Glucose transporter isoform-3 mutations cause early pregnancy loss and fetal growth restriction

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Submitted 13 July 2006; accepted in final form 1 January 2007

Glucose transporter isoform-3 mutations cause early pregnancy loss and fetal growth restriction. Am J Physiol Endocrinol Metab 292: E1241–E1255, 2007. First published January 9, 2007; doi:10.1152/ajpendo.00344.2006.—Glucose transporter isoform-3 (GLUT3) is the trophoblastic facilitative glucose transporter. To investigate the role of this isoform in embryonic development, we created a novel GLUT3-null mouse and observed arrested early embryonic development and loss at neurulation stage when both alleles were mutated. This loss occurred despite the presence of other related isoforms, particularly GLUT1. In contrast, when a single allele was mutated, despite increased embryonic cell apoptosis, adaptive changes in the subcellular localization of GLUT3 and GLUT1 in the preimplantation embryo led to postimplantation survival. This survival was compromised by decreased GLUT3-mediated transplacental glucose transport, causing late-gestation fetal growth restriction. This yielded young male and female adults demonstrating catch-up growth, with normal basal glucose, insulin, insulin-like growth factor-I and IGF-binding protein-3 concentrations, fat and lean mass, and glucose and insulin tolerance. We conclude that GLUT3 mutations cause a gene dose-dependent early pregnancy loss or late-gestation fetal growth restriction despite the presence of embryonic and placental GLUT1 and a compensatory increase in system A amino acid placental transport. This critical life-sustaining functional role for GLUT3 in embryonic development provides the basis for investigating the existence of human GLUT3 mutations with similar consequences during early pregnancy.

Creation of GLUT3-Null Mouse Lines

Isolation of 129 Ola genomic GLUT3 clones. A P1 library (Genome Systems, St. Louis, MO) was screened (Genome Systems) by polymerase chain reaction using mouse GLUT3 specific oligonucleotides (forward primer 5′-ttccacctctcagattat-3′ and reverse primer 5′-tgctcaagctgattc-3′). A GLUT3-positive clone was sequenced (Promega, Madison, WI) using a GLUT3-specific primer to confirm the presence of GLUT3 sequences. To establish that the entire GLUT3 coding region was present, the P1 clone was restricted with BamH1, EcoR1, HindIII, Kpn1, Pst1, Sac1, SalI, Kpn1/BamH1, Kpn1/Sac1, SalI/BamH1, SalI/EcoR1, SalI/Pst1, and SalI/Sac1 enzymes, blotted, and probed with end-labeled oligonucleotides that anneal 30 base pairs either upstream of the transcriptional start site within exon 2 or within exon 10. The P1 clone was then shotgun cloned into pGEM3zf+ by digestion with either HindIII or SalI. Subclones were probed with exon-specific end-labeled oligonucleotides on Southern blot analysis to map each fragment. A 12.5-kb HindIII fragment containing exons 1–6 and an 8-kb HindIII fragment containing exons 7–10 were identified. All exons and the ends of each clone were sequenced for confirmation. The 12.5-kb clone contained 4.5 kb of the 5′-flanking sequence, and the 8-kb fragment contained 2.2 kb of the 3′-flanking sequence.

GLUT3 knockout targeting vector. The pKO Scrambler NTK 1903 vector (Stratagene, La Jolla, CA) was used to generate a mouse GLUT3 knockout targeting construct. Left and right targeting arms were designed to delete a 3.5-kb fragment that would completely remove exons 7, 8, and 9 and the coding region of exon 10. The left arm was a 3.2-kb SalI/HindIII genomic fragment starting in intron 3 and ending in intron 6. The right arm was generated by PCR with Xhol/EcoR1 overhanging ends beginning at 500 bp downstream of the 5′-end of the GLUT3 coding sequence. The PCR products were blunt-end ligated into pGEM3zf+ and digested with EcoR1 and HindIII.

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E1241

Adapted from http://ajpendo.physiology.org/ by 10.220.33.1 on June 15, 2017
Southern blot analysis of tail genomic DNA. Animals (and their agouti offspring were tested for germ line transmission by ES cell clones was injected into C57BL/6 wild-type (WT) females, in accordance with the guidelines set by the National Institutes of Health. Each of the four correctly targeted heterozygous recombination events were identified in four clones (73, 74, 80, 86) genomic DNA probed with a GLUT3 DNA fragment that hybridizes 500 bp downstream of the stop codon in exon 10. WT endogenous GLUT3 allele yields a 6.5-kb band, whereas the mutated (KO) allele migrates at 3.6 kb. C: representative Southern blot of EcoR1 predigested tail genomic DNA obtained from heterozygous mating using the probe spanning exons 2–5 of the GLUT3 gene. The GLUT3 mutated (KO) allele demonstrates loss of the EcoR1 site yielding a 12.6-kb DNA fragment, whereas the WT allele retains the EcoR1 site, resulting in an 8.5-kb DNA fragment. W/WT, wild type; H/Het, heterozygote.

Heterozygous null embryonic stem cell lines. The 129 embryonic stem (ES) cell line LW1 was cultured as described previously (24). Following electroporation with the targeting vector, 287 clones resistant to G418 (200 µg/ml concentration) were selected, and homologous recombination events were identified in four clones (73, 74, 80, 86) of the first 100 clones screened by Southern blot analysis, as described below, following restriction with either EcoR1 or HindIII (Fig. 1B).

GLUT3 knockout mouse lines. All mouse studies were approved by the Animal Research Committee of the University of California, Los Angeles, in accordance with the guidelines set by the National Institutes of Health. Each of the four correctly targeted heterozygous ES cell clones was injected into C57BL/6 wild-type (WT) females, and their agouti offspring were tested for germ line transmission by Southern blot analysis of tail genomic DNA. Animals (+/-) for the targeted allele were initially intercrossed and then back-crossed to create the F10 generation and beyond with a homogeneous C57/BL6 background gene. Exons are numbered boxes.

Body composition. Initially, body weights were measured using an Ohaus LS 2000 top-loading balance with an accuracy of 1 g. Whole body fat and lean masses were assessed in unanesthetized, freely moving male and female 10-wk-old WT and GLUT3 heterozygous null mice by use of 1H magnetic resonance spectroscopy (Bruker minispectroscopy analyzer; Echo Medical Systems, Houston, TX).

