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Prevention of Hepatocellular Adenoma and Correction of Metabolic Abnormalities in Murine Glycogen Storage Disease Type Ia by Gene Therapy

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Abstract

Glycogen storage disease type Ia (GSD-Ia), characterized by impaired glucose homeostasis and chronic risk of hepatocellular adenoma (HCA), is caused by deficiencies in the endoplasmic reticulum (ER)-associated glucose-6-phosphatase- α (G6Pase- α or G6PC) that hydrolyzes glucose-6-phosphate (G6P) to glucose. G6Pase- α activity depends upon the G6P transporter (G6PT) that translocates G6P from the cytoplasm into the ER lumen. The functional coupling of G6Pase- α and G6PT maintains interprandial glucose homeostasis. We have previously shown that gene therapy mediated by AAV-GPE, an adeno-associated virus (AAV) vector expressing G6Pase- α directed by the human *G6PC* promoter/enhancer (GPE), completely normalizes hepatic G6Pase- α deficiency in GSD-Ia (G6pc^{-/-}) mice for at least 24 weeks. However, a recent study showed that, within 78 weeks of gene deletion, all mice lacking G6Pase- α in the liver develop HCA. We now show that gene therapy mediated by AAV-GPE maintains efficacy for at least 70–90 weeks for mice expressing more than 3% of wild type hepatic G6Pase- α activity. The treated mice displayed normal hepatic fat storage, normal blood metabolite and glucose tolerance profiles, reduced fasting blood insulin levels, maintained normoglycemia over a 24-hour fast, and had no evidence of hepatic abnormalities. After a 24-hour fast, hepatic G6PT mRNA levels in G6pc^{-/-} mice receiving gene therapy were markedly increased. Since G6PT transport is the rate-limiting step in microsomal G6P metabolism it may explain why the treated G6pc^{-/-} mice could sustain prolonged fasts. The low fasting blood insulin levels and lack of hepatic steatosis may explain the absence of HCA.

Conclusion—These results confirm that AAV-GPE-mediated gene transfer corrects hepatic G6Pase- α deficiency in murine GSD-Ia and prevents chronic HCA formation.

Keywords

Adeno-associated virus; glucose-6-phosphatase- α ; glucose-6-phosphate transporter; blood insulin; glucose homeostasis

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Introduction

Glycogen storage disease type Ia (GSD-Ia or von Gierke disease, MIM232200) is caused by a deficiency in glucose-6-phosphatase- α (G6Pase- α or G6PC), an enzyme that is expressed primarily in the liver, kidney, and intestine.¹ G6Pase- α catalyzes the hydrolysis of glucose-6-phosphate (G6P) to glucose and phosphate in the terminal step of glycogenolysis and gluconeogenesis. Patients affected by GSD-Ia are unable to maintain glucose homeostasis and present with fasting hypoglycemia, growth retardation, hepatomegaly, nephromegaly, hyperlipidemia, hyperuricemia, and lactic acidemia.¹ There is no cure for GSD-Ia. Hypoglycemia can be managed using dietary therapies^{2,3} that enable patients to attain near normal growth and pubertal development. However, the longer term clinical complications, and their underlying pathological processes, remain uncorrected. One of the most significant chronic risks is hepatocellular adenoma (HCA), that develops in 70–80% of GSD-I patients over 25 year-old.^{1,4,5} HCAs in GSD-Ia patients are small, multiple, and nonencapsulated, with complications including local compression and intratumoral hemorrhage. In 10% of GSD-Ia patients, HCAs undergo malignant transformation to hepatocellular carcinoma (HCC).^{1,5,6}

G6Pase- α is a hydrophobic protein anchored in the endoplasmic reticulum (ER) by 9 transmembrane helices.¹ Gene therapy studies using adeno-associated virus (AAV) vectors carrying G6Pase- α have been performed in animal models of GSD-Ia and, in short term studies, demonstrated efficacy in the absence of toxicity.^{reviewed in 7} We have previously shown that systemic administration of AAV-GPE, an AAV pseudotype 2/8 (AAV8) vector containing the human G6Pase- α coding sequence along with ~ 3 kb of the *G6PC* gene 5'-flanking promoter/enhancer region (GPE) delivered the G6Pase- α transgene to the liver of G6Pase- α -null (G6pc^{-/-}) mice and restored wild type levels of hepatic G6Pase- α activity for the entire duration of a 24-week study.⁸ The infused G6pc^{-/-} mice exhibited normal blood glucose and metabolite profiles, normal hepatic glycogen and fat storage, and the absence of any apparent immune response. This raised the possibility that if chronic complications of GSD-Ia were also resolved, the AAV-GPE therapy might be a viable clinical option. The AAV vectors have low immunogenicity, exhibit few toxic effects, and are not known to be associated with any human disease.^{7,9,10} However, in addition to the chronic risk of HCA and HCC in GSD-Ia, Donsante et al.¹¹ have also shown an increased incidence of HCC associated with the use of an AAV vector in mice. In this long-term gene therapy study, we examine this combined vector and disease risk for hepatic neoplasia.

