kg, i.v.), and echocardiography was performed 3–4 months later. In both control and Akita mice, gene transfer increased ejection fraction (EF) and the velocity of circumferential fiber shortening (VCFc, corrected for HR), a measure of left ventricular contractility. Left ventricular end-diastolic and end-systolic diameters were reduced (EDD, ESD). Heart rate (HR) showed no group difference. *Ucn2* gene transfer increased systolic function similarly in normal and in Akita mice. Data are presented as mean ± SE. p values are from Student t test (unpaired, two-tailed).

leakage can lead to adverse effects on the health and function of retinal cells. Functional studies indicated that the observed anatomic differences after *Ucn2* gene transfer translate to improved retinal function (Figure 5C). The extent of beneficial effect of the *Ucn2* gene transfer is superior to other systemic treatments of diabetic retinopathy.[4,25,26]

Finally, we saw reduced mortality in the present study (Figure 5D). The deaths were unwitnessed and likely related to metabolic derangement.[19] At 9 months (7 months after gene transfer), we saw reduced mortality in Akita mice that had received *Ucn2* gene transfer (Akita + Ucn2: 18% mortality; Akita + saline: 94% mortality; $p < 0.0001$; Figure 5D). Urine albumin-to-creatinine ratio was reduced in Akita mice after *Ucn2* gene transfer ($p = 0.01$; Figure 2E) but serum creatinine was unchanged (Figure 2F) and kidney histology (Figure S1) did not show group differences in mesangial expansion, the hallmark lesion in diabetic nephropathy. Nephropathy in the Akita mouse depends upon what line the *Ins2*^{Akita} line is crossed with, and C57BL/6J tends to have minimal renal abnormalities.[20,27]

The physiological mechanisms by which *Ucn2* gene transfer affects glucose disposal resides primarily in its augmentation of skeletal muscle insulin sensitivity and subsequent glucose uptake in Akita mice, effects which were shown in hyperinsulinemic euglycemic clamp studies (Figures 3G–3L). In addition, in Akita mice, *Ucn2* gene transfer increased insulin release *in vivo* (Figure 4), owing to a direct effect of Ucn2 on islet insulin secretion (Figure 4D). Importantly, insulin's stimulation of whole-body glucose clearance was restored. Finally, we saw reduced hepatic glucose production after *Ucn2* gene transfer in Akita mice before clamp. During clamp, hepatic glucose production was reduced by 78%, an absolute reduction of 14.2 mg/kg/min. These data indicate increased hepatic insulin sensitivity. But the key group difference was in skeletal muscle, which became a significant contributor to glucose homeostasis after *Ucn2* gene transfer.

Insulin action involves increased activation of Akt and AMPK, which promotes Glut4 translocation to the plasma membrane and subsequently increases glucose disposal. In skeletal muscle from Akita mice that received *Ucn2* gene transfer, we saw increased Akt phosphorylation (Figure 6A) at both Ser473 ($p = 0.005$) and Ser308 ($p = 0.005$). Additionally, this was associated with increased AMPK phosphorylation at Thr172 ($p = 0.036$; Figure 6B). Akt and AMPK activation in skeletal muscle were associated with similar increases in skeletal muscle glucose uptake *in vivo* in Akita mice that had received *Ucn2* gene transfer (Figure 3L). In contrast to Akita mice, *Ucn2* gene transfer did not affect Akt or AMPK activity in skeletal muscle from C57BL/6J mice (Figure 6C). Finally, we showed that Ucn2 gene transfer increases basal and glucose-stimulated insulin release and insulin-induced glucose disposal *in vivo* in Akita mice (Figures 4A–4C). This reflects increased Ucn2-stimulated islet insulin release, which we demonstrated in isolated islets (Figure 4D). Thus, Ucn2 peptide directly stimulates islet insulin release, and, even when plasma Ucn2 is substantially and chronically elevated *in vivo* by *Ucn2* gene transfer, isolated islets still respond to stimulation by Ucn2 peptide, indicating that islets do not become refractory to Ucn2-stimulated insulin secretion.