Glucose and insulin tolerance tests. Following an overnight fast, 10-wk-old WT or GLUT3 heterozygous null male and female mice received intraperitoneally either D-glucose (1 g/kg body wt) or insulin (0.5 U/kg body wt of pharmaceutical grade; Novo-Nordisk, Clayton, NC). Tail vein blood samples were collected at 0, 15, 30, 45, 60, 75, 105, 120, and 135 min, and blood glucose concentrations were assessed using the Hemocue system (Mission Viejo, CA).

Adult blood glucose, insulin, and IGFBP3 measurements. After 10-wk-old mice were euthanized, intracardiac blood samples were collected, and plasma was separated. Plasma glucose concentrations were measured using a Beckman glucose analyzer, and plasma insulin concentrations were assessed with ELISA kits (Linco Research, St. Charles, Mo). IGF-I (R&D Systems, Minneapolis, MN) and IGFBP3 concentrations were also measured by ELISA, as described previously (47, 48).

Transplacental glucose and system A amino acid uptake. Following heterozygote mating at 10 wk of age, pregnant female mice at 18.5 days gestation were intraperitoneally injected with a bolus of 12 µCi of 2-deoxy-[1-14C]glucose (PerkinElmer, Boston, MA). An hour later, the injection blood was collected from the jugular vein, and glucose concentration and radioactivity were assessed. Soon after the mice were euthanized with pentobarbital sodium (100 mg/kg ip), the fetuses and placentas were collected separately. A small portion of preweighed placenta was employed for genotyping, and the remaining placenta and whole fetus were hydrolyzed separately in 1 M NaOH at 60°C for 45 min and then neutralized with 1 M HCl. One aliquot (200 µl) of the neutralized lysates was added to 1 ml of HClO4, and another aliquot (200 µl) was added to Ba(OH)2/ZnSO4. After centrifugation, the supernatants (800 µl) were used to assess radioactivity in a scintillation counter. Protein and glucose concentrations were estimated on the placental/fetal lysates as well. Glucose uptake by the placenta and fetus was calculated as a ratio between specific activities of glucose in the placenta/fetus and specific activity of glucose in...
maternal blood (13, 25, 44). The glucose uptake (HClO₄-precipitated supermatant) by the placenta signified glucose transport and phosphorylation [Ba(OH)₂/ZnSO₄ precipitate] in this tissue, whereas glucose uptake by the fetus signified fetal glucose transport and phosphorylation as a consequence of transplacental glucose transport.

In the case of amino acid uptake, 3.5 μCi/100 μl [¹⁴C]methylnosobutyric acid (MeAIB; New England Nuclear, NEC-671; specific activity of 50.5 mCi/mmol) in saline was administered via the tail vein to 18.5-gestation-day 10-wk-old pregnant mice. At 5 min following the tracer administration, placenta and fetus were dissected following hysterotomy. Tissue lysis was carried out overnight at 55°C in Biosol (National Diagnostics, Atlanta, GA) using a volume of 4 ml for the fetus and 0.5 ml for the placenta. Measured aliquots were used to assess radioactivity that was expressed per gram of tissue (7).

Tissue RNA studies. Poly(A⁺)-enriched RNA was extracted per the manufacturer’s instructions, using an oligotex mRNA extraction mini-kit (Qiagen, Valencia, CA) from various tissues obtained from genotyped WT and GLUT3 heterozygous progeny. Extracted mRNA (2 μg) was subjected to Northern blot analysis as previously described (37). Following electrophoresis overnight of RNA on a 1% agarose gel, transfer to nylon membranes (Amersham, Hybond N⁺), and UV cross-linking, the blots were prehydrized for 2 h at 42°C and subsequently hybridized overnight at 42°C with the probe. The same ³²P-labeled murine GLUT3 cDNA described above served as the probe. After washing and exposure to X-ray film as described above, the same blots were stripped in 0.1% SDS at 100°C for 3–5 min and rehybridized to the full-length murine GLUT1 cDNA (11) and later to a full-length GAPDH probe that served as the internal control to standardize interlane loading variability. mRNA band density was assessed by densitometry using the Scion Image software program after ensuring that the resultant optical density was linear to the loading mRNA concentrations.

Tissue protein studies. Tissue homogenates were solubulized in 50 mM Tris, pH 6.8, containing 2% SDS and protein concentration determined by the Bio-Rad dye-binding assay. Western blot analysis was carried out as described previously (36, 37, 39). Briefly, the solubulized protein homogenates (20 μg) were subjected to electrophoresis on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Transblot; Bio-Rad, Hercules, CA). The primary antibody consisted of an affinity-purified rabbit anti-mouse GLUT3 or GLUT1 IgG that was generated against a keyhole limpet hemocyanin-linked terminal 17 amino acids of the mouse GLUT3 or GLUT1 peptide, the isoform specificity of which has been previously characterized by us (36, 41). The primary anti-mouse GLUT3 and the anti-mouse GLUT1 antibodies were used at a 1:1,000 dilution for 2 h at room temperature. The secondary antibody consisted of a horseradish peroxidase-conjugated antibody (1:4,000) that allowed detection of the immunoreactive protein bands by enhanced chemiluminescence. The intensity of the protein bands was assessed by densitometry using the Scion Image software program after ensuring that the resultant optical density was linear to the protein concentration.

Immunolocalization of GLUT3 and GLUT1 in adult tissues. Heterozygous and WT tissues obtained from 10-wk-old mice consisting of the 18.5-gestation-day placenta, testis, and brain were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, and subjected to immunohistochemistry. Fixed placenta and testis were placed in 20% sucrose in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.02% sodium azide, and embedded in OCT compound (Tissue-Tek, Torrance, CA). Specimens were rapidly frozen in liquid nitrogen and then sectioned (8-μm thickness) with a Frigo-Cut (Leica Microsystems, Nussloch, Germany). Perfused brains in 4% paraformaldehyde were embedded in 2% agar and sectioned (30-μm thickness) using a Leica VT 1000S vibrotome (Nussloch, Germany). Immunofluorescence staining was carried out as previously described (40). Double immunofluorescence staining for GLUT3 and GLUT1 was carried out by preincubating these sections with 5% normal donkey serum in PBS followed by incubation for 1 h with a mixture of rabbit anti-mouse GLUT3 antibody at 1:500 dilution (36, 41) and guinea pig anti-mouse GLUT1 antibody at 1:500 (kindly provided by K. Takata, Gunma University Medical School, Japan) for 1 h. After a washing with PBS, the sections were incubated with a mixture of Texas red-labeled donkey anti-rabbit IgG, FITC-labeled donkey anti-guinea pig IgG (Jackson Immunoresearch, West Grove, PA), and 4′,6-diamidino-2-phenyindole dihydrochloride (DAPI; Sigma Chemical), a nuclear stain, for 45 min. The sections were washed with PBS, mounted using an antibleaching mounting medium (40), and examined with a Nikon E-600 microscope (Nikon, Melville, NY) equipped with a cooled, charge-coupled device camera (CoolSNAP HQ Monochrome; Roper Scientific, Tucson, AZ). Negative controls consisted of normal serum or primary antibody in the presence of peptides used to generate the antibodies that entirely abolished the immunoreaction.

