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Generation of Mice with a Conditional Allele for *G6pc*

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Summary

Glucose-6-phosphatase- α (G6Pase- α or G6PC) catalyzes the hydrolysis of glucose-6-phosphate to glucose and is a key enzyme in interprandial glucose homeostasis. Mutations in the human *G6PC* gene, expressed primarily in the liver, kidney, and intestine, cause glycogen storage disease type Ia (GSD-Ia), an autosomal recessive disorder characterized by a disturbed glucose homeostasis. For better understanding of the roles of G6Pase- α in different tissues and in pathological conditions, we have generated mice harboring a conditional null allele for *G6pc* by flanking exon 3 of the *G6pc* gene with *loxP* sites. We confirmed the null phenotype by using the *Ell1a-Cre* transgenic approach to generate mice lacking exon 3 of the *G6pc* gene. The resulting homozygous *Cre*-recombined null mice manifest a phenotype mimicking G6Pase- α -deficient mice and human GSD-Ia patients. This *G6pc* conditional null allele will be valuable to examine the consequence of tissue-specific G6Pase- α deficiency and the mechanisms of long-term complications in GSD-Ia.

Introduction

Glucose-6-phosphatase- α (G6Pase- α also known as G6PC) is a key enzyme in glucose homeostasis catalyzing the hydrolysis of glucose-6-phosphate to glucose and phosphate in the terminal steps in gluconeogenesis and glycogenolysis (Chou *et al.*, 2002). The expression of G6Pase- α is tissue-specific, restricted primarily to the liver, kidney, and intestine (Pan *et al.*, 1998). Deleterious mutations in the *G6PC* gene causes glycogen storage disease type Ia (GSD-Ia), an autosomal recessive metabolic disorder characterized by hypoglycemia, hepatomegaly, nephromegaly, hyperlipidemia, hyperuricemia, lactic acidemia, and growth retardation (Chou *et al.*, 2002). Currently, there is no cure for GSD-Ia, but many of the disease symptoms can be managed or improved using dietary therapies (Greene *et al.*, 1976; Chen *et al.*, 1984) that maintain normoglycemia. This strategy enables patients to attain near normal growth and pubertal development, with fewer complications as they age. Despite intensive dietary therapy, the long-term complications of GSD-Ia - renal disease and hepatocellular adenomas with risk for malignant transformation - remain (Chou *et al.*, 2002). Moreover, GSD-Ia patients suffer from intermittent diarrhea that worsens with age (Visser *et al.*, 2002). Therefore, the metabolic disruption by G6Pase- α deficiency has tissue-specific consequences. We have previously generated a null allele for G6Pase- α (*G6pc*^{-/-}) by knocking out exon 3 and shown that the *G6pc*^{-/-} mice manifest a phenotype virtually identical to that of human GSD-Ia patients (Lei *et al.*, 1996). Despite administering a glucose therapy to prevent hypoglycemic seizures, the *G6pc*^{-/-} mice rarely live to 3

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months of age. Consequently, we have been unable to study the molecular mechanisms leading to long-term complications of GSD-Ia. While liver and/or kidney transplantation have been proposed for the treatment of late stage liver and renal disease in GSD-Ia (Matern *et al.*, 1999; Labrune, 2002), their effectiveness is not known. To understand whether such transplants are viable, and whether single or double transplant is necessary, we need to understand the importance of G6Pase- α expression in the liver and kidney, individually, and whether wild-type G6Pase- α expression at one site is sufficient to compensate for lack of expression at the other.

To address the role of G6Pase- α in maintaining normal liver, kidney, and intestinal functions, we report here the generation of mice harboring a conditional null allele for *G6pc* using DNA homologous recombination in embryonic stem cells and the Cre-*loxP* and FLPe-*Frt* strategies. These *G6pc* conditional null mice should facilitate the study of the individual consequence of G6Pase- α deficiency in the liver and kidney, and help clarify whether a deficiency in one can affect the other. The conditional mice could also be used to study the consequence of G6Pase- α deficiency in the intestine, an organ also capable of endogenous glucose production (Croset *et al.*, 2001).

