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Targeted disruption of inducible 6-phosphofructo-2-kinase results in embryonic lethality

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Abstract

Inducible 6-phosphofructo-2-kinase (iPFK-2; PFKFB3) produces fructose-2,6-bisphosphate (F2,6BP), which is a potent allosteric activator of 6-phosphofructo-1-kinase (PFK-1), the rate-limiting step in glycolysis. iPFK-2 functions as an activator of anaerobic glycolysis within the hypoxic microenvironment of growing tumors. The early embryo is challenged similarly since the process of vasculogenesis does not begin until after embryonic day 7. We hypothesized that iPFK-2 expression is essential for the survival of the growing embryo. First, we cloned the mouse homolog of iPFK2 and found that it is abundantly expressed in cortical neurons, epithelial cells, and secretory cells of the choroid plexus, pancreas, and adrenal gland of the adult mouse. Using gene targeting, we then disrupted exons 3–7 of the mouse iPFK2 gene, which encode the substrate binding site. No full-term homozygous iPFK-2$^{-/-}$ progeny were produced from 11 F7 iPFK-2$^{+/-}$ crosses and no homozygous iPFK-2$^{-/-}$ embryos were detected after 8 days of embryogenesis.

Keywords: Glycolysis; Cancer; 6-Phosphofructo-2-kinase; Embryogenesis; Lipopolysaccharide

Glycolytic metabolism is essential for the supply of anabolic precursors and ATP during cellular division and differentiation. Glycolytic flux is rate-limited by 6-phosphofructo-1-kinase (PFK-1), which is regulated by several allosteric effectors, including ATP, citrate, H^+ ions, and fructose-2,6-bisphosphate (F2,6BP) [1,2]. F2,6BP has recently emerged as a key regulator of glycolytic flux during neoplastic transformation and the response to hypoxic challenge [3–8]. F2,6BP is both synthesized and degraded by a family of bifunctional enzymes called 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK2/FBPase), which are encoded by four separate genes, PFKFB1-4 [9,10]. The PFKFB3 gene encodes for the inducible 6-phosphofructo-2-kinase (iPFK2) [3,4,6] which is rapidly induced by inflammatory stimuli [4] and hypoxia [7,8] (also termed placental PFK2 [11,12], ubiquitous PFK2 [13–15], and PGR1 [16]). iPFK2 is constitutively expressed by human solid tumors and has been found to be essential for the shunting of glycolytic intermediates into the synthesis of 5-phosphoribosyl-1-pyrophosphate [3,4].

The mouse embryo requires glucose for energy and anabolic precursor production at the early blastocyst stage [17–19]. Glucose is believed to be essential during the blastocyst stage since an acceleration of cell differentiation and proliferation occurs during this stage and glycolysis provides not only ATP but also a ready supply of key amino acid and nucleic acid precursors. iPFK2 mRNA has been detected at the blastocyst stage of embryogenesis [20], and we hypothesize that the

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production of F2,6BP by this particular isozyme is essential to enable the activation of PFK-1 despite the inhibitory effects of high intracellular ATP and H^+ ions. In the following study, we describe the cloning and initial characterization of the mouse homolog of iPFK2 and the results of targeted disruption of the mouse iPFK2 gene on embryogenesis.

Materials and methods

Cloning of mouse iPFK2. The rat brain PFK2/FBPase cDNA sequence (GenBank Accession No. D87244) was aligned with the human iPFK2 cDNA sequence (AF056320) using DNAstar MegAlign. Several regions of 100% identity were observed and the following primers were designed to hybridize with both the rat and human cDNA species, and then used to RT-PCR amplify potential iPFK2 cDNAs from mouse spleen: 5′-CAGAAGCCATGGACGACTTC-3′, 5′-GGCATC TCCTCTGCACTCTT-3′. A 683 bp amplicon was observed, then ligated into the pCR2.1-TOPO vector (Invitrogen), and sequenced. This fragment shared 98% identity with the human iPFK2 cDNA and the complete cDNA was then amplified by 3′- and 5′-rapid amplification of cDNA ends using a spleen cDNA library (Invitrogen). Three separate PCR amplicons were produced using Platinum PCR *Taq* DNA polymerase (Invitrogen), cloned, and sequenced. All three amplicons shared 100% identity and the sequence was submitted to GenBank (Accession No. AF294617). Subsequent to this GenBank submission, the National Institutes of Health Mammalian Gene Collection (MGC) Program also sequenced the iPFK2 gene and the sequence was found to be 100% identical.