A previous study reported that Ucn3 peptide reduced glucose-stimulated islet insulin release due to Ucn3-CRHR2 activation on islet delta cells, which increased somatostatin release, which, in turn, reduced beta cell insulin release.[28] We found that Ucn2 peptide has the opposite effect—glucose-stimulated islet insulin release was increased (Figure 4D), and there was no change in somatostatin release. Both peptides bind CRHR2 with similar affinities. However, unlike the case with Ucn3, islet somatostatin levels were unchanged by Ucn2 peptide. We speculate that the difference may reflect an inverse agonist effect of Ucn2, which may impede Ucn3's normal effects on CRHR2-mediated somatostatin release from delta cells. Testing this will require studies in delta-cell specific CRHR2-deleted mice. Delineating the molecular basis for this difference in action of Ucn2 versus Ucn3 on islet insulin release is a focus of ongoing studies in our laboratory.

Our data indicate that there are two key mechanisms for the beneficial effects of *Ucn2* gene transfer in Akita mice: (1) increased Akt and AMPK activation leading to increased skeletal muscle glucose uptake; and (2) increased insulin release from islets. These actions are directly dependent upon increased plasma levels of Ucn2 peptide, and are

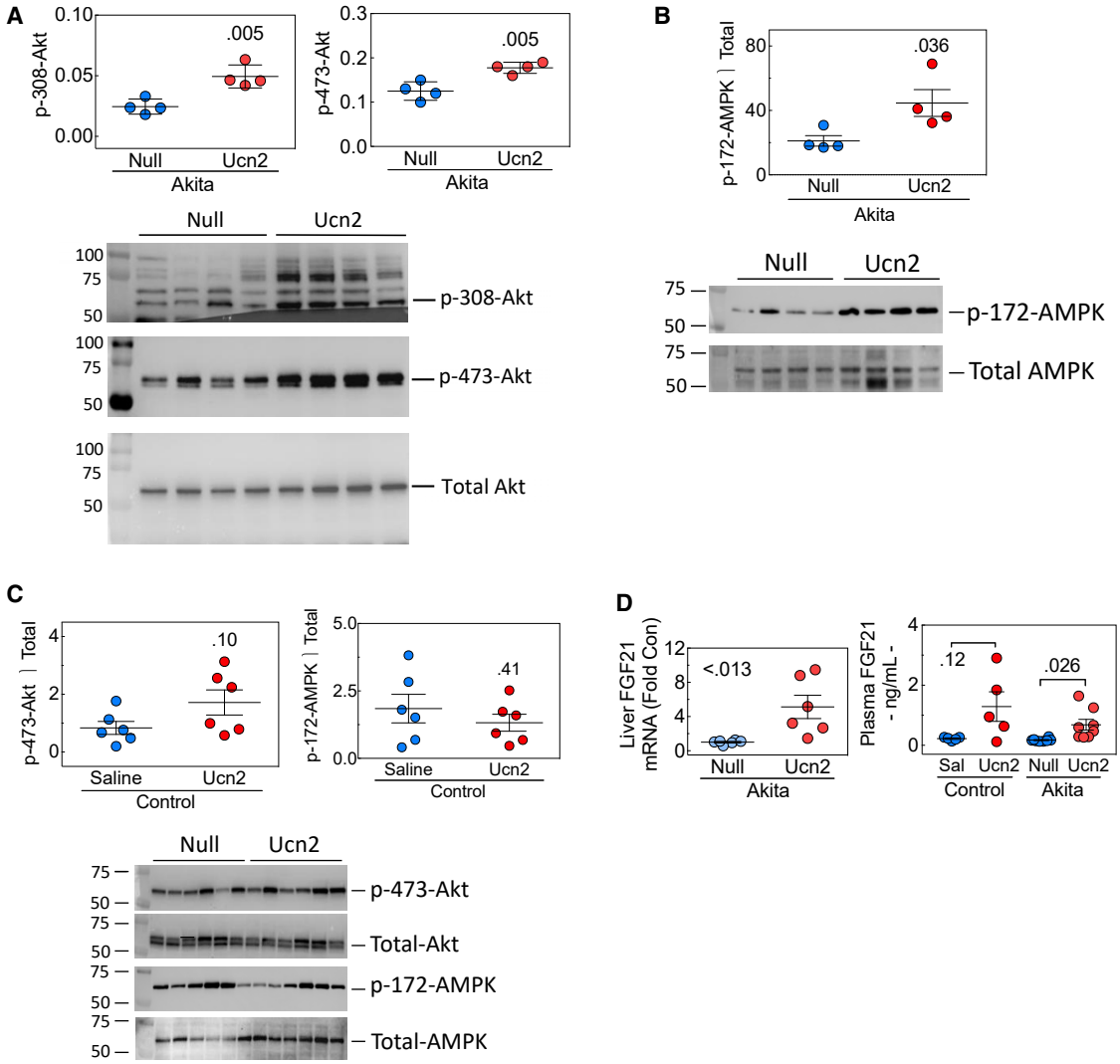

Figure 6. *Ucn2* Gene Transfer: Effects on Skeletal Muscle Akt Activation and Plasma Concentrations of FGF21

(A and B) Akita mice (2 months old) received AAV8.*Ucn2* or AAV8.Null, and assessment was performed 3 months later. Quadriceps femoris muscle from animals that had received *Ucn2* gene transfer showed increased activities of Akt (Ser-308, p = 0.005; Ser473, p = 0.005; repeated 3 times each) and AMPK (Thr172, p = 0.036; repeated 4 times). (C) Control mice (C57BL/6J), 2.5 months old, received i.v. saline or AAV8.