Phenotypic Characterization of Preimplantation Embryos

Preimplantation embryo recovery. Embryos were recovered as previously described (5, 32, 38). In brief, 4- to 6-wk-old female heterozygous mice were superovulated with an intraperitoneal injection of 10 IU/animal of pregnant mare serum gonadotropin (Sigma Chemical) followed later by 10 IU/animal of human chorionic gonadotropin (Sigma Chemical). Mating with male GLUT3 heterozygous mice occurred, and conception in the female GLUT3 heterozygous mice was confirmed by identification of a vaginal plug. Animals were euthanized on day 3.5 (implantation occurs between days 4.5 and 5.5), 96 h after human chorionic gonadotropin administration and mating. Blastocysts were obtained by flushing dissected uterine horns and oviduct as described previously (5, 32). The embryos were then immediately placed in potassium-rich simplex optimization medium (KSOM) (Speciality Media, Phillipsburg, NJ) and cultured at 37°C in an atmosphere of 5% CO₂-5% O₂-90% N₂.

Localization of GLUT3 and GLUT1 proteins and detection of apoptosis by TUNEL assay in blastocysts. Blastocysts were fixed onto glass slides with 3% paraformaldehyde and permeabilized with 0.1% Tween. The embryos were then washed and incubated with a primary rabbit anti-mouse GLUT3 antibody or a primary murine anti-rat GLUT1 antibody for 1 h at room temperature (20 μg/ml). All antibodies were peptide purified and used in the same concentration as the peptide-purified preimmune serum that served as the negative control. The embryos were then washed and incubated with a secondary antibody, goat anti-rabbit FITC-labeled antibody, for 1 h. Certain embryos were incubated with fluorescent-labeled dUTP and terminal transferase in the dark for 1 h at 37°C to label fragmented 3′ DNA (TUNEL, Cell Death In Situ Kit; Roche Molecular Biochemicals, Indianapolis, IN) as previously described (5, 32). Counterstaining of nuclear DNA was achieved by incubating embryos in either propidium iodide at a concentration of 0.01 mg/ml (red channel) or To-Pro-3 iodide (Molecular Probes, Eugene, OR) at a concentration of 4 μM (blue channel) for 20 min. After extensive washing, the embryos were visualized using confocal immunofluorescent microscopy (Nikon C1 laser scanning microscope) at ×63 magnification, as previously described (5, 32, 44). The GLUT3 and GLUT1 immunoreaction was scored by an independent observer on a scale from 0 to 3, with 0 representing no staining and 3 representing the most intense staining. A Z-series was performed on each blastocyst to determine the total number of nuclei and the number of apoptotic or TUNEL-positive nuclei per total nuclei per embryo.

Postimplantation Embryos

Early postimplantation embryos. Genomic DNA isolated from embryos spanning days 6.5 to 9.5 were subjected to PCR using gene-specific primers for GLUT3 corresponding to the deleted region in the mutant mouse (exons 7–10) (GLUT3-del), neomycin resistance gene (NEO), which was inserted to create a deletion in the endogenous GLUT3 gene, and GAPDH gene as an internal control. These primers were as follows: GLUT3-del forward primer (+9950 bp)
5'-ggagactgccctatagat-3', reverse primer (+10908 bp) 5'-ctaggcca-cataaacag-3'; NEO forward primer (+50 bp) 5'-agagcttcatgctat-gae-3', reverse primer (+744 bp) 5'-ctagctgctgaatccgaggg-3'; and GAPDH gene forward primer (+1 bp) 5'-ccggaattcgaaggtcgg-3', reverse primer (+230 bp) 5'-cacaacagctgggaa-3'. PCR was performed using the Herculase Hot Start enzyme (Invitrogen) with 20–25 cycles and an annealing temperature of 55°C. One hundred nanograms of DNA template, 0.2 mM dNTPs, 1 pmol of primers, and 5 units of the enzyme were mixed in 4% DMSO. The relative intensities of the gel electrophoresis-separated amplification products were assessed with the image J software program and expressed as intensity of either the GLUT3-del or NEO DNA product/GAPDH DNA product.

Localization of GLUT3 and GLUT1 in early postimplantation embryos. This was undertaken by immunohistochemistry using the avidin-biotin peroxidase complex (ABC) as previously described (14, 39). GLUT3-null heterozygous pregnant females that had been mated with heterozygous male counterparts were euthanized at 6.5 and 8.5 days of gestation. Intact deciduas containing the embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), washed in PBS, dehydrated with increasing concentrations of ethanol, cleared in xylene, and embedded in paraffin. Paraffin sections (8 μm) were rehydrated, blocked in 5% normal donkey serum for 1 h in PBS, and then incubated overnight with the primary antibody (rabbit anti-mouse GLUT3 and GLUT1) at 1:500 dilution. After a washing with PBS, the sections were treated with biotinylated goat anti-rabbit IgG (1:500) and ABC mixture (Vector Lab) for 1 h. The sections were finally washed with PBS and incubated with 0.03% hydrogen peroxide plus 0.05% diaminobenzidine tetrahydrochloride in 0.1 M PBS for a color reaction. The embryos were counterstained with hematoxylin and eosin. Specimens were dehydrated through a graded series of ethanol, mounted in Vecta Mount (Vector Lab), and subjected to light microscopy (11, 14, 39). For morphological analysis, fixed embryos were dehydrated in graded ethanol and acetone, embedded in paraffin, and sectioned (8-μm thickness) with a Leica RM2125 microtome (Nussloch, Germany), and then hematoxylin-eosin staining was carried out as previously described (11, 40).