Fluorescence in situ hybridization. A BAC clone (F1199) containing bp 1721–2814 of the 3′-UTR of the mouse iPFK2 homolog was labeled with digoxigenin dUTP by nick translation (Incyte Genomics). Labeled probe was combined with sheared mouse DNA and hybridized to normal metaphase chromosomes derived from mouse embryo fibroblast cells in a solution containing 50% formamide, 10% dextran sulfate, and 2× SSC. Specific hybridization signals were detected by incubating the hybridized slides in fluoresceinated antidigoxigenin antibodies followed by counterstaining with DAPI. Initially, specific labeling of the proximal portion of a large chromosome with the appearance of chromosome 2 was observed. A specific probe for the telomeric region of chromosome 2 was then co-hybridized with clone F1199 and double signal was observed. A total of 80 metaphase cells were analyzed with 78 exhibiting similar labeling.

In situ hybridization. The antisense and sense RNA probes for mouse iPFK-2 were 983 bp in length (corresponding to nucleotides 2343–3326; GenBank Accession No. AF294617). The probes were synthesized with T7 polymerase using ^{35}S-CTP and alkali hydrolyzed before use so as to generate probes of ~200–300 nucleotides in length. Tissue sections were deparaffinized with xylene and then pretreated with proteinase K at 37 °C for 15 min. The sections were then incubated with 0.1 M triethanolamine buffer and acetylated with 0.12% acetic anhydride in 0.1 M triethanolamine buffer to reduce the nonspecific binding of the probe. Prehybridization was performed with hybridization solution containing 0.3 M NaCl, 0.5 mM EDTA (pH 8.0), 10 mM Tris–Cl (pH 7.4), 0.1% BSA, 0.02% Ficoll, 0.2% polyvinylpyrrolidone, 5 mM DTT, 50% deionized formamide, and 50 μg/ml mRNA for 2 h at 45 °C. Hybridization was performed with ^{35}S-labeled sense or antisense RNA probes at 1.6×10^5 cpm/μl in hybridization solution containing 10% dextran sulfate for 16 h at 45 °C. After hybridization, the sections were washed in 2× SSC/1 mM EDTA/5 mM DTT for 15 min at room temperature and then in 50% formamide/1× SSC/0.5 mM EDTA for 15 min at 45 °C. The slides were washed three times in 2× SSC/1 mM EDTA/0.1% Triton X-100/5 mM DTT for 15 min at 60 °C and twice in 0.1× SSC/1 mM EDTA/5 mM DTT for 15 min at 60 °C. The slides were then incubated for 40 min in 25 μg/ml RNase A and 0.25 U/μl RNase T1 at 37 °C. Finally, the slides were washed twice in 2× SSC/1 mM EDTA/5 mM DTT at 60 °C, dehydrated, dipped in NTB-3 emulsion autoradiography (Eastman Kodak, Rochester, NY), allowed to dry, and exposed in the dark at 4 °C for 3–10 days. The emulsion was developed with D19 developer (Eastman Kodak), counterstained with H&E, and observed under the microscope (light and dark field).

LPS activation studies. Twenty gram C57BL/6 mice were injected intraperitoneally with 50 μg LPS (*Escherichia coli* 0111:B4; Sigma) and after 3, 7, and 21 h, peritoneal exudate cells (predominantly macrophages) were removed by intraperitoneal lavage with 5 ml RPMI. The peritoneal cells were then washed twice, and iPFK2 mRNA and protein expression was analyzed by RT-PCR and Western blot analysis using an anti-iPFK2 polyclonal antibody raised against full-length human iPFK2 (97% identity with mouse iPFK2) as previously described [3,4]. Total intracellular F2,6BP was also determined using the method described by Van Schaftingen et al. [21].