*Ucn2*, and assessment was performed 1 month later. In contrast to Akita mice, in control mice, *Ucn2* gene transfer did not affect activities of Akt or AMPK. Experiments repeated 2 times. (D) Control and Akita mice (2 months old) received i.v. saline or AAV8.*Ucn2* and FGF21 expression assessed 2 months later. Liver FGF21 mRNA expression was increased 5.1-fold (left panel, p < 0.013). *Ucn2* gene transfer did not increase plasma FGF21 concentration in control mice (p = 0.12), but a 3.9-fold increase in plasma FGF21 was seen after Ucn2 gene transfer in Akita mice (right panel, p = 0.026). (A–D) Student's t test (unpaired, two-tailed) with Bonferroni correction in (D); group sizes are indicated in graphs. Graphs display data normalized to total Akt (A), vinculin (B), or GAPDH (C) as shown. Data are presented as mean ± SE.

mediated through Ucn2's cognate receptor, CRHR2, which is expressed in skeletal muscle.[14] We previously showed that Ucn2 peptide increases Glut4 translocation as effectively as insulin in skeletal myotubes via AMPK activation.[13] The favorable effects of *Ucn2* gene transfer on glucose disposal are mediated through CRHR2, since deletion of this receptor prevents these effects.[13]

Ucn2 gene transfer increased liver expression of FGF21 in Akita mice (p < 0.013; Figure 6D), and increased plasma FGF21 concentration in Akita mice (p = 0.026; Figure 6D). FGF21 has beneficial effects in animal models of diabetes, obesity, and lipid metabolism and has been proposed as a potential clinical therapeutic in metabolic disorders.[29] It may be of mechanistic importance in Ucn2's effects on glucose disposal, although FGF21 effects are on adipocytes and increased insulin sensitivity in liver—Ucn2's effects, in contrast, are on insulin sensitivity in skeletal muscle. A recent study showed beneficial effects of FGF21 gene transfer in type 2 diabetes,[30] but long-acting FGF21 formulations have not been

effective in lowering glucose in clinical trials, although they appear to reduce weight.[31]