Culturing early postimplantation embryos. Pregnant females at 7.5–8.5 days post vaginal plug were anesthetized with isoflurane, and the uterus was removed and placed in DMEM with high glucose (25 mM) and 10% fetal calf serum. Each embryo was removed from its decidua and placed in a separate well of a 96-well microtiter plate containing DMEM, teased apart with sterile forceps from its yolk sac, and incubated at 37°C, 5% CO2, for 4 days. For genotyping of the yolk sac, genomic DNA was extracted by adding 150 μl of a digestion buffer (100 mM EDTA, 1% SDS, 75 mM NaCl, 5 mg/ml proteinase K, 10 mM Tris, pH 7.5) and incubating at 37°C for several minutes. The lysates were collected and stored at −80°C. Genomic DNA was extracted by adding 0.3 volumes of saturated NaCl and mixing by inversion for 10–15 s and then adding 1 volume of chloroform and mixing by inversion for 10–15 s. The tubes were incubated on ice for 10 min before centrifugation at 10,000 g for 5 min. The aqueous phase was collected, 2.5 volumes of 100% ethanol were added, and the samples were centrifuged at 10,000 g for 15 min, washed with 70% ethanol, and air dried. DNA pellets were resuspended in 50 μl of TE buffer (pH 8.0). Forty-four microliters of DNA from each sample were mixed in 4% parafomaldehyde in PBS for 15 min, washed in PBS three times, treated with 50 mM glycine for 15 min, and washed again with PBS three times. The coverslips were incubated for 1 h with rabbit anti-mouse GLUT3 primary antibody (1:500) followed by donkey anti-rabbit secondary antibody conjugated with Texas red, as described above. Samples were visualized using a Nikon fluorescent microscope (40, 41).

Characterization of mid- and late-stage postimplantation embryos. Pregnancies were interrupted at 12.5, 14.5, 16.5, 18.5, and 20.5 days for genotyping by Southern blot analysis and assessment of GLUT3 and GLUT1 protein in placenta and the whole body or head regions separately by Western blot analysis. In addition placental, whole fetal body (12.5 to 20.5 days), and birth weights were measured using a Mettler AB104 precision balance with an accuracy of 0.01 mg.

Data Analysis

Results are shown as means ± SE. All statistical analyses were performed using SigmaStat 3.5 software (Systat, Point Richmond, CA). Comparison between age- and sex-matched (only in adults) WT and GLUT3-null mice was performed by Student’s t-test in the presence or the nonparametric Mann-Whitney rank sum test in the absence of a normal distribution, and significance was assigned for a P < 0.05. Coefficients of correlation were derived by linear regression analysis.

RESULTS

GLUT3-Null Mouse Lines

The GLUT3 knockout targeting vector was designed to remove exons 7–9 and the coding region of exon 10 (Fig. 1A), thereby deleting the glucose-transporting functional domain. Homologous recombination events were identified in four clones (73, 74, 80, 86) (Fig. 1B). Three of these ES clones (73, 80, 86) that were microinjected yielded chimeric mice, of which two clones supported germ line transmission. Clone 73 established GLUT3 knockout mouse line 1 and clone 86 established mouse line 10.

Embryonic Lethality of GLUT3-Null Mice

The average litter size of heterozygous mating was 4.97 ± 0.17 in line 1 and 4.3 ± 0.6 in line 10, in contrast to 6 ± 1.29 of WT mating. Heterozygous mating led to 2.48 ± 0.13 heterozygotes per litter and 1.73 ± 0.12 wild types per litter, the genotype confirmed by Southern blot analysis (Fig. 1C). The total ratio of (+/+) to (+/-) to (−/−) was 1:1.5:0 (n = 763), being 1:1.6:0 in males (n = 390) and 1:1:4:0 in females (n = 376), with the latter demonstrating an ~13% decrease in (+/-) vs. the former. The male-to-female ratio per litter was 0.95 ± 0.18. The homozygous genotype was uniformly lethal in both mouse lines and through all generations. As WT mating led to an average litter size of 6, the Mendelian ratio of (+/+) to (+/-) to (−/−) is expected to be 1:5:3:1.5. Loss of all homozygous mice would produce a ratio between (+/+) to (+/-) to (−/−) of 1:5:3:0 or 1:2:0. Instead, the ratio we observed was 1:1:5:0 (P < 0.0001), supporting some loss of heterozygous mice estimated on an average to be ~0.5 pup/litter.

Phenotype of GLUT3 Heterozygote

Body weight and plasma assays. Body weights of male and female adult heterozygous animals were different from the sex-matched WT mice at 10 wk of age (Table 1). No difference in adult fasting blood glucose concentrations was observed in
the males and females (Table 2). Similarly, no change in basal plasma insulin concentrations was observed in either males or females (Table 2). Plasma IGF-I and IGFBP3 measured in males and females were no different between the GLUT3 heterozygous null mice and age-matched WT mice (Table 2).

**Body composition.** No change in total, fat, or lean mass was observed at 10 wk of age in either males or females in the GLUT3 heterozygous null vs. the WT mice (Fig. 2A).

**Glucose and insulin tolerance tests.** Both males and females at 10 wk of age were glucose (Fig. 2, B and C) and insulin (Fig. 2, D and E) tolerant in the two genotypes with no difference.

**Placental and fetal body weights.** No difference in placental weight was noted at 12.5, 14.5, 16.5, 18.5, and 20.5 days gestation between the WT and GLUT3 heterozygous null genotypes (Table 1). Although no statistically significant difference in fetal body weight at 12.5 and 14.5 days was observed, an ~7% decline at 16.5 days gestation, an ~19% decline at 18.5 days gestation and an ~22% decline at 20.5 days gestation in the GLUT3 heterozygous fetal body weight was consistent with late-gestation fetal growth restriction. At birth, the pups demonstrated ~12% growth restriction (Table 1).

**Placento-embryonic glucose and amino acid transport.** Transplacental glucose transport into the 18.5-days-gestation fetus was decreased by ~31% (P < 0.05) in the heterozygous compared with the WT (Fig. 3A) mice. Paralleling these changes in glucose transport, a comparable decrease in fetal glucose phosphorylation was also observed. In contrast, other than a trend toward a decline, there was no change in intraplacental glucose transport or phosphorylation at the same gestational age in the heterozygous vs. the WT mice (Fig. 3A). Of note was a compensatory increase in intra- and transplacental system A amino acid transport reflected in both the placenta and fetus (Fig. 3B).