While G6pc^{-/-} mice exhibit a clinical phenotype that mimics the symptoms of human GSD-Ia,¹² they have a short lifespan that complicates studies on the chronic risk of HCA development. Recently, Mutel et al.¹³ generated liver-specific G6pc-null mice that exhibited a markedly improved survival rate compared to the global knockout mice. They showed that the aged liver-specific G6pc-null mice developed hepatomegaly and marked hepatic steatosis. The first detectable HCA occurred 36 weeks after gene deletion, with all mice developing HCA within 78 weeks of gene deletion.¹³ In this study, we examine the safety and efficacy of gene therapy mediated by AAV-GPE in murine GSD-Ia throughout a 90-week study and demonstrate that this vector not only corrects metabolic abnormalities in murine GSD-Ia but also shows no evidence for HCA formation.

Materials and Methods

Infusion of G6pc^{-/-} Mice with AAV-GPE

All animal studies were conducted under an animal protocol approved by the NICHD Animal Care and Use Committee. The AAV-GPE vector⁸ was infused into G6pc^{-/-} mice¹² via the retro-orbital sinus. Age-matched G6pc^{+/+}/G6pc^{+/-} as well as 6- to 10-week-old

G6pc^{-/-} mice were used as controls. For the virus-infused mice, glucose therapy¹² was terminated immediately after infusion.

Glucose tolerance testing of mice consisted of fasting for 6 hours, prior to blood sampling, followed by intraperitoneal injection of a glucose solution at 2 mg/g body weight, and repeated blood sampling via the tail vein for 2 hours.

Phosphohydrolase and Microsomal G6P Uptake Assays

Microsome isolation, phosphohydrolase assays, enzyme histochemical analysis of G6Pase- α , and microsomal G6P uptake assays were performed as described previously.^{8,12}

Quantification of Vector DNA

Total DNA was isolated from liver tissues using the GenElute™ Mammalian Genomic DNA Miniprep Kits (Sigma-Aldrich, St Louis, MO, USA). The vector genome numbers were quantified by real-time RT-PCR in an Applied Biosystems 7300 Real-Time PCR System (Foster City, CA). The following TagMan probes were used: human *G6PC* gene, Hs00609178_m1 and mouse β -Actin, Mm00607939_s1. Data were analyzed using the SDS v1.3 software (Applied Biosystems) and normalized to β -actin DNA. Plasmid DNA corresponding to 0.01 to 100 copies of human *G6PC* gene was used in a standard curve. To determine the vector genome copy number, the Ct values of sample were compared to the standard curve.

Phenotype Analyses

Mice were first examined for hepatic nodules by ultrasound using the Vevo 2100 system (VisualSonics, Ontario, Canada) and blood samples were collected from the tail vein. Blood glucose, total cholesterol, and uric acid were analyzed using kits obtained from Thermo Electron (Louisville, CO); triglycerides, by a kit from Sigma Diagnostics (St Louis, MO); lactate, by a kit from Trinity Biotech (St. Louis, MO); and insulin, by an ultra sensitive mouse insulin ELISA kit from Crystal Chem (Downers Grove, IL). Hepatic glycogen contents were measured as described previously.⁸ To determine hepatic triglyceride, glucose, and G6P contents, liver tissues were homogenized in RIPA buffer (50 mM Tris HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% Na-deoxycholate, and 0.1% SDS) (Thermo Scientific, Rockford, IL), and triglycerides were measured using a kit from Sigma Diagnostics, glucose, by a kit from Thermo Electron, and G6P, by a kit from BioVision (Mountain View, CA).

For hematoxylin and eosin (H&E) and oil red O staining,⁸ liver sections were preserved in 10% neutral buffered formalin and sectioned at 4–10 microns thickness. The stained sections were visualized using the Axioskop2 plus microscope and the AxioVision 4.5 software (Carl Zeiss, Thornwood, NY).

Quantitative Real-Time RT-PCR and Antibody Analysis

Total RNAs were isolated from liver tissues using the TRIzol Reagent (Invitrogen, Carlsbad, CA). The mRNA expression was quantified by real-time RT-PCR in an Applied Biosystems 7300 Real-Time PCR System using Applied Biosystems TaqMan probes (Foster City, CA). Data were analyzed using the Applied Biosystems SDS v1.3 software and normalized to β -actin RNA. Antibodies against human G6Pase- α were detected by Western-blot analysis as described previously.⁸

Statistical Analysis

The unpaired t test was performed using the GraphPad Prism Program, version 4 (San Diego, CA). Values were considered statistically significant at $P < 0.05$.