The Akita and STZ models of insulin deficiency each have advantages and disadvantages. Insulin deficiency in Akita mice results from apoptotic destruction of islet beta cells because of abnormal insulin folding due to a point mutation in *Ins2*. Residual insulin production (10% of normal), persists for several months,[17] enabling long term studies of therapeutic interventions. Although homozygous mice die early, heterozygotes have sufficient lifespans for studies of 6–7 months.[19] Female Akita mice develop less severe hyperglycemia, so males are generally used. Likewise, although STZ is an effective islet toxin in male mice, it does not have an equivalent effect in females. Therefore, the present studies were, by necessity, limited to male mice. Will *Ucn2* gene transfer be effective in females? We have shown previously that *Ucn2* gene transfer increases insulin sensitivity and normalizes glucose disposal equally well in male and female db/db mice,[13] and that *Ucn2* gene transfer increases plasma Ucn2 levels in a dose-related manner in normal male and female mice (unpublished data). In addition to an 87% reduction in plasma insulin and a flat insulin response to glucose, Akita mice showed insulin resistance. Glucose flux analysis during the clamp showed that untreated Akita mice exhibited liver and peripheral insulin resistance, which was also documented in a previous study.[32] Studies in STZ-induced insulin-deficient mice demonstrated similar beneficial effects of *Ucn2* gene transfer on glycemic indices to those found in the Akita model (Figure 5), indicating that the benefits of *Ucn2* gene transfer in insulin deficiency is not model-limited. Although both Akita and STZ are reliable models that mimic the metabolic features of clinical T1D, they do not involve an autoimmune etiology to insulin deficiency as seen in clinical T1D. However, *Ucn2* gene transfer is not proposed as a means to prevent the autoimmune effects that lead to islet destruction, but rather as a treatment for impaired glucose disposal once present, regardless of etiology.

Crossbreeding leptin-expressing transgenic mice and Akita mice yielded improved glycemic indices and reduced mortality.[19] However, the generation of a transgenic cross is not a viable clinical therapy option, and leptin therapy (metreleptin peptide administration) was not effective in a subsequent T1D clinical trial.[33] In the present study, plasma leptin concentration was unaltered by *Ucn2* gene transfer. Insulin gene transfer has been used to circumvent the difficult dosing requirements otherwise required for insulin use. Although such efforts have been successful,[34,35] hypoglycemia has been problematic,[34] and no clinical trials using this approach have been initiated. Pancreas or islet transplantation, which are effective, require life-long immunosuppression, which, in addition to other problems, compromises kidney function. Consequently, islet transplantation currently is not suitable for a majority of patients.[36] Stem-cell approaches can circumvent problems associated with immunosuppression, and preclinical studies have shown the efficacy of cell-based therapy for insulin deficiency.[37] However, large randomized trials have not been conducted. *Ucn2* gene transfer could forestall the need for islet transplantation or stem cell therapy, and, once performed, could promote insulin release from transplanted or newly formed islets and increase insulin's effectiveness.

Based on the present data, we speculate that, in clinical settings, *Ucn2* gene transfer might increase insulin effectiveness and reduce insulin requirements, potentially achieving better glucose control while reducing insulin's adverse effects. It would be applicable as a sole therapy in patients with residual islet function, and as a co-therapy with exogenous insulin in those with more advanced disease. The proposed therapy is a one-time i.v. injection, circumventing daily or weekly dosing, a unique and desirable feature for patients and clinicians. Because of its beneficial effects on cardiac function, it is anticipated to reduce cardiomyopathy associated with T1D and to be particularly effective in patients with diabetes and concomitant heart failure. AAV8 vectors, in general, at the doses we would propose based on the current studies, have been well-tolerated in non-human primate and clinical trials.[38–40] i.v. delivery of AAV8.*Ucn2* provides sustained elevation in plasma Ucn2.[13,23,41] Pre-existing anti-AAV8 antibodies are not as prevalent in humans (19%) as are AAV1 and AAV2 (50%–59%).[42]

In summary, *Ucn2* gene transfer may be a viable therapy both for new onset T1D with detectable endogenous insulin, and for later stage T1D to reduce exogenous insulin needs. *Ucn2* gene transfer could potentially provide tight glycemic control with a 1-time administration. Such an approach, if successful would add a new option for use in a disease that is treated only with insulin, an agent that, despite nearly 100 years of use, has considerable shortcomings.