**Adult tissue GLUT3 and GLUT1 expression.** By examination of adult tissues, GLUT3 mRNA and protein were expressed in WT 18.5-gestation-day placenta, testis, and brain but not in liver, kidney, and lung. In the heterozygote 18.5-gestation-day placenta, testis, and brain, ~50% decreases in GLUT3 mRNA (Fig. 4A) and protein (Fig. 4B) concentrations were noted. GLUT1 mRNA existed in WT 18.5-gestation-day placenta, liver, testis, and brain, with low amounts in kidney and lung. No difference was noted in GLUT1 mRNA (Fig. 4A) and protein (Fig. 4B) concentrations in any of these tissues except for an increase in brain in the heterozygote. The decline in heterozygous null placental GLUT3 with no change in GLUT1 protein concentrations was associated with a trend toward decreased intraplacental glucose transport (~23%) (Fig. 3A).

**GLUT3 and GLUT1 immunolocalization.** Despite the overall decline in GLUT3 immunoreaction, there were no differences in the pattern of distribution between the heterozygous and WT 18.5-gestation-day placenta, brain, and testis (Fig. 5, a–l). Similarly, the level of GLUT1 immunoreaction and localization were no different between the two genotypes in the three tissues (Fig. 5, a–f).

**Preimplantation Embryos.**

GLUT3 immunostaining allowed differentiation between the genotypic GLUT3 WT/heterozygous and homozygous embryos. Absence of GLUT3 in the trophectoderm was reliably associated with highest percentage of TUNEL-positive staining in the blastocyst (Fig. 6C). Presence of GLUT3 immunoreaction could be categorized into two types: classical, consisting of GLUT3 expressed on the apical surface of trophectoderm, which was associated with a normal rate of minimal TUNEL-positive cells (Fig. 6A), and atypical, consisting of GLUT3 on the apical and basolateral surfaces of the trophectoderm, which was associated with a moderate increase in the percentage of TUNEL-positive cells (Fig. 6B). In addition, the intensity of GLUT3 immunoreaction was different between these two types, being greater in the former than in the latter. On the basis of the type of GLUT3 staining pattern and the level of expression, we could categorize the embryos into WT if they expressed GLUT3 immunoreaction on the apical surface of the trophectoderm only (Fig. 6A), heterozygous if GLUT3 was observed on the apical and basolateral surfaces of the trophectoderm (Fig. 6B), and homozygous if no GLUT3 immunoreaction was noted (Fig. 6C). The GLUT3 staining intensity [(+/+):(+/-):(-/-) = 2.7 ± 0.2:1.6 ± 0.3:0.3 ± 0.01; n = 3–8/genotype, *P < 0.01] and TUNEL stain scoring (2 ± 2:12.5 ± 5*:35 ± 7*: n = 3–8-genotype, *P < 0.01) revealed

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Values are means ± SE in grams. WT, wild type; Het, heterozygous. *P < 0.05 vs. WT (+/+) (Table 2).

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<th>Table 2. Plasma glucose, insulin, IGF-I, and IGFBP-3 concentrations</th>
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Values are means ± SE. IGF-I, insulin-like growth factor I; IGFBP-3, IGF-binding protein-3.
a reciprocal relationship ($r = 0.99$, $r^2 = 0.97$, $P < 0.01$). GLUT1 immunoreaction was observed on the basolateral surface of trophectoderm (32) in WT (Fig. 6D) with partial redistribution to the apical surface in heterozygous embryos (Fig. 6E). The homozygous embryos, however, demonstrated punctate cytoplasmic distribution (Fig. 6F).

**Postimplantation Embryos**

The mean litter size noted at gestational ages 9.5 day to 20.5 day was no different from the litter size of live born mice produced by heterozygous mating. Furthermore, while WT and heterozygous embryonic genotypes were noted there were no homozygous embryos. In contrast, the mean litter size noted between 6.5 day and 8.5 gestation-day was 8.8 ± 0.86 vs. 5.2 ± 0.08 at 9.5 day to 20.5 gestation-day ($P < 0.001$). Genomic DNA obtained at 6.5 day, 8.5 day, and 9.5 gestation-day revealed the presence of WT (GLUT3-del+, NEO−, GAPDH+), heterozygous (GLUT3-del+, NEO+, GAPDH+), and homozygous (GLUT3-del−, NEO+, GAPDH+) embryos at 6.5 days (Fig. 7A) and 8.5 days (Fig. 7B), but the homozy-
glous embryos were absent at 9.5 days (Fig. 7C). Histology along with GLUT3 and GLUT1 immunoreaction of 6.5-day embryos revealed the presence of GLUT3 in the apical and basolateral cell layers of the visceral endoderm (+/+ or +/−; Fig. 8A, a). GLUT1 immunoreaction was observed in all layers of the embryonic ectoderm and most of the cytotrophoblastic cells in all 6.5-day embryos examined (Fig. 8A, c). In certain embryos, no GLUT3 immunoreaction was detected (Fig. 8A, b) with an unchanged GLUT1 distribution (−/−; Fig. 8A, d). Although GLUT3-containing embryos had a well-defined morphology, embryos lacking GLUT3 were associated with infiltration by GLUT1-containing cells (Fig. 8A, d). GLUT3-lacking 8.5-day embryos demonstrated a developmental arrest and appeared amorphous in certain cases (Fig. 8B, b and d), whereas the WT embryos demonstrated the forebrain vesicle and neural tube structures with GLUT3 immunoreaction restricted to the Reichert’s membrane (Fig. 8B, a) and select trophoblastic cells (Fig. 8B, e). In contrast, GLUT1 was observed in the neuroepithelium lining the forebrain vesicle, neural tube, somites, blood cells, Reichert’s membrane (Fig. 8B, c), and the placental labyrinthine region (Fig. 8B, f). This in vivo observation was confirmed further in 7.5-day-to 8.5-day-old cultured embryos. The GLUT3-expressing embryos (Fig. 8C, f) demonstrated progression of growth and development in vitro (Fig. 8C, b) generating adequate numbers of dispersed cells (Fig. 8C, d), whereas the GLUT3-lacking embryos (Fig. 8C, e) demonstrated arrested development (Fig. 8C, a) and generated sparse, dispersed cells (Fig. 8C, c). Examination of subsequent stages of development spanning 12.5 to 18.5 days of gestation revealed a significant increase in placental GLUT3 concentrations between 12.5- and 14.5-gestation days, followed by a plateau between 14.5- and 18.5-gestation day in the (+/+ genotype. In contrast, placental GLUT3 protein concentrations peak at 16.5-gestation day in the (+/− genotype. Placental GLUT1 concentrations in the GLUT3 (+/+) and (+/−) genotypes were no different, increasing between 12.5 and 14.5 days followed by a plateau between 14.5 and 18.5 gestation days (Fig. 8D). In contrast to the placenta, fetal head and body regions demonstrated GLUT1 predominantly with no GLUT3 expression at 14.5 and 18.5 gestation days, with minimal GLUT3 expression only at 18.5 gestation days (+/+) in the fetal head (brain) (Fig. 8E).