Results

AAV-GPE Infusion Directs Long-term Hepatic G6Pase- α Expression

We infused 2- or 4-week-old G6pc^{-/-} mice (n = 18) with varying doses of AAV-GPE [5×10^{12} to 3×10^{13} viral particles (vp)/kg] predicted to restore and maintain 3% to 100% of wild type hepatic G6Pase- α activity. We also infused one 15-week-old (5×10^{12} vp/kg) and one 30-week-old (1×10^{13} vp/kg) G6pc^{-/-} mouse. The low survival rate of GSD-Ia mice under glucose therapy severely restricted the numbers of adult mice available to study.¹² Metabolic and histological profiles of the 20 infused animals were monitored across 70–90 weeks and all measurements compared to those of their G6pc^{+/+} and G6pc^{+/-} littermates. The phenotype of both G6pc^{+/+} and G6pc^{+/-} mice are indistinguishable from wild type.¹²

There were no premature deaths of G6pc^{-/-} mice receiving gene therapy. Hepatic G6Pase- α activity and glycogen content, were assessed in mice sacrificed after a 24 hour fast. The mean fasting hepatic G6Pase- α activity of 70- to 90-week-old wild type mice (n = 20) was 185.8 ± 12.7 nmol/mg/min (Fig. 1A). As planned, there was a range of hepatic G6Pase- α activities restored in the treated mice. Of the 20 AAV-GPE-treated G6pc^{-/-} mice sacrificed at age 70 to 90 weeks, 6 mice had low levels (3% to 9% of wild type activity) of hepatic G6Pase- α activity and were designated AAV-L, 9 mice had medium levels (22% to 63% of wild type activity) of hepatic G6Pase- α activity, designated AAV-M, and 5 mice had high levels (81% to 128% of wild type activity) of hepatic G6Pase- α activity, designated AAV-H (Fig. 1A). Real-time RT-PCR analysis showed a linear relationship between hepatic G6Pase- α mRNA expression and G6Pase- α activity (Fig. 1B). Likewise, hepatic G6Pase- α activity also increased with hepatic vector genome copy numbers (Fig. 1C).

Enzyme histochemical analysis showed that G6Pase- α in wild type mice was distributed throughout the liver with significantly higher levels in proximity to blood vessels. There was no stainable G6Pase- α activity in the liver sections of untreated G6pc^{-/-} mice (Fig. 1D). In AAV-GPE-treated G6pc^{-/-} mice, G6Pase- α was also distributed throughout the liver but with foci, not related to blood vessels, containing markedly higher levels of enzymatic activity (Fig. 1D). The uneven distribution of hepatic G6Pase- α in the treated G6pc^{-/-} mice suggests that a substantial proportion of hepatocytes harbored low or little G6Pase- α , including AAV-H livers expressing 81–128% of wild type G6Pase- α activity. Uniform hepatic G6Pase- α expression is not required for rescue of the GSD-Ia phenotype.

AAV-GPE Infusion Corrects Metabolic Abnormalities in GSD-Ia

GSD-Ia is characterized by hypoglycemia, hypercholesterolemia, hypertriglyceridemia, hyperuricemia, and lactic acidemia.¹ Blood glucose levels in AAV-H mice expressing wild type hepatic G6Pase- α activity were indistinguishable from those of the control littermates (Fig. 2A). AAV-M and AAV-L expressing 22–63% and 3–9% normal hepatic G6Pase- α activity, respectively also maintained euglycemia (~ 100 mg/dl) state¹⁴ but their blood glucose levels were consistently lower than the control littermates (Fig. 2A). All treated G6pc^{-/-} mice exhibited normal serum profiles of cholesterol and triglyceride, while serum levels of uric acid and lactic acid in the treated G6pc^{-/-} mice were lower than those in the control littermates (Fig. 2A).

The average body weights of female and male AAV-GPE-treated mice at age 70–90 weeks were 70% and 62%, respectively of their age- and sex-matched control mice (Fig. 2B).

However, the average body lengths of the treated G6pc^{-/-} mice were 90% of the controls (Fig. 2B). Consequently, body mass index (BMI) values¹⁵ of the treated G6pc^{-/-} mice were significantly lower than those of the control littermates (Fig. 2B). While BMI values of both mouse groups signify normal growth,¹⁵ the treated G6pc^{-/-} mice were considerably leaner. The liver weights in wild type mice were relatively constant (Fig. 2C). In AAV-GPE-infused mice, liver weights were inversely correlated to the hepatic G6Pase- α activity restored (Fig. 2C). When liver weights were expressed as percent of body weight, the treated G6pc^{-/-} mice had significantly higher values because of their lower body weights. However, when absolute liver weights were compared directly, there was no significant difference between AAV-M, AAV-H, and control littermates (Fig. 2C). AAV-L mice, however, continued exhibiting hepatomegaly. AAV-GPE delivers little or no transgene to the kidney.⁸ However, the infused mice expressing higher hepatic G6Pase- α activity had lower kidney weights (data not shown), suggesting good hepatic metabolic control normalized nephromegaly.