Targeted disruption of iPFK2. The targeting vector was constructed by using a 1100 bp DNA fragment as the short arm, which consists of a PCR fragment from site PFKSA1 that is located in intron 2 close to exon 2, with a sequence of 5′-CCCATCAGATGTAACTAGCC-3′, to PFKSA2 which is located between intron 2 and exon 3, with a sequence of 5′-ACACTGGACAGGAAAGACAAGGG-3′. The long arm was a 7.5 kb genomic fragment that starts from the *Cla*I site downstream of exon 7. In this design, the iPFK2 mRNA transcription will stop inside the Neomycin gene cassette and also exons 3–7 will be deleted. Ten micrograms of targeting vector was linearized by *Not*I and then transfected by electroporation of 129 SV embryonic stem cells. After selection in G418, surviving colonies were expanded, and PCR analysis was performed to identify clones that had undergone homologous recombination. PCR was performed using primer pairs PFKSA7 and Neo1: primer PFKSA7 is located in the short arm, 130 bp outside of PFKSA1, with a sequence of 5′-GCCAGGTCTGA AGGCTAGTAGCAG-3′; primer Neo1 is located in the 5′-promoter region of the neo gene cassette and has the sequence 5′-TGCGAGGC CAGAGGCCACTTGTGTAGC-3′. The positive clones give rise to a 1.3 kb PCR fragment. The correctly targeted ES cell lines were then microinjected into C57BL/6 host blastocysts. Resultant chimeras were outbred to C57BL/6 mice, and tail DNA was isolated and genotyped using the REDextract system (Sigma) and the following primers: 5′-G AGTGCTGTCCTTGGTCTGG-3′ (intron 2; forward) and either 5′-C TCTCATGGCTTCCTCATTG-3′ (exon 3; reverse) or 5′-GGAGTAG AAGGTGGCGCGAA-3′ (neomycin; reverse). Male iPFK2$^{+/-}$ mice were then outbred with C57BL/6 female mice.

Real-time RT-PCR. Amplified DNA from mouse iPFK2 was quantitated by spectrophotometry (OD_{260nm}) and the cDNA copies/ml were calculated based on Avogadro's number (6.02205×10^{23}/mol). SYBR green real-time PCR (Cepheid SmartCycler) was conducted with mouse iPFK2 specific primers using 1×10^4 to 10 cDNA copies/ml as template. A standard curve was produced based on the threshold cycle number of the tested concentrations of mouse iPFK2 double-stranded DNA and plotted compared to the threshold cycle number of the cDNA preparations examined. The primers used for real-time PCR of mouse iPFK2 were: 5′-TCTAGAGGAGGTGAGATCAG-3′ and 5′-CCTGCCACTCTTATCTTCTG-3′.

Southern blotting. Genomic DNA was prepared from lung fibroblasts using a DNA extraction kit (QIAGEN) following the manufacturer's instructions. For the detection of the iPFK2 genomic DNA by Southern blotting, a probe was amplified from genomic DNA using primers 5′-AGGTCTGAAGGCTAGTAGCA-3′ and 5′-AAAGGAA AAGTCCCATGGAG-3′. This 150-bp fragment includes sequence from intron 2 of the PFKFB3 gene that is not part of the targeting construct. Probe was then labeled with [α-^{32}P]dCTP using the Megaprime DNA labeling system (Amersham). For each sample, 8 μg of DNA was digested with *sbf*I and *sfi*I restriction enzymes successively, which should produce an 11 kb fragment of the wild-type allele and a

0.8 kb fragment of the recombined neomycin allele. Digested DNA was separated on 0.8% agarose overnight. The DNA was denatured and blotted onto a nylon membrane (Schleicher & Schuell Bioscience). Blots were prehybridized with ExpressHyb solution (Clontech) for 20 min at 68 °C with shaking. Labeled probe was added, and the blot was hybridized in ExpressHyb solution for 2 h at 68 °C with gentle shaking. Non-specific binding was removed by successive 10-min washes in: 2× SSC/0.1% SDS, 2× SSC/0.1% SDS, and 1× SSC/0.1% SDS (at room temperature), followed by 0.2%SSC/0.1% SDS at 65 °C. Hybridizing bands were visualized by phosphor-imaging (Amersham).