MATERIALS AND METHODS

AAV8.*Ucn2* Vector Production

An AAV8 vector encoding murine *Ucn2* with a chicken β-actin (CBA) promoter (Figure 1C) was produced as detailed previously.[23] Plasmid pRep2/Cap8 was obtained from the University of Pennsylvania Vector Core.

Animal Use

The Principles of Laboratory Animal Care (NIH publication no. 85–23, revised 1985) were followed and the Animal Use and Care Committees of the VA San Diego Healthcare System approved the studies. See Table S7 for summary of animal use. We used 222 male mice (Jackson Laboratories, Bar Harbor, ME) including 108 heterozygous Akita mice (C57BL/6J-*Ins2*^{Akita}; age 10.1 ± 0.3 weeks, weight 20.7 ± 0.2 g) and 114 male C57BL/6J mice (age 10.1 ± 0.1 weeks, weight 25.6 ± 0.2 g). Nine C57BL/6J mice were excluded in the STZ study due to failure to reach pre-specified hyperglycemia levels; 1 Akita mouse died during anesthesia prior to treatment, and 19 Akita mice died during the 7 month survival study (16 of 17 that received saline; 3 of 17 that received *Ucn2* gene transfer). Animal numbers and additional information are included in figure legends and tables. Mice were provided (*ad libitum*) a cereal-based diet (Harlan Teklad Lab, Madison, WI, USA) for the entire study, regardless of intervention. Duration of these studies ranges from 1.5–7.5 months and are stipulated in the results. Mice were housed (20–21°C) with lights

off from 6 PM to 6 AM daily. Food consumption and body weights were recorded on 2- to 3-day intervals.

Streptozotocin Model of Insulin Deficiency

Normal male mice received 5 daily injections of STZ (50 mg/kg/d, intraperitoneal, i.p.) following previously published reports for induction of stable insulin deficient mice. These mice were used to determine whether *Ucn2* gene transfer was effective in a second mouse model of insulin deficiency.[22] Normal C57BL/6J male mice that were given buffer but no STZ were simultaneously studied. Three weeks after the final dose of STZ, glycemic indices were obtained (12 h fasting glucose, glucose tolerance tests, HbA1c). Mice then were assigned, based on their fasting glucose, to receive i.v. AAV8.*Ucn2* (n = 9) or saline (n = 8), such that the two groups had similar pre-treatment mean fasting glucose levels. Mice that failed to respond sufficiently to STZ (12 h fasting glucose <120 mg/dL) were excluded from further study. Repeat glycemic indices were obtained weekly for 4 weeks after randomization.

AAV8.*Ucn2* Delivery

Under anesthesia (1.5% isoflurane via nose cone) a small incision was made to expose the jugular vein for i.v. delivery. Mice were injected with AAV8.*Ucn2* or AAV8.*Null* (2×10^{13} gc/kg in 50 μL PBS) or an equivalent volume of saline. Vector dose was 2×10^{13}gc/kg, i.v., in all studies.

Glucose Tolerance Test

Mice were fasted for 12 h and received glucose (2 g/kg, i.p.). Blood glucose levels were measured before and 30, 60, 90, and 120 min after glucose injection. Blood was collected via a small tail incision and glucose was measured using Contour Blood Glucose Meter via Blood Glucose Test Strips (Bayer, Whippany NJ).

Hemoglobin A1c

HbA1c was measured in nonfasted mice using a DEC system.