Absence of Histological Abnormalities, Steatosis, or HCA in AAV-GPE-infused G6pc^{-/-} Livers

To determine the presence of HCA nodules in AAV-GPE-treated G6pc^{-/-} mice, we first conducted ultrasound analysis, followed by extensive examination of the livers and histological analysis of liver biopsy samples, using 5 or more separate sections per liver. Ultrasound and morphological analyses detected no hepatic nodules in wild type (n = 20) and the transduced G6pc^{-/-} (n = 20) mice that lived to age 70–90 weeks. The AAV-GPE-treated G6pc^{-/-} mice infused at age 2 or 4 weeks (n = 18) exhibited no hepatic histological abnormalities (Fig. 3A) except increased glycogen storage (Fig. 3A and 3B). The 84-week-old mouse infused at age 15 weeks, which expressed 6% of normal hepatic G6Pase- α activity, exhibited elevated glycogen storage and a few necrotic foci in one liver section (Fig. 3C). While most of the liver tissue sections of the 90-week-old mouse infused at age 30 weeks, which expressed 38% of normal hepatic G6Pase- α activity, exhibited no histological abnormalities, one liver section did have many necrotic foci (Fig. 3C). As necrotic foci are a characteristic hepatic pathology seen in untreated GSD-Ia mice age 6 weeks or older,¹⁶ it is quite likely that the necrotic foci had developed before initiation of gene therapy at age 15 or 30 weeks.

The livers of liver-specific G6pc-null mice were reported to develop HCA with marked steatosis.¹³ While a few wild type mice had increased hepatic fat storage, there was little or no fat storage in the livers of AAV-GPE-treated G6pc^{-/-} mice (Fig. 3A). Moreover, hepatic triglyceride contents in the treated G6pc^{-/-} mice (n = 20) were not statistically different from those in wild type mice (Fig. 3D). Oil red O staining confirmed that lipid contents in the treated animals (n = 20) were similar to that in the controls (Fig. 3E).

It has been well established that cyclooxygenase-2 (COX-2) is a marker that is over-expressed in many pre-malignant and malignant cancers, including HCC.¹⁷ Quantitative RT-PCR analysis showed that similar levels of hepatic COX-2 message were expressed in AAV-GPE-treated G6pc^{-/-} and control littermates (Fig. 3F).

AAV-GPE-treated G6pc^{-/-} Mice Exhibit Normal Fasting Glucose and Glucose Tolerance Profiles

The mean blood glucose levels of wild type mice (n = 20) before commencing fasting were 165.0 $\pm$ 3.0 mg/dl (zero time) which decreased to 113.3 $\pm$ 6.5 mg/dl after 24 hours of fasting (Fig. 4A). The fasting blood glucose profiles of AAV-L and AAV-M mice paralleled those of the control mice but blood glucose levels were consistently lower (Fig. 4A), while the fasting glucose profile of AAV-H mice was indistinguishable from that of the control mice. In sharp contrast, untreated G6pc^{-/-} mice, exhibited marked hypoglycemia within 60 to 75

min of fasting (Fig. 4B), a hallmark of GSD-Ia.¹ In summary, AAV-GPE-treated G6pc^{-/-} mice no longer suffered from the fasting hypoglycemia characteristic of GSD-Ia.¹

Blood glucose tolerance profiles in AAV-M and AAV-H mice were indistinguishable from those of wild-type littermates (Fig. 4C). In AAV-L mice, following intraperitoneal glucose injection blood glucose levels declined at a faster rate than the wild type controls.

Reduced Fasting Blood Insulin Levels in AAV-GPE-treated G6pc^{-/-} Mice

Insulin signaling regulates hepatic glucose and lipid metabolism.¹⁸ After 24 hours of fasting, blood insulin levels in 70–90-week-old wild type (n = 20) and the treated G6pc^{-/-} mice (n = 20) were 1.84 ± 0.29 and 0.56 ± 0.09 ng/ml, respectively (Fig. 5A). Both were within the normal range,¹⁹ although fasting blood insulin levels in the AAV-GPE-treated G6pc^{-/-} mice were more close to the normal average values.¹⁹ While fasting blood insulin levels in the treated G6pc^{-/-} mice did not correlate with hepatic G6Pase- α restored, insulin levels in wild type and the treated G6pc^{-/-} mice exhibited a linear relationship to their body weights (Fig. 5A).

The transcriptional effect of insulin is mediated by sterol regulatory element binding protein-1c (SREBP-1c).¹⁸ Quantitative RT-PCR analysis showed that similar levels of hepatic SREBP-1c transcripts were expressed in the 24-hour-fasted AAV-GPE-treated G6pc^{-/-} and control mice (Fig. 5B). Glucokinase is a glucose sensor.²⁰ Hepatic glucokinase activity decreases when blood insulin levels are low, as when fasting.²⁰ As was seen with blood insulin, after 24 hours of fast, hepatic glucokinase transcripts in the treated G6pc^{-/-} mice were significantly lower than that in the control littermates (Fig. 5C). Interestingly, levels of hepatic glucokinase mRNA and fasting blood insulin exhibited a linear relationship in both wild type and AAV-GPE-treated G6pc^{-/-} mice (Fig. 5C).