Results

Cloning of the Mus musculus iPFK2 homolog

Prior to completion of the mouse genome sequencing project, we RT-PCR amplified portions of mouse iPFK2 using primers specific for regions of homology in the previously sequenced rat and human iPFK2 cDNA sequences and then obtained a full-length 4579 bp cDNA using 5′- and 3′-rapid amplification of cDNA ends (GenBank Accesssion No. AF294617). We aligned the open reading frames of human iPFK2, mouse PFK2, and the previously sequenced isozyme mouse kidney PFK2 and found that the residue sequence from the previously unidentified cDNA sequence shared 97% identity with the human iPFK-2 (Fig. 1). We then conducted chromosomal localization of the mouse iPFK2 gene using fluorescent in situ hybridization. A mouse iPFK2-positive probe and a probe specific for the telomeric region of chromosome 2 were co-hybridized and double signal was observed which indicated that the iPFK2 containing probe hybridized at a position that is immediately adjacent to the

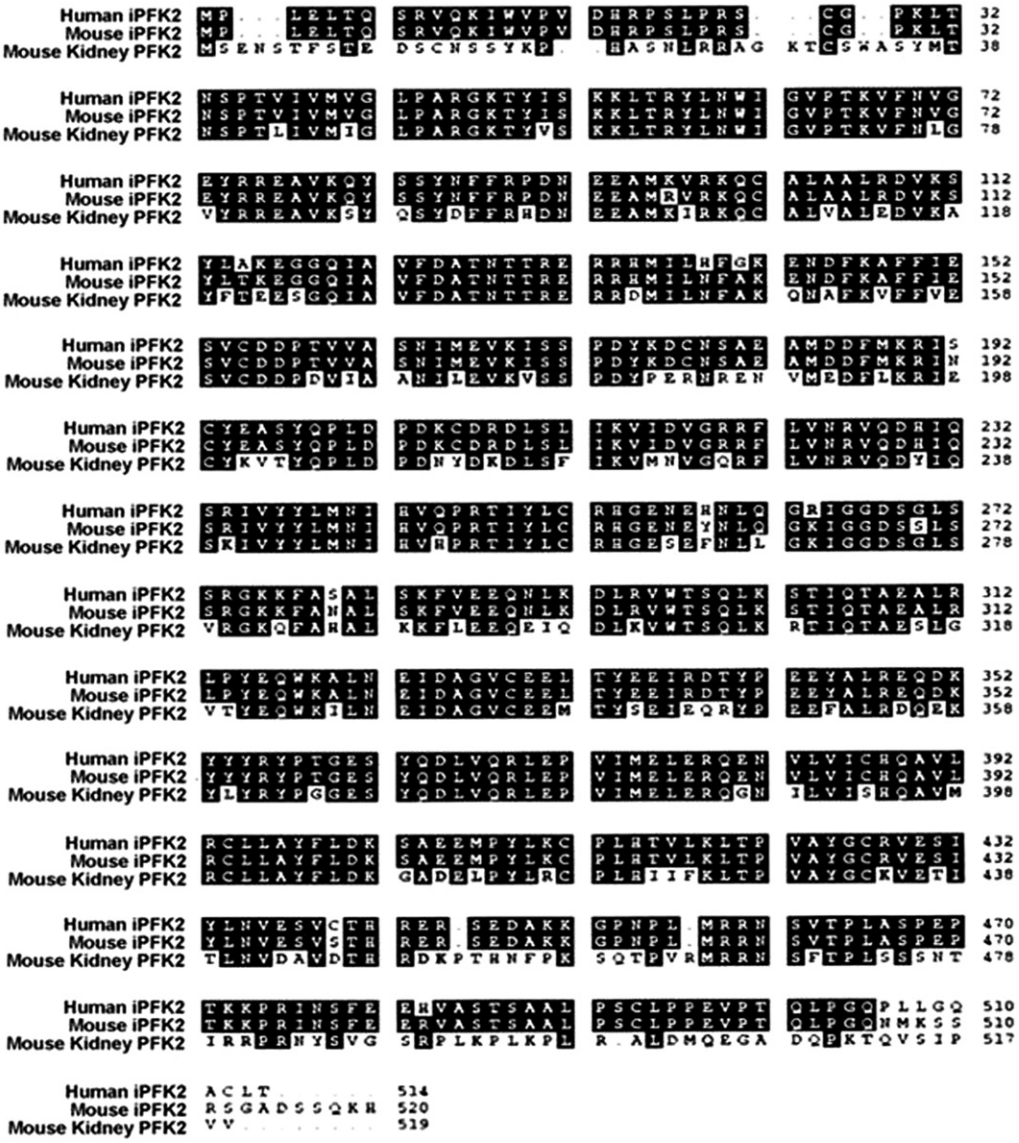

Fig. 1. Residue alignment of human and mouse iPFK2 with mouse kidney PFK2. The open reading frames of human and mouse iPFK2 and mouse kidney PFK2 were translated and then aligned using DNAstar MegAlign.

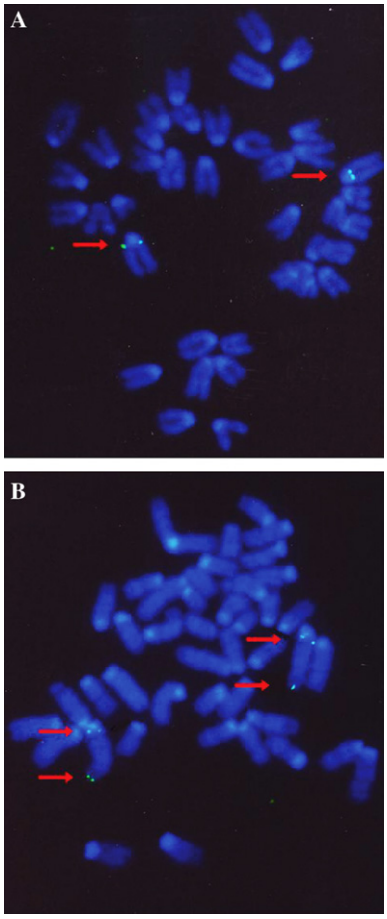

Fig. 2. Localization of mouse iPFK2 to chromosome 2. (A) Digoxigenin-labeled iPFK2 genomic hybridization signals were detected by incubating the hybridized slides in fluoresceinated antidigoxigenin antibodies followed by counterstaining with DAPI. (B) A probe specific for the telomeric region of chromosome 2 was cohybridized with the iPFK2 probe (red arrows indicate the locations of the hybridized probes). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