Insulin Release

Fasted mice (12 h) received glucose (oral gavage, 2.5 mg/kg). Blood before and sequentially after glucose delivery was taken for 2 h. Plasma insulin was measured using insulin-specific ELISA.

Insulin Tolerance Test

Fasted mice (5 h) received insulin (0.75 units/kg, i.p.) and blood glucose levels were measured before and 30, 60, 90, and 120 min later.

Hyperinsulinemic-Euglycemic Clamps

Hyperinsulinemic-euglycemic clamps were performed by the Vanderbilt Mouse Metabolic Phenotyping Center to assess glucose disposal rate, tissue glucose uptake, and hepatic glucose production. Akita mice (2.5 months old, male) received AAV8.*Ucn2* (n = 7) or saline i.v. (n = 8). 4 months later, unrestrained mice underwent hyperinsulinemic euglycemic clamp studies to quantitate glucose disposal and insulin sensitivity, using a continuous insulin infusion (4 mU/kg/min) in mice after 5 h fast.[43]

Islet Insulin Release

Male C57BL/6J mice (25.6 ± 0.4 g; 2 months old) received i.v. saline (n = 8) or i.v. AAV8.*Ucn2* (2×10^{13} gc/kg, n = 8). 6 weeks later, pancreata were perfused *in situ* with collagenase P digestion solution (1.119 g/L collagenase P; Sigma, St. Louis MO) in Hank's balanced salt solution (HBSS, HyClone, Logan, UT), excised, digested, washed with HBSS containing 0.3% BSA (Thermo Fisher, San Diego CA) and 0.5 g dextrose (Thermo Fisher, San Diego CA). Two pancreata were pooled so that there were 4 data points per condition in each of the two groups. Purified islets from two mice within each group were divided into two culture conditions and cultured overnight (37°C, 5% CO_2) in RPMI 1640 (Waltham, MA) without Ucn2 peptide or with 200 nM of Ucn2 peptide. On day 2, a glucose-stimulated insulin secretion assay was performed. 100 islet equivalents (IEQ) were hand-picked and placed into a 12 μm Millicell cell culture insert (MilliporeSigma, Burlington, MA), rinsed and incubated for 1 h (37°C, 5% CO_2) in 2.8 mM glucose Krebs Buffer (0.115 M NaCl, 25 mM HEPES, 5 mM KCl, 24 mM $NaHCO_3$, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, pH 7.4) containing no peptide or 200 nM Ucn2 peptide. The same 100 IEQ islet Millicell was then transferred to fresh low glucose (2.8 mM) Krebs buffer with or without Ucn2 peptide for 1 h (37°C, 5% CO_2). Culture media were collected and stored at −20°C. The same islets then were transferred to high glucose (28 mM) Krebs buffer with or without Ucn2 peptide for 1 h (37°C, 5% CO_2) and culture media were collected and stored at −20°C. Secreted insulin was measured using Mercodia Mouse Insulin ELISA (Uppsala, Sweden); media somatostatin was assessed using mouse somatostatin ELISA (Ray Biotech, Peachtree Corner, GA).

Urocortin 2, Insulin, GLP-1, Glucagon, Triglyceride

Plasma Ucn2 was measured as previously reported,[41] using a mouse Ucn2 enzyme immunoassay kit (Kamiya Biomedical, Seattle WA). Plasma insulin was measured using the mouse insulin ELISA Kit (Crystal Chem, Downers Grove, IL). Fasting plasma GLP-1 was measured by ELISA (Crystal Chem, Elk Grove Village, IL). Fasting plasma glucagon concentrations were measured before and 2 h after glucose stimulation (2.5 g/kg, oral) by an ELISA (Crystal Chem, Downers Grove, IL). Random plasma triglyceride was determined using a colorimetric/fluorometric kit (Biovision, San Francisco CA).