Generation of a Conditional Allele of *G6pc*

The murine *G6pc* gene consists of 5 exons spanning approximately 12 kb of mouse genomic DNA. To generate a conditional allele for *G6pc*, a targeting vector was constructed using plasmid pPNT4 which contains the neo gene flanked by *Frt* sites and one *loxP* site at its 5' region, and the HSV-tk gene (Conrad *et al.*, 2003). The neo gene provides a positive selection for insertion of the targeting sequences in ES cells in the presence of G418, and the HSV-tk gene for negative selection in the presence of gancyclovir. The neo gene, along with the flanking *Frt* and *loxP* sites, was inserted at the *SacII* site within intron 3 of the *G6pc* gene. An additional DNA sequence containing a *loxP* site and three diagnostic restriction sites (*AvaI*, *SspI*, and *EcoRV*) was inserted at the *StuI* site within intron 2 of the *G6pc* gene. The resulting targeting vector contained *loxP* sites to flox the *G6pc* exon 3, and *Frt* sites to flank the neo gene (Fig. 1a). The targeting vector was introduced into J1 ES cells and the cells, subjected to selection with the drugs G418 and gancyclovir. Homologous recombinants were identified by Southern hybridization of *EcoRV* digested genomic DNA using two probes corresponding to specific 5' or 3' fragment located outside of the targeted region (Fig. 1a and 1b). One of the *G6pc*-targeted ES clones was used for the production of chimeric animals. The chimeric mice were then mated with C57BL/6 mice and germline transmission of the *G6pc* targeted allele in F1 heterozygous (fn/+; fn denotes *floxP-neo*) mice was confirmed by PCR analysis (Fig. 1c). The fn/+ mice were mated and homozygous (fn/fn) progenies were identified by PCR analysis (Fig. 1c). The *G6pc*^{fn/fn} mice were phenotypically indistinguishable from the wild-type mice.

The *Frt*-flanked neo gene was deleted *in vivo* by breeding the *G6pc*^{fn/fn} mice with the FLPeR mice which express the enhanced version of the site-specific recombinase FLPe ubiquitously (Farley *et al.*, 2000). The resulting *G6pc*^{fx/+} (fx denotes *floxP* with neo-deleted) conditional mice which have *loxP* sites flanking *G6pc* exon 3 and one *Frt* site in the *G6pc* allele were confirmed by PCR analysis (Fig. 1d). The *G6pc*^{fx/+} mice were mated and the resulting homozygous *G6pc*^{fx/fx} conditional mice were viable and fertile, and they did not display any recognizable phenotype. To demonstrate that the *loxP* flanked *G6pc* exon 3 could be deleted *in vivo*, the *G6pc*^{fx/fx} mice were crossed with the *Elia-Cre* transgenic mice, where Cre is expressed under the control of the adenoviral *Elia* promoter that targets expression of the Cre recombinase during preimplantation development (Lakso *et al.*, 1996). The exon 3- and neo-deleted (*G6pc* ^{Δ/Δ}) mice were confirmed by PCR analysis. There were no detectable G6Pase enzymatic activity in the liver and kidney of the *G6pc* ^{Δ/Δ} mice (data

not shown), confirming that the targeted mutation in exon 3 had resulted in a null *G6pc* locus *in vivo*.