heterochromatic–euchromatic boundary on chromosome 2 (band 2A2–2A3) (Fig. 2). These data have been independently confirmed by the National Institutes of Health Mammalian Gene Collection (MGC) Program.

In situ hybridization of mouse tissues using an iPFK-2 specific probe

Human iPFK2 (PFKFB3) has been reported to be ubiquitously expressed [14] as well as selectively expressed in astrocytes [22], placental cells [11,12], transformed cells [3,4,16], epithelial cells [3,16], and activated monocytes/macrophages [3,4]. We produced an in situ hybridization probe for mouse iPFK-2 that was specific for the 3′-UTR of this isozyme and then screened formalin-fixed tissue specimens from mouse brain, skin, muscle, heart, intestine, spleen, kidney, lung adrenal gland, pancreas, and liver. We observed a low level of ubiquitous mRNA expression in the epithelial cells of all organs examined (data not shown). Interestingly, we found that cortical neurons and the secretory cells of the choroid plexus, adrenal cortex and medulla, and pancreas express the greatest constitutive levels of mouse iPFK2 mRNA (Fig. 3A). Epithelial cells of the pulmonary alveoli and bronchioles, adipocytes, glomeruli, and splenic megakaryocytes and lymphocytes constitutively express moderate levels of iPFK2 mRNA (Fig. 3B).