Metabolic Studies

The Comprehensive Laboratory Animal Monitoring System (CLAMS, Columbus Instruments, Columbus, OH) was used to assess oxygen consumption and activity level in 12 male control mice (C57BL/6J) and 12 male Akita mice. Half of each group (n = 6) were randomized to receive AAV8.*Ucn2* and half received saline, and all underwent studies 8 weeks later. Mice were kept in metabolic cages for 6.5 days (6 light, 7 dark cycles) while measurements were continuously acquired.[13] In addition, assessment of water intake, food consumption, and body weight were conducted every 2–3 days for 8 weeks following treatment.

Evaluation of Retinopathy and Retinal Function

Mice were anesthetized with ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). Eyes were topically anesthetized with 0.5% proparacaine (Bausch + Lomb, Rochester, NY) and dilated with 1.5% tropicamide (Alcon Labs, Fort Worth, TX) and 2.5% phenylephrine (Akorn, Lake Forest, IL). Anesthetized animals were injected with 1% (w/v) fluorescein sodium (4 μL/g, i.p.) and placed on an adjustable stage, so that the Micron III (Phoenix Labs, Pleasanton, CA) objective could be stereoscopically adjusted to contact with the cornea through an ophthalmic lubricant gel. Fluorescein angiographs were captured using a filter with excitation (486 nm) and emission (536 nm) controlled by a commercial software using the same acquisition parameters for all mice. The vascular area (occupied by green fluorescence) was quantified in ImageJ. Retinal function was evaluated through electroretinography (ERG) following established protocols for mice.[44] Mice were dark-adapted for 12 h and anesthetized with ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). Mice were given a dilating eye drop of 1.5% tropicamide (Alcon Labs, Fort Worth, TX), 2.5% phenylephrine (Akorn, Lake Forest, IL), and a drop of 0.5% proparacaine (Bausch + Lomb, Rochester, NY) as analgesic. Mice were examined with a full-field Ganzfeld bowl setup (Diagnosys, Lowell, MA), with electrodes placed on each cornea, a subcutaneous ground needle electrode placed in the tail, and a reference electrode in the mouth (Grass Telefactor, West Warwick, RI). Lubricant (Goniovisc 2.5%, HUB Pharmaceuticals, Rancho Cucamonga, CA) was used to provide contact of the electrodes with the eyes. Amplification (1–1,000 Hz bandpass, without notch filtering), stimuli presentation, and data acquisition were programmed and performed using the UTAS-E 3000 system (LKC Technologies, Gaithersburg, MD). For scotopic ERG, the retina was stimulated with a xenon lamp at −2 and −0.5 log cd·s/m^2. For photopic ERG, mice were adapted to a background light of 1 log cd·s/m^2, and light stimulation was set at 1.5 log cd·s/m^2. Recordings were collected and averaged in manufacturer's software (Veris, EDI, Milpitas, CA) and processed in Microsoft Excel.

Kidney Function

Urine was collected for 3 h. Albumin concentration was determined using an ELISA assay kit and creatinine concentration determined using a colorimetric assay kit (both from Abcam, Cambridge, MA). Serum creatinine was measured using liquid chromatography tandem mass spectrometry.

RT-PCR and Immunoblotting

RT-PCR and immunoblotting were conducted as previously described.[41] Skeletal muscle underwent evaluation of Akt and AMPK activation using antibodies obtained from Cell Signaling Technology (Danvers, MA): p-308-Akt (#4056), p-473-Akt (#4058), total Akt (# 9272), p-172-AMPK (#2535), and GAPDH (# 51332S). Antibody to vinculin was obtained from Sigma (St. Louis, MO; SAB4200080).