Glucose Homeostasis in the Liver of AAV-GPE-infused G6pc^{-/-} Mice

During fasting, blood glucose homeostasis is maintained by endogenous glucose produced in the liver from hydrolysis of G6P by the G6PT/G6Pase- α complex in the terminal step of gluconeogenesis and glycogenolysis.¹ G6pc^{-/-} mice, lacking a functional G6Pase- α , are incapable of producing endogenous glucose in the liver, kidney, or intestine. After 24 hours of fast, hepatic free glucose levels in wild type mice (n = 20) were 389 ± 17 nmol/mg protein (Fig. 6A) and in AAV-L (n = 6), AAV-M (n = 9), and AAV-H (n = 5) mice were 61%, 68% and 90%, respectively, of that in wild type mice (Fig. 6A). Intracellular G6P levels in the fasted AAV-L and AAV-M livers were 2.9- and 1.6-fold, respectively higher than wild type livers but intracellular G6P levels in the fasted AAV-H livers were statistically similar to that of wild type livers (Fig. 6A)

Hepatic G6P participates in several metabolic pathways, including glycogen synthesis, glycolysis, the pentose-phosphate pathway, in the cytoplasm, and endogenous glucose production in the ER lumen (Fig. 7). We examined hepatic expression of several key enzymes involved in the above mentioned pathways in mice after a 24-hour fast. These included: the cytosolic phosphoenolpyruvate carboxykinase (PEPCK-C) that catalyzes the first committed step in hepatic gluconeogenesis;²¹ fructose-1,6-bisphosphatase (FBPase-1) that converts fructose-1,6-bisphosphate to fructose-6-phosphate;²² phosphoglucomutase (PGMase) that catalyzes the reversible conversion of G6P and glucose-1-P in glycogenolysis and glycogen synthesis;²² phosphofructokinase-1 (PFK-1) that catalyzes the irreversible rate-limiting step in glycolysis by converting fructose-6-P to fructose-1,6-diphosphate;²² G6P dehydrogenase (G6PDH) that catalyzes the first reaction in the pentose-phosphate pathway by converting G6P to 6-phosphogluconolactone;²³ and G6PT that transports cytoplasmic G6P into the ER lumen¹ (Fig. 7). Quantitative real-time RT-PCR analysis

showed that in the fasted livers, PEPCK-C and PGMase transcripts were unchanged while FBPase-1 transcripts were increased in the AAV-GPE-treated G6pc^{-/-} mice, compared to the controls (Fig. 6B). Both PFK-1 and G6PDH transcripts in AAL-L livers were increased although in AAV-M and AAV-H livers they were still statistically similar to that of wild type livers (Fig. 6C). Interestingly, hepatic G6PT mRNA levels in the treated G6pc^{-/-} mice were 2.2-fold higher than the wild-type controls (Fig. 6B), regardless of the levels of hepatic G6Pase- α activity restored. The G6PT-mediated hepatic microsomal G6P uptake activity is the rate-limiting in endogenous glucose production,²⁴ but is co-dependent upon G6Pase- α activity.¹² Hepatic microsomes prepared from G6pc^{-/-} mice, with an intact G6PT, exhibit markedly lower G6P uptake activity compared to wild type hepatic microsomes,¹² which can be reversed if G6Pase- α activity is restored via gene transfer.²⁵ In AAV-L, AAV-M, and AAV-H livers, microsomal G6P uptake activities were 43%, 50%, and 72%, respectively of wild type activity (Fig. 6D), reflecting the increase in hepatic G6Pase- α activity (Fig. 1A) that paralleled hepatic free glucose levels (Fig. 6A).

Absence of Immune Response Against Human G6Pase- α

To determine whether a humoral response directed against human G6Pase- α is generated in the infused mice, we performed Western blot analysis using the sera obtained from the 70–90-week-old control and AAV-GPE-treated G6pc^{-/-} mice (Fig. 6E). A monoclonal antibody against human G6Pase- α that also recognizes murine G6Pase- α ⁸ was used as a positive control (lane 1). No antibodies directed against G6Pase- α were detected in any of the infused G6pc^{-/-} (lanes 2, 4, 6, 8, 10, and 12) or wild type control (lanes 3, 5, 7, 9, 11, and 13) mice that lived to age 70 to 90 weeks.