The *G6pc*^{Δ/Δ} Mice Manifest Disturbed Glucose Homeostasis

GSD-Ia patients (Chou *et al.*, 2002) and *G6pc*^{-/-} mice (Lei *et al.*, 1996) manifest a phenotype of disturbed glucose homeostasis characterized by hypoglycemia, hepatomegaly, nephromegaly, hypercholesterolemia, hypertriglyceridemia, hyperuricemia, lactic acidemia, and growth retardation. The *G6pc*^{Δ/Δ} mice exhibit similar characteristics. Metabolic analysis reveals a fasting hypoglycemia typical of GSD-Ia. The mean serum glucose level in *G6pc*^{Δ/Δ} mice (43 ± 11 mg/dl) was significantly lower than those in wild-type (220 ± 8 mg/dl) littermates (Fig. 2a). The *G6pc*^{Δ/Δ} mice suffered from hypoglycemic seizures requiring glucose therapy, like the *G6pc*^{-/-} mice (Lei *et al.*, 1996). Additionally, serum cholesterol, triglyceride, uric acid, and lactic acid levels in *G6pc*^{Δ/Δ} mice averaged 7.2-, 15.9-, 1.6- and 1.6-fold higher, respectively, than that in the control littermates (Fig. 2a), like the *G6pc*^{-/-} mice (Lei *et al.*, 1996). The *G6pc*^{Δ/Δ} mice are growth retarded, and exhibit hepatomegaly and nephromegaly (Fig. 2b) similar to that observed in GSD-Ia patients (Chou *et al.*, 2002) and *G6pc*^{-/-} mice (Lei *et al.*, 1996). The average liver and kidney weights of 3-week-old *G6pc*^{Δ/Δ} mice were elevated to 15.6% and 2.6% of body weight, respectively, compared to the wild-type value of 4.6% and 0.7%. Hematoxylin-eosin (H&E) staining revealed glycogen accumulation in the hepatocytes of the liver and tubular epithelial cells of the kidney in *G6pc*^{Δ/Δ} mice (Fig. 2c), like the *G6pc*^{-/-} mice (Lei *et al.*, 1996).

In conclusion, we have generated a conditional null *G6pc* allele and shown that homologous exon 3 deletion leads to a phenotype of disturbed glucose homeostasis mimicking that of *G6pc*^{-/-} mice and human GSD-Ia patients. The mice carrying the floxed *G6pc* allele are now excellent candidates to study tissue-specific role of the *G6pc* gene and the mechanism of long term complications in GSD-Ia.

METHODS

Gene Targeting and Generation of a *G6pc*-Floxed Allele

A bacterial artificial chromosome (BAC) containing the murine *G6pc* gene was obtained by screening a BAC library in the 129/svJ mouse strain (Invitrogen, CA) with a *G6pc* cDNA probe. The targeting construct was generated in two steps from the neo- and HSV-tk-containing plasmid pPNT4 where the neo gene is flanked by *Frt* sites and one *loxP* site at its 5' region (Conrad *et al.*, 2003). First, the 3.5-kb *SacII*-*EcoRI* fragment of the murine *G6pc* gene containing 3' region of intron 3, intron 4, exon 4 and 5' region of exon 5 was inserted into the *KpnI*-*EcoRI* sites of pPNT4 downstream of the neo gene, to create the construct pPNT4-*G6pc*-SE. In a second step, the 5.8-kb *G6pc* *KpnI*-*SacII* fragment containing exons 2, 3, introns 1, 2, and 5' region of intron 3, including an additional *loxP* site plus three diagnostic restriction sites, *AvaI*, *SspI*, and *EcoRV* at the *StuI* site within intron 2, was inserted into *SalI* and *NotI* sites of pPNT4-*G6pc*-SE plasmid upstream of the neo gene, yielding the targeting construct. The final targeting construct contained a neo gene flanked by two *Frt* sites, and exon 3 of the *G6pc* gene flanked by two *loxP* sites (fig. 1a).

The targeting-construct, linearized at the *SfiI* site, was introduced into J1 ES cells by electroporation and the cells subjected to selection with G418 and gancyclovir (Mansour *et al.*, 1988). Homologous recombinants were identified by Southern blot hybridization of *EcoRV* digested genomic DNA using two probes corresponding to specific 5' or 3' fragment located outside of the targeting region. Twenty-one correctly targeted clones were obtained after screening 318 G418 resistant clones. The *G6pc*-targeted ES cells were microinjected into blastocysts from C57BL/6 mice for the production of chimeric embryos, which then

was implanted into the uterine horn of pseudopregnant FVB/N foster mice for the production of chimeric animals. The chimeric mice were mated with C57BL/6 mice and germline transmission of the exon 3-floxed *G6pc* targeted allele in F1 heterozygous (*G6pc^{fn/+}*) mice was confirmed by PCR analysis. The *Frt*-flanked neo gene was deleted *in vivo* by breeding the homozygous *G6pc^{fn/fn}* mice with the FLPeR mice (Farley *et al.*, 2000). Exon 3 of the *G6pc* gene was deleted by crossing the *G6pc^{fx/fx}* mice with the *Elia-Cre* transgenic mice (Lakso *et al.*, 1996).