Selective Transcript Screening

Gene Array

Total RNA from liver and skeletal muscle was reverse-transcribed into cDNA and amplified in PCR. Data shown are from insulin signaling and glucose metabolism gene arrays (QIAGEN, Germantown, MD), which have 92 gene-specific primer sets in each 96-well plate. Data are displayed only if: (1) >1.5-fold or <0.7-fold change in mRNA (AAV8.*Ucn2* versus AAV8.Null) or (2) $p < 0.05$ regardless of fold-change. Skeletal muscle, liver, and pancreas mRNA expression. Skeletal muscle, liver, and pancreas RNA were isolated and reverse transcribed into cDNA (Tables S3–S5). Genes of interest were detected using their specific primers in qPCR. GAPDH was used in each 96-well plate for an internal control during PCR. Results were normalized to GAPDH and expressed as fold change. Data in Tables S2–S5 are from 6 mice per group (AAV8.Null, n = 6; AAV8.*Ucn2*, n = 6).

Necropsy and Histology

2-month-old male control mice received i.v. saline (n = 6) or i.v. AAV8.*Ucn2* (n = 6); age-matched male Akita mice received i.v. AAV8.Null (n = 8) or i.v. AAV8.*Ucn2* (n = 8). Mice were killed 3 months later (5 months of age) and underwent necropsy. Body, liver, kidney, lung, and LV weight (including septum) and tibial length were recorded. A short-axis midwall LV ring and samples of liver, kidney, and pancreas were obtained and portions quickly frozen in liquid nitrogen and stored at −80°C or fixed in formalin and embedded in paraffin. 5 μm sections were mounted and counterstained with H&E and with Masson's trichrome and examined for fibrosis and inflammation. To evaluate fibrosis of liver and transmural LV, we scanned and quantified slides (Zeiss Axio Scan; Carl Zeiss, Germany). Kidney histopathology was examined after Periodic acid-Schiff (PAS) staining.

Finally, pancreatic islet number was assessed. Akita mice (2 months old) received i.v. saline (n = 4) or i.v AAV8.*Ucn2* (n = 4). Six weeks later, pancreata were perfused *in situ*, imbedded in OCT, frozen and sectioned (10 μm). Insulin was detected using rabbit anti-insulin antibody (Abcam, Cambridge, MA), and donkey anti-rabbit immunoglobulin G (IgG) secondary antibody (Alexa Fluor 488; Thermo Scientific, San Diego, CA). Islets were counted and normalized to area of tissue section (islets per mm^2), and islet insulin intensity using NIH ImageJ V1.52 software. Images were obtained with a 20X objective at 488 nm and 509 nm excitation and emission wavelengths, respectively, using a Zeiss Observer.Z1 microscope (Carl Zeiss, Oberkochen, Germany), equipped with ApoTome.2 system.

Statistical Analysis

Analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA). Treatment (*Ucn2* gene transfer versus saline or AAV8.Null) was randomized. Those collecting and analyzing data obtained from physiological experiments and histological comparisons were blinded to group identity. One scientist not involved in the study recorded group/treatment identity. Once unblinded, no

exclusions of data were permitted. Group size (minimum n = 5) was estimated based on the mean coefficient of variation of key efficacy endpoints (20%) and desired detectability threshold (40%). Power calculations assumed a β-error of 0.10 (90% power) and an α-error of <0.05. The mean group size was n = 7. Statistical tests for each experiment are described in figure legends. Data represent mean ± SE; group differences were tested for significance using Student's t test (unpaired, two-tailed), repeated-measures ANOVA or two-way ANOVA. When multiple tests were made on the same dataset, corrections were made, as indicated. In glucose tolerance tests, the trapezoidal rule was applied to determine area under the curve (AUC). The null hypothesis was rejected when $p < 0.05$. Akita mice and mice rendered insulin deficient by STZ both have a stronger phenotype in male versus female mice, which necessitated our use of male mice. Groups were composed of animals of similar age, weight, and absence of confounding diseases. The AAV vectors used were made at the University of Pennsylvania Vector Core. Plasmids underwent tests to determine: concentration, A260/280 ratio, detection of endotoxin, and plasmid identity. Real-time PCR was used to determine genome copies to assess AAV particles with full genome content. Vectors were sequenced and purity determined by electrophoresis.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

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CONFLICTS OF INTEREST

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