Discussion

Previous short-term studies have shown that AAV-GPE completely normalizes metabolic abnormalities associated with hepatic G6Pase- α deficiency in murine GSD-Ia.⁸ While these studies have shown promise, they do not address the issue whether AAV-GPE-mediated gene transfer can prevent the development of HCA, a severe long-term complication of GSD-Ia¹ and a potential complication of AAV-mediated gene therapy.¹¹ In this study we show that AAV-GPE-treated G6pc^{-/-} mice, expressing low levels (3% to 9%, AAV-L), medium levels (22% to 63%, AAV-M), and high levels (81% to 128%, AAV-H) of normal hepatic G6Pase- α activity, grow normally for 70–90 weeks, display no hepatic abnormalities, exhibit normalized blood metabolite and glucose tolerance profiles, and have no detectable anti-G6Pase- α antibodies. The treated G6pc^{-/-} mice exhibit no hepatic steatosis and have normal levels of hepatic triglyceride. In contrast to GSD-Ia patients¹ and untreated G6pc^{-/-} mice (this study), which cannot tolerate a short fast, the treated G6pc^{-/-} mice can sustain 24 hours of fast. Supporting this, significant levels of endogenous glucose are produced in the liver of AAV-GPE-treated G6pc^{-/-} mice after a 24-hour fast, enabling them to maintain interprandial glucose homeostasis. While 100% of the conditional liver-specific G6pc-null mice developed HCA at 78 weeks following gene deletion,¹³ none of the treated G6pc^{-/-} mice that lived to age 70–90 weeks developed HCA. Our data demonstrate, for the first time, that AAV-GPE-mediated gene transfer not only corrects hepatic G6Pase- α deficiency on a long-term basis, but is not associated with a chronic risk of HCA.

During a fast, endogenous glucose is primarily produced in the liver via hydrolysis of G6P in the terminal step of gluconeogenesis and glycogenolysis (Fig. 7). This process is catalyzed by two ER transmembrane proteins, G6PT and G6Pase- α .¹ G6PT transports G6P from the cytoplasm into the ER lumen and G6Pase- α , with its active site inside the lumen,¹ hydrolyzes intraluminal G6P to glucose (Fig. 7). These two activities are co-dependent and together they maintain interprandial glucose homeostasis. When untreated G6pc^{-/-} mice are fasted, hepatic G6P is transported by G6PT into the ER lumen, albeit at a markedly slower

rate than in wild type mice,¹² due to the lack of G6Pase- α coupling. As a result, G6P slowly accumulates in the ER lumen. Because of G6Pase- α deficiency, no glucose is released back to the circulation, leading to fasting hypoglycemia, the hallmark of GSD-Ia¹ (Fig. 7). In contrast, AAV-GPE-treated G6pc^{-/-} mice, exhibiting 3–128% of normal hepatic G6Pase- α activity can sustain 24 hours of fast. It is intriguing that after a 24-hour fast, the AAV-L mice expressing as little as 3% of wild type hepatic G6Pase- α activity are capable of hepatic glucose production averaging 61% of wild type levels. The expression of several key enzymes in gluconeogenesis (PEPCK-C, FEBase-1), glycogenolysis (PMGase), glycolysis (PFK-1), and the pentose phosphate pathway (G6PDH) (Fig. 7) are not significantly altered but hepatic G6PT mRNA expression in AAV-GPE-treated G6pc^{-/-} mice is 2.2-fold higher than the wild type level. A similar increase in hepatic G6PT mRNA expression is also observed in untreated G6pc^{-/-} mice (data not shown). The rate-limiting step in hepatic microsomal glucose production is G6P uptake,²⁴ mediated by G6PT, and it is notable that in the presence of a reduced G6Pase- α activity there appears to be a feedback mechanism that partially compensates by increasing G6PT mRNA expression. The net result is an increase in the rate-limiting microsomal G6P uptake activity that enables AAV-L mice to maintain glucose homeostasis during prolonged fasts. This extends our understanding of the nature of functional co-dependence of the two components of the G6PT/G6Pase- α complex that maintains interprandial blood glucose homeostasis. In addition to a functional coupling,¹² a transcriptional component may also be involved.

In AAV-GPE-treated G6pc^{-/-} mice, G6Pase- α was distributed unevenly through the liver, with many hepatocytes exhibiting minimal activity, but foci containing markedly higher levels of activity. Although it is not clear if the upregulation of G6PT expression and activity is limited to specific cells, the similar increase in G6PT mRNA expression seen in untreated G6pc^{-/-} mice suggests that it is characteristic of all hepatic cells with reduced G6Pase- α activity. The ability to compensate by feedback is a very attractive feature of this therapy as it provides for a greater biological tolerance for the efficiency of gene therapy. Clearly there are additional levels of control over hepatic blood glucose than previously recognized.