**DISCUSSION**

We have created a GLUT3-null mouse that in the homozygous state demonstrates early embryonic lethality during neurulation and in the heterozygous state develops fetal growth restriction, survives birth, and demonstrates “catch-up” growth in the young adult male and female, seen as normal body weight and composition, glucose, and insulin tolerance. Embryonic lethality in response to a lack of GLUT3 despite the presence of other glucose transporter isoforms (GLUT1, GLUT2, GLUT8, GLUT9, GLUT12) (5, 6, 32, 38, 50) supports a critical role for this isoform in embryonic development. The homozygote survives the postimplantation stage spanning days 6.5 to 8.5 but not days 9.5 to 20.5. Culturing of 7.5- to 8.5-day homozygous embryos in a nutrient-rich medium failed to rescue the arrested growth and development.

Studies involving preimplantation embryos demonstrated increased apoptosis in GLUT3-deficient blastocysts resulting in subsequent postimplantation embryonic demise. This apoptotic event is the result of preimplantation embryos being highly reliant on glucose as an alternate substrate in a relatively hypoxic milieu (32). The same effect was seen in blastocysts that were homozygous for the GLUT1 antisense transgene that culminated in fetal loss (17). Absence of GLUT3 on the apical trophoderm that is in direct contact with the maternal milieu resulted in successful implantation in our present study but to the demise of postimplantation embryos in the neurulation phase (8.5 days) (35). This observation supports the critical need for a threshold level of glucose transport into the blasto-

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Fig. 3. A: deoxy-[14C]glucose uptake consisting of glucose transport and phosphorylation into (+/+) and (+/−) 18.5-gestation-day placenta and fetus. Each measurement in (+/−) placenta and embryo was compared by Mann-Whitney rank sum test to the corresponding measurement in (+/+). B: [14C]methylaminoisobutyric acid (MeAIB) uptake into (+/+) and (+/−) 18.5-gestation-day placenta and fetus. Each measurement in (+/−) placenta and embryo was compared with (+/+) placenta and embryo by Mann-Whitney rank sum test. *P < 0.05 vs. corresponding tissue in the (+/+ group.

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**AJP-Endocrinol Metab • VOL 292 • MAY 2007 • www.ajpendo.org**
cyst-stage embryo to ensure well being and postimplantation survival.

Closer investigation of GLUT3-deficient homozygous preimplantation embryos revealed an absence of GLUT1 in basolateral surfaces of the trophoderm and inner cell mass, where it normally resides in WT embryos (32). Instead, GLUT1 was present intracellularly in cytoplasmic vesicles with minimal cell surface expression. This is similar to that seen in blastomeres of precompaction embryos prior to the establishment of polarity when totipotent blastomeres differentiate into trophodermal epithelium (38). This suggests that some degree of GLUT3 expression is required to trigger polarization and successful establishment of a healthy blastocyst. GLUT1 and GLUT3 may need to be coordinately expressed to allow adequate glucose uptake by blastocysts. Despite these changes in the homozygous preimplantation embryos, the process of implantation proceeded, and embryos developed until the neurulation phase (35). This observation in vivo is contrary to previous observations in vitro that supported a differentiating role for GLUT3 in blastocysts. Antisense GLUT3 oligonucleotides led to a developmental arrest at the preimplantation stage (33).

Heterozygous blastocysts demonstrated decreased GLUT3 immunoreaction; however, both GLUT1 and GLUT3 were seen at basolateral and apical locations, demonstrating their ability to polarize correctly, even though both transporters overlapped in their localization. These adaptive changes allowed the survival of heterozygous embryos into and beyond the postimplantation stage with only a minimal loss of ~0.5 pup/litter compared with WT littermates. The heterozygous

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**Fig. 4.** A: **top:** representative Northern blots that demonstrate 3.9-kb GLUT3 mRNA (left) or 2.8-kb GLUT1 mRNA (right) in (+/+), (+/-), and (-/-) 18.5-gestation-day placentas, 10-wk-old testis, brain, liver, kidney, and lung. Only in the placenta is a truncated GLUT3 mRNA species seen in the heterozygote. GAPDH mRNA, the internal control. **Bottom:** densitometric quantification of GLUT3 and GLUT1/GAPDH mRNA as percent WT value (n = 3 per genotype). B: **top:** representative Western blots showing 50-kDa GLUT3 (left) or 45-kDa GLUT1 (right) protein band in (+/+), (+/-), and (-/-) brain, 18.5-gestation-day placenta, and testis. Vinculin protein band, the internal control. **Bottom:** densitometric quantification of GLUT3 and GLUT1/vinculin protein (n=3 per genotype) as percent WT value. Student’s t-test demonstrated differences as shown, *P < 0.01.
embryos not only survived the pre- and postimplantation period but progressed through late gestational life, demonstrating fetal growth restriction (~20% peak decline in body weight vs. WT) only during late gestation. This phenotypic presentation was observed whether the maternal genotype was WT or heterozygous, provided the genotype of the placenta-embryonic unit was heterozygous. The 12.5- to 20.5-day placenta expresses both GLUT1 and GLUT3 and so does the 6.5- to 8.5-day ectoplacental cone. Fetal growth restriction observed by us may relate to moderately increased apoptosis observed in totipotent heterozygous blastocysts. However, since there was no difference in fetal weight at 12.5 and 14.5 gestation days, the more likely reason may be diminished placental glucose transfer that is unable to meet the demands of the late-gestation fetus at its peak growth phase. 