PCR, Genotype, and Phenotype Analyses

The introduced *loxP* site in intron 2 was identified using a primer pair, 1S (5'-CGGGCAATGGCTAGTTAGAG-3') and 2AS (5'-CAGGCTGCTAGGAAGGACAC-3'), expected to amplify a fragment of 103 bp in wild-type alleles and a fragment of 162 bp in the mutant allele. The FLPeR allele was identified using the primer pair, FS (5'-CCCATTCCATGCGGG GTATCG -3') and FAS (5'-GCATCTGGG AGATCACTGAG -3'), expected to amplify a fragment of ~700 bp. A neo-specific primer pair, neoS (5'-GATCGGCCATTGAACAAGAT-3') and neoAS (5'-ATACTTCTCGGCAGGAGCA-3') is expected to amplify a fragment of 345 bp in the neo gene of the targeted allele. The FLPeR-mediated deletion of the neo cassette was identified using the primer pair, 3S (5'-CAGTCGGGTGCTCTTACCTG-3') and 4AS (5'-AGCCTGTTCTCTCTGCTTGG-3'), expected to amplify a fragment of 166 bp in wild-type alleles and a fragment of 262 bp in the neo-deleted mutant allele. The primer pair 1S/4AS, expected to amplify a fragment of 289 bp, was used to identify *Elia-Cre*-mediated deletion of floxed exon 3.

Serum glucose, total cholesterol, and uric acid were analyzed using kits obtained from Thermo Electron (Louisville, CO), triglycerides using a kit from Sigma Diagnostics (St Louis, MO), and lactic acid using a kit from Trinity Biotech (St. Louis, MO). For H&E staining, tissues were preserved in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 4–6 micron thickness.

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FIG. 1.

Conditional disruption of the murine *G6pc* gene. (a) The *G6pc* locus is presented along with the targeting construct, the targeted allele (*G6pc*^{fn}), the *G6pc* conditional allele (*G6pc*^{fx}) and the anticipated outcome of the *G6pc* null allele (*G6pc*^Δ). Introns are denoted as lines, exons as boxes. K, *KpnI*; R, *EcoRI*; RV, *EcoRV*; St, *StuI*; Sa, *SacII*; Sf, *SfiI*. The PCR primers used to verify the conditional alleles are denoted by arrows. (b) Southern-blot analysis of *EcoRV* digested mouse genomic DNA from wild-type (+/+) and targeted ES (+/-) clones using 5' and 3' external probes. The wild-type locus hybridizes with either probe to give a 16 kb band while the disrupted locus hybridizes with the 5' probe to give a 5.3 kb band, and with the 3' probe to give a 12.6 kb band. (c) PCR analysis of genomic DNA of the targeted allele (*G6pc*^{fn}). The primer pair, 1S/2AS is expected to amplify a fragment of 103 bp in wild-type alleles and a fragment of 162 bp in the mutant allele. The primer pair Neo1/NeoAs is expected to amplify a fragment of 345 bp in the neo gene of the targeted allele. (d) PCR analysis of genomic DNA of the *G6pc* conditional allele (*G6pc*^{fx}). The primer pairs, 1S/2AS and 3S/4AS are expected to amplify fragments of 103 and 166 bp, respectively, in wild-type alleles and fragments of 162 and 262 bp, respectively, in the mutant allele. Deletion of the neo gene is confirmed by the absence of a 345 bp fragment predicted to amplify the neo gene by the primer pair NeoS/NeoAS.

FIG. 2.

The *G6pc*^{Δ/Δ} mice manifest a phenotype of disturbed glucose homeostasis. Metabolic functions were analyzed in 3-week-old *G6pc*^{Δ/Δ} and wild-type mice. (a) Serum glucose, cholesterol, triglyceride, uric acid, and lactic acid levels. Data are presented as mean ± SEM. ***p* < 0.005. (b) The body weight and the weights of the liver and kidney relative to total body weight. Data are presented as mean ± SEM. ***p* < 0.005. (c) H&E stained liver and kidney sections from *G6pc*^{Δ/Δ} and wild-type mice with magnifications of ×200.