GSD-Ia patients under dietary therapy exhibit moderate glucose intolerance, delayed insulin response, and increased lipogenesis.²⁶ This is most likely related to the manner of glucose intake by frequent meals of low glycemic index carbohydrate diets and/or nocturnal intragastric feeding which, by consistently elevating blood glucose levels, may lead to glucose intolerance and reduced peripheral insulin sensitivity. Insulin signaling regulates hepatic glucose and lipid metabolism that is essential for the maintenance of energy homeostasis.¹⁸ Insulin signaling associated with carbohydrate flux promotes *de novo* lipogenesis in the liver, but also turns off β -oxidation of fatty acids. Over the long term this may contribute to the development of hepatic steatosis^{18,27} seen in GSD-Ia patients. Consistent with this, mice with a liver-specific knockout of G6Pase- α activity are reported to develop HCAs and exhibit marked hepatic steatosis.¹³ In contrast, the AAV-GPE-treated G6pc^{-/-} mice do not manifest hepatic steatosis and have normal hepatic triglyceride. Both wild type and the treated G6pc^{-/-} mice exhibit normal fasting blood insulin levels, although the treated G6pc^{-/-} mice with lower fasting blood insulin levels indicate a better metabolic control. In AAV-GPE-treated G6pc^{-/-} mice, hepatic free glucose levels, an indicator of endogenous glucose production, are consistently lower than those in wild type mice, leading to reduced fasting blood insulin in the treated mice. Supporting this, fasting blood insulin levels in the treated G6pc^{-/-} and control mice correlate with their body weights and the treated G6pc^{-/-} mice are considerably leaner. The low fasting blood insulin levels and lack of hepatic steatosis may explain why AAV-GPE-treated G6pc^{-/-} mice do not develop HCA.

In summary, we have demonstrated that AAV-GPE-mediated gene therapy in *G6pc*^{-/-} mice that restores 3% to 128% of normal hepatic G6Pase- α activity corrects hepatic G6Pase- α deficiency and is not associated with HCA formation. Our results suggest that G6Pase- α gene transfer may offer a therapeutic approach to the management of human GSD-Ia.

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List of Abbreviations

AAV	adeno-associated virus
ER	endoplasmic reticulum
FBPase-1	fructose-1,6-bisphosphatase
G6P	glucose-6-phosphate
G6Pase-α	glucose-6-phosphatase- α
G6PDH	G6P dehydrogenase
G6PT	glucose-6-phosphate transporter
GSD-Ia	glycogen storage disease type Ia
HCA	hepatocellular adenoma
HCC	hepatocellular carcinoma
GPE	human <i>G6PC</i> promoter and enhancer

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Fig. 1. Hepatic G6Pase- α activity and mRNA expression in 70- to 90-week-old wild type and AAV-GPE-treated G6pc^{-/-} mice following a 24-hour fast. Seven 2-week, eleven four-week, one 15-week (◆) and one 30-week (◆◆) old G6pc^{-/-} mice were studied. (A) Hepatic G6Pase- α activity is shown at the indicated ages in weeks (W). The mice are grouped based on their G6Pase- α activity relative to wild type activity as low (AAV-L), medium (AAV-M) and high (AAV-H). (B) Hepatic G6Pase- α mRNA expression and its relationship to G6Pase- α activity in the treated G6pc^{-/-} mice. Data are presented as mean $\pm$ SEM. * P < 0.05, ** P < 0.005. (C) The relationship between hepatic G6Pase- α mRNA expression and vector genome copy numbers in the treated G6pc^{-/-} mice. (D) Histochemical analysis of

hepatic G6Pase- α activity. Freshly sectioned liver specimens were analyzed for G6Pase- α activity using the method of lead trapping of phosphate generated by G6P hydrolysis.⁸ Each image represents an individual mouse, so two mice are shown for each treatment. Treatments are indicated by: (-/-), untreated G6pc^{-/-} mice; (+/+), wild type mice; (-/- AAV), G6pc^{-/-} mice infused with various dosages of AAV-GPE. AAV-L (n = 6), AAV-M (n = 9), AAV-H (n = 5) are AAV-GPE-treated G6pc^{-/-} mice expression 3–9%, 22–63%, and 81–128% normal hepatic G6Pase- α activity, respectively.

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Fig. 2.

Phenotype analysis of AAV-GPE-treated $G6pc^{-/-}$ mice at age 70 to 90 weeks. (A) Blood glucose, triglyceride, cholesterol, uric acid, and lactic acid levels. (B) Body weight, body length, and BMI. F, females; M, males. (C) Liver weight. Treatments are indicated as: (+/+), wild type mice; (-/- AAV), $G6pc^{-/-}$ mice infused with various dosages of AAV-GPE. AAV-L (n = 6), AAV-M (n = 9), AAV-H (n = 5) are AAV-GPE-treated $G6pc^{-/-}$ mice expressing 3–9%, 22–63%, and 81–128% normal hepatic G6Pase- α activity, respectively. Data are presented as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.005$.

Fig. 3.

Histological, glycogen, lipid, and COX-2 analyses in the liver of 24-hour fasted wild type and AAV-GPE-infused $G6pc^{-/-}$ mice at age 70 to 90 weeks. Treatments are indicated as: ($-/-$), untreated $G6pc^{-/-}$ mice; ($+/+$), wild type mice ($n = 20$); ($-/-$ AAV) AAV-GPE-treated $G6pc^{-/-}$ mice ($n = 20$). AAV-L ($n = 6$), AAV-M ($n = 9$), AAV-H ($n = 5$) are the treated $G6pc^{-/-}$ mice expressing 3–9%, 22–63%, and 81–128% normal hepatic G6Pase- α activity, respectively. (A) H&E stained liver sections at original magnifications of $\times 200$ in the treated $G6pc^{-/-}$ mice infused at age 2 or 4 weeks. Each plate represents an individual mouse, so two mice are shown for each treatment. (B) Hepatic glycogen contents. (C) H&E stained liver sections at original magnifications of $\times 200$ in AAV-GPE-treated $G6pc^{-/-}$ mice infused at

age 15 or 30 weeks. Duplicated plates are shown for each mouse. (D) Hepatic triglyceride contents. (E) Oil red O staining at original magnifications of $\times 400$. Each plate represents an individual mouse, so two mice are shown for each treatment. (F) Quantification of COX-2 mRNA by real-time RT-PCR. Data are presented as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.005$.