Prior investigations involving creation of intrauterine growth restriction (IUGR) demonstrated a decrease in the placental exchange surface and GLUT3 protein concentrations (9, 30), supporting an association. In contrast, maternal glucocorticoid-induced fetal growth restriction is associated with a compensatory increase in placental GLUT3 concentrations (29) to meet fetal demands. These observations collectively support an important role for placental GLUT3-mediated transplacental nutrient supply in IUGR. What was not clear from these investigations was whether the change in placental GLUT3 concentrations was the cause or the effect of fetal growth restriction. More recent studies in the IGF-II heterozygous null mouse demonstrated a decline in transplacental system A amino acid transfer to the fetus, with an adaptive increase in placental GLUT3-mediated transplacental glucose transport in response to fetal signals of impaired growth (7). Thus, in the face of decreased transplacental amino acid transport, the fetal demands led to increased GLUT3-mediated glucose transport (7). This is in contrast to our present GLUT3 heterozygous null mice, where decreased transplacental GLUT3-mediated glucose transport led to an adaptive increase in transplacental system A amino acid transport in response to signals imposed by impaired fetal growth. 

Further existing controversy regarding the role of placental GLUT3 in mediating transplacental glucose transport in the face of large amounts of placental GLUT1 remained unresolved. GLUT3 resides on the maternal facing surface of syncytiotrophoblastic layer I lining the labyrinthine region that is responsible for materno-fetal glucose exchange (40) in the rodent trichorial placenta. GLUT1 resides on both the maternal and fetal facing surfaces of syncytiotrophoblastic layers II and I, respectively (40). In the epithelio-chorial ovine placenta, a similar collaboration between GLUT3 and GLUT1 ensures an adequate glucose supply to the developing conceptus (8, 9). The presence of GLUT3 at the apical surface and its lower $K_m$ may more efficiently trap circulating maternal glucose in trophoblast cells surrounding fetal vasculature. On the other hand, GLUT1 may mediate transplacental glucose transport to the fetus. Contrary to these associative observations, our present investigations are the first to mechanistically demonstrate the critical role of GLUT3 in transplacental glucose transport. Murine hemochorial placental GLUT3 deficiency led to a decrease in transplacental glucose transport and fetal glucose

![Immunohistochemical localization of GLUT3 and GLUT1.](image)
uptake that was more than the trend toward a decrease in intraplacental glucose uptake. This decrease in transplacental glucose transport was substantial despite normal amounts of GLUT1 in the labyrinthine nutrient exchange region, supporting GLUT3’s higher affinity for glucose.

The other controversy concerns whether insufficient glucose per se can adversely affect fetal growth. Maternal protein restriction alone has a potent negative impact on fetal growth. Our present study demonstrated that GLUT3-mediated transplacental glucose deficiency caused fetal growth restriction similar in severity to that seen with maternal protein restriction (49). Embryonic growth restriction and developmental delays were also observed in the antisense GLUT1 transgenic (17) and GLUT1-null homozygous mice (46), with some variations in embryonic malformations, depending on GLUT1 gene dosage (17, 46). The more severe form of GLUT1 absence encountered in the GLUT1-null homozygous mouse led to abnormal rostral embryonic pole development observed at 13 days (46). The less severe antisense GLUT1 transgenic mouse expressed neural tube defects, caudal regression, a headless state, and microphthalmia at 16.5 gestation days (17). The least severe GLUT1-null heterozygous mouse demonstrated no embryonic or postnatal growth abnormalities or malformations (46). Unlike GLUT1 deficiency, GLUT3 deficiency was not associated with gross embryonic malformations. This may relate to isoform specificity, since the neuroepithelium expresses GLUT1 whereas GLUT3 expression appears in mature neuronal cells at later stages (3, 14, 25). Furthermore, the separate but distinct cellular location of individual glucose transporter isoforms in the preimplantation blastocyst may lead to apoptosis and loss of different cell types. This may, in turn, contribute toward the ultimate phenotype in GLUT1-inner cell mass) vs. GLUT3 (trophectoderm) deficiency. In addition, the null mutation of GLUT1 or the antisense GLUT1 transgene led to embryonic demise at ~14 days or 18.5 days, respectively, subsequent to formation of the mature placenta (17, 46), unlike GLUT3 deficiency (8.5 to 9.5 days). The GLUT1 heterozygous null mice expressed normal intrauterine and postnatal growth (46), in contrast to the GLUT3 heterozygous null mice that presented with late-gestation fetal growth restriction. Thus, there are significant differences in the embryonic presentation supporting differing functionalities of the two isoforms. Our studies demonstrate that GLUT3 is important for early embryonic development (neurulation) and contributes to ongoing late-gestation fetal growth and development. This global embryonic effect is reflective of a direct consequence secondary to GLUT3’s critical function in the trophoderm/placenta rather than alterations in certain cell types of the developing embryo.

Fig. 6. Preimplantation embryos demonstrate dual immunohistochemical staining for GLUT3 and TUNEL. A: representative WT; B: heterozygous (Het); C: homozygous (KO) embryo demonstrating GLUT3 (green) and TUNEL (red) along with nuclear DAPI (blue) staining. WT demonstrates apical distribution (arrow); heterozygous embryo demonstrates punctate distribution of GLUT3 on apical and basolateral surfaces of the trophoderm (arrow); homozygous embryos demonstrate no GLUT3. TUNEL staining progressively increases from WT to homozygous embryos. D: representative WT; E: heterozygous; F: homozygous embryos expressing GLUT1 (green) staining. WT demonstrates basolateral distribution (arrow); heterozygous GLUT3-null embryos demonstrate basolateral and apical distribution of GLUT1 (arrow); homozygous GLUT3-null embryos demonstrate punctate vesicular distribution of GLUT1 (arrow).
GLUT1, on the other hand, plays an innate role in embryonic organogenesis/morphogenesis that is perhaps not a consequence of its function in placental trophoblasts but rather due to GLUT1’s important function in selective cell types of the developing embryo (17, 19, 46). This therefore results in greater longevity of embryonic survival with no effect on late-gestation fetal growth (17, 46). Thus, our present investigation has for the first time allowed the unrelavong of crucial functional differences between GLUT3 and GLUT1 that impact embryonic development.