Lipopolysaccharide induction of mouse iPFK2

A hallmark of the human iPFK2 isozyme is induction of expression in macrophages after activation by lipopolysaccharide (LPS) [4]. In order to provide evidence that the expression of the homologous mouse iPFK2 isozyme is controlled similarly, we intraperitoneally injected LPS into C57Bl/6 mice and then collected peritoneal macrophages after 3–21 h. We observed a marked induction of mouse iPFK2 mRNA and protein within 3 h of LPS injection (Fig. 4). We also explanted mouse liver, skeletal muscle, spleen, and brain 21 h after LPS injection and found that iPFK2 mRNA expression was markedly induced in the cortical neurons of the brain (Fig. 4D). We did not observe any iPFK2 induction in the liver, skeletal muscle, and spleen (data not shown).

Targeted disruption of the mouse iPFK2 gene

We constructed a targeting vector that introduced a neomycin resistance cassette containing a stop codon in substitution for exons 3–7 that encode for the substrate (fructose-6-phosphate) binding site and the Mg^{2+} binding site (Fig. 5A). After selection for homologously recombined embryonic stem cells, blastocyst injection, and uterine implantation, resultant chimeras were outbred to C57Bl/6J mice, and offspring were screened using primers selective for the recombined iPFK2/neomycin gene and the wild-type iPFK2 gene. During the first seven serial generations of outbred mice, no homozygous iPFK2 knockout mice were identified (data not shown). Eleven $iPFK2^{+/-}$ crosses of F7 mice resulted in 95 offspring of which none were $iPFK2^{-/-}$ (Fig. 5B). We then examined 8-day-old embryos for the iPFK2 genotype from five separate $iPFK2^{+/-}$ F7 crosses and found that the lethality occurs prior to this stage of embryogenesis (Fig. 5B). RT-PCR analysis of iPFK2 mRNA expression in day 7, 11, 15, and 17 embryos indicated high expression throughout these stages of embryogenesis (data not shown). Quantitative real-time RT-PCR analysis for iPFK2 mRNA expression in lung tissue explanted from $iPFK2^{+/+}$ and $iPFK2^{+/-}$ mice demonstrated a 50% reduction in