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Fig. 4.

Fasting blood glucose and glucose tolerance profiles. (A) Fasting blood glucose profiles in wild type and AAV-GPE-treated G6pc^{-/-} mice at age 70 to 90 weeks. (B) Fasting blood glucose profiles in untreated G6pc^{-/-} mice at age 6–8 weeks. (C) Glucose tolerance profiles in wild type and the treated G6pc^{-/-} mice at age 70 to 90 weeks. Wild type or the infused G6pc^{-/-} mice were fasted for 6 hours, injected intraperitoneally with 2 mg/g of dextrose, and then sampled for blood every 30 minutes via the tail vein. Data are presented as mean ± SEM. (+/+), wild type mice; (-/-), untreated G6pc^{-/-} mice. AAV-L (n = 6), AAV-M (n = 9), AAV-H (n = 5) are AAV-GPE-treated G6pc^{-/-} mice expression 3–9%, 22–63%, and 81–128% normal hepatic G6Pase-α activity, respectively.

Fig. 5. Blood insulin and hepatic mRNA levels for SREBP-1c and glucokinase in 70 to 90 week-old wild type and AAV-GPE-treated $G6pc^{-/-}$ mice after 24 hours of fast. (A) Fasting blood insulin levels and its relationship to body weights of the animals. (B) Quantification of SREBP-1c mRNA by real-time RT-PCR. (C) Quantification of glucokinase mRNA and the relationship of fasting blood insulin to hepatic glucokinase mRNA levels. (+/+, $\circ$), wild type mice ($n = 20$); (-/- AAV, $\bullet$) AAV-GPE-treated $G6pc^{-/-}$ mice ($n = 20$). Data are presented as mean $\pm$ SEM. ** $P < 0.005$.

Fig. 6.

Glucose homeostasis and analysis of anti-G6Pase- α antibody in wild type and AAV-GPE-treated G6pc^{-/-} mice. Hepatic levels of free glucose, G6P, mRNA for PEPCK-C, FBPase-1, PGMase, G6PT, PFK-1, and G6PDH were determined in the liver of wild type and the treated G6pc^{-/-} mice after a 24-hour fast at age 70 to 90 weeks. (A) Hepatic free glucose and G6P levels. (B) (C) Quantification of PEPCK-C, FBPase-1, PGMase, G6PT (B), PFK-1, and G6PDH (C) mRNA levels by real-time RT-PCR. (D) Microsomal G6P uptake activity. Data are presented as mean $\pm$ SEM. * P < 0.05, ** P < 0.005. (E) Analysis of anti-G6Pase- α antibody in the sera of 70- to 90-week-old wild type and AAV-GPE-treated G6pc^{-/-} mice. Microsomal proteins from Ad-human G6Pase- α infected COS-1 cells were electrophoresed

through a single 12% polyacrylamide-SDS gel and transferred onto a PVDF membrane.⁸ Membrane strips, representing individual lanes on the gel were individually incubated with the appropriate serum. A monoclonal antibody against human G6Pase- α that also recognizes murine G6Pase- α ⁸ was used as a positive control (lane 1). Treatments are indicated as: (+/+), wild type mice; (-/- AAV), G6pc^{-/-} mice infused with various dosages of AAV-GPE. AAV-L (n = 6), AAV-M (n = 9), AAV-H (n = 5) are the treated G6pc^{-/-} mice expressing 3–9%, 22–63%, and 81–128% normal hepatic G6Pase- α activity, respectively.

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Fig. 7.

Pathways for G6P metabolism in normal and GSD-Ia liver during fasting. During fasting, G6P, the end product of gluconeogenesis and glycogenolysis, is transported from the cytoplasm into the lumen of the ER by G6PT.¹ Inside the ER, G6P is hydrolyzed by G6Pase-α and the resulting glucose transported back into the cytoplasm then released into the circulation. In the GSD-Ia liver, which lacks a functional G6Pase-α, ER-localized G6P cannot be converted to glucose, leading to hypoglycemia following a short fast.¹ The GLUT2 transporter, responsible for the transport of glucose in and out of the cell, is shown embedded in the plasma membrane. The G6PT, responsible for the transport of G6P into the ER, and G6Pase-α, responsible for hydrolyzing G6P to glucose and phosphate, are shown embedded in the ER membrane.