Our studies demonstrate the importance of placental GLUT3 distinct from GLUT1 in mediating transplacental glucose transport that is necessary for fueling fetal growth. Fetal GLUT3 deficiency independent of transplacental glucose transport makes a negligible contribution toward the decrease in fetal glucose transport, since GLUT3 expression noted only in the 18.5-day fetal brain is barely detectable (25). The predominant isoform in most embryonic tissues at this stage is GLUT1. Thus decreased fetal glucose uptake mainly reflects diminished transplacental glucose transport. The implications of our observations in mouse to humans are potentially important. In the human term hemochorial placenta, IUGR is associated with no change in GLUT1 concentrations but a decline in GLUT3 expression (22, 42). This suggests a critical role for placental GLUT3 in human fetal growth. GLUT3 protein in term human placenta is observed in fetal vascular endothelial cells contributing toward materno-fetal glucose transport (2). Since GLUT1 is the predominant glucose transporter isoform in most embryonic tissues at this stage is GLUT3 remaining undefined.

However, GLUT3 has been observed in the early human placental cytotrophoblast and endothelial cells, supporting a role in early human embryonic development (16, 28). Thus human GLUT3 mutations comparable to what we have described in the mouse have the propensity to interrupt early pregnancy, resulting in embryonic loss and/or fetal growth restriction. The lack of GLUT3 in term human placentomes may relate to the stage of trophoblastic development. GLUT3 is expressed in proliferating cytotrophoblasts...
Fig. 8. A and B: longitudinal sections. A: 6.5-day embryos (E) in situ within maternal deciduas and uterine cavity demonstrate the presence (+/+)(a) consistent with a WT phenotype or absence (b) consistent with a GLUT3-null homozygous phenotype of GLUT3 immunoreaction (arrows) on the embryonic visceral endoderm (EmVe), seen under higher magnification (*) in inset a. GLUT1 immunoreaction (arrows) is seen in GLUT3 WT (+/+)(c) and GLUT3-null homozygous (−/−)(d) embryos in all embryonic layers and the embryonic ectoderm (EmEc) shown under high magnification (*) in inset c surrounding the preamniotic cavity (PrC) and in the ectoplacental cone (Epc) (c). In contrast to the WT (+/+), the GLUT3-null homozygous embryo (−/−) demonstrates cellular infiltration into the cephalad portion of the embryo (d). Scale bar, 100 μm. B: 8.5-day embryos (E) in situ demonstrate GLUT3 immunoreaction (arrows) in Reichert’s membrane (r), also shown under higher magnification (*) in inset a and select cells within the labyrinthine (La) region of the placenta of the WT (+/+)(c) and not in the GLUT3-null homozygous (−/−) embryos (E) that appear amorphous (b). GLUT1 immunoreaction (arrows) is seen in WT Reichert’s membrane (r), forebrain vesicle (f), and neural tube (nt), also seen under high magnification (*) in inset e and somites (s) (c) in the uterus (u) and placenta, also seen under higher magnification (*) in inset d of the (−/−) embryos. WT placenta demonstrates GLUT3 in select cells, also seen under higher magnification (*) in inset e, and GLUT1 is widely seen in cells of the labyrinthine (La) region, also shown under higher magnification in inset f. Arrows, immunoreaction; scale bar, 100 μm. C: representative cultured 8.5-day embryos that lack GLUT3 (a, c, e) and age-matched embryos (b, d, f) from the same litter of heterozygous mating that express GLUT3 (Texas red). Arrows, embryo, cells, and GLUT3 immunoreaction; scale bar, 10 μm. D: representative Western blot analysis depicting GLUT3, GLUT1, and vinculin (internal control) protein in 12.5- to 20.5-gestation-day (+/+) and (−/−) placenta. E: representative Western blot analysis of 14.5- and 18.5-gestation-day (+/+) and (−/−) fetal body and head regions demonstrating GLUT3, GLUT1, and vinculin (internal control) protein bands. Although distinct bands for GLUT1 and vinculin are noted in both fetal body and head of (+/+) and (−/−) mice, only a smear with no distinct GLUT3 protein band is observed, consistent with no GLUT3 expression at these stages of development, except in the 18.5-gestation-day (+/+) fetal head.
rather than differentiated syncytiotrophoblasts (18, 40). The mouse term placenta may contain more proliferating trophoblasts than the human term placenta.

Similarly to placenta, the adult testicular decrease in GLUT3 in the heterozygous mouse led to no change in GLUT1 mRNA or protein concentrations detected by Northern and Western blot analyses. Immunohistochemical analysis revealed GLUT3 as the major glucose transporter isoform localized to Sertoli cells, spermatagonia, primary spermatocytes, and spermatids, whereas minimal GLUT1 was detected. Others have found GLUT3 in Sertoli cells, peritubular myoid cells, macrophage-like interstitial cells, testicular endothelial cells, and early spermatocytes in human, mouse, and rat (12, 26, 27). Abdominal testis led to an 80–90% decline in testicular GLUT3.
concentrations with degeneration of spermagonia in rat (12). In our present investigation, a 50% GLUT3 deficiency did not affect testicular morphology or the procreative function. In addition to the placenta and testis, GLUT3 is the neuronal glucose transporter isoform (14, 31). The decline in brain (neuronal) GLUT3 caused a compensatory increase in GLUT1 expression, thereby not affecting gross brain weight or neurologic function.

In summary, our present in vivo studies demonstrate a critical role for GLUT3 in normal embryonic growth and development. On the basis of these novel findings, GLUT3 mutation forming the basis of early human pregnancy loss and/or fetal growth restriction is a real possibility and needs to be investigated.

ACKNOWLEDGMENTS

We acknowledge the assistance of Melvin Chen and Shanika Boyce in mouse breeding, colony maintenance, and genotyping. Dr. Jiang Meishang in mouse breeding, colony maintenance, and genotyping, Dr. Camille Fung in mouse brain sectioning. We thank Dr. Pinchas Cohen (UCLA) for the mouse IGF-I and IGFBP3 on preimplantation embryos, and Dr. Camille Fung in mouse brain sectioning.

GRANTS

This work was supported by the National Institute of Child Health and Human Development Grants HD-46079, HD-33997, and HD-41230.

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