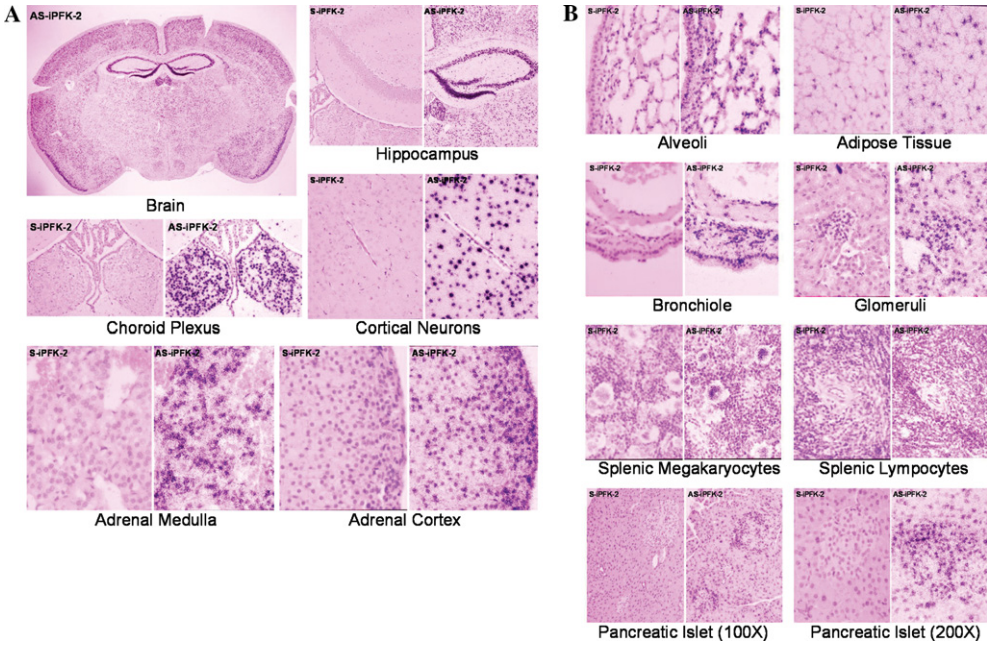

Fig. 3. In situ hybridization analyses of mouse iPFK2 mRNA expression. (A) Brain and adrenal gland. (B) Lung, adipose tissue, kidney, spleen, and pancreas. The indicated tissues were formalin-fixed and then subjected to in situ hybridization using sense (S-iPFK2) and anti-sense (AS-iPFK2) riboprobes specific for the mouse iPFK2 isozyme. Photomicrographs were captured under light field.

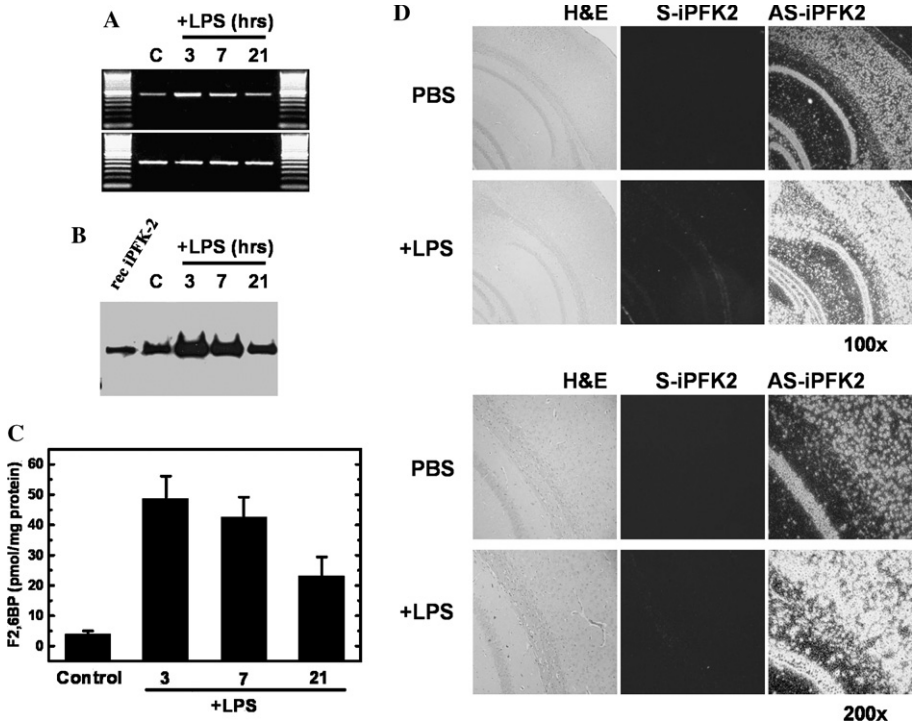

Fig. 4. Expression of iPFK2 after intraperitoneal injection of LPS. C57Bl/6 mice were injected i.p. with 50 μg LPS and after 3, 7, and 21 h, peritoneal macrophages were collected by lavage and analyzed for iPFK-2 expression by RT-PCR (A), Western blot (B), and activity (C). After 21 h, the brain (D), liver, skeletal muscle, and spleen were also removed and analyzed for iPFK-2 expression by in situ hybridization (dark field). No induction was observed in the liver, skeletal muscle, and spleen (data not shown).

mRNA expression in iPFK2$^{+/-}$ mice (Fig. 5C). This reduction in iPFK2 mRNA expression was coincident with a reduction in steady-state F2,6BP concentration in lungs (iPFK2$^{+/+}$, 9.237 ± 1.32 pmol/mg protein; iPFK2$^{+/-}$, 5.71 ± 0.9 pmol/mg protein). We observed a similar reduction in iPFK2 mRNA and F2,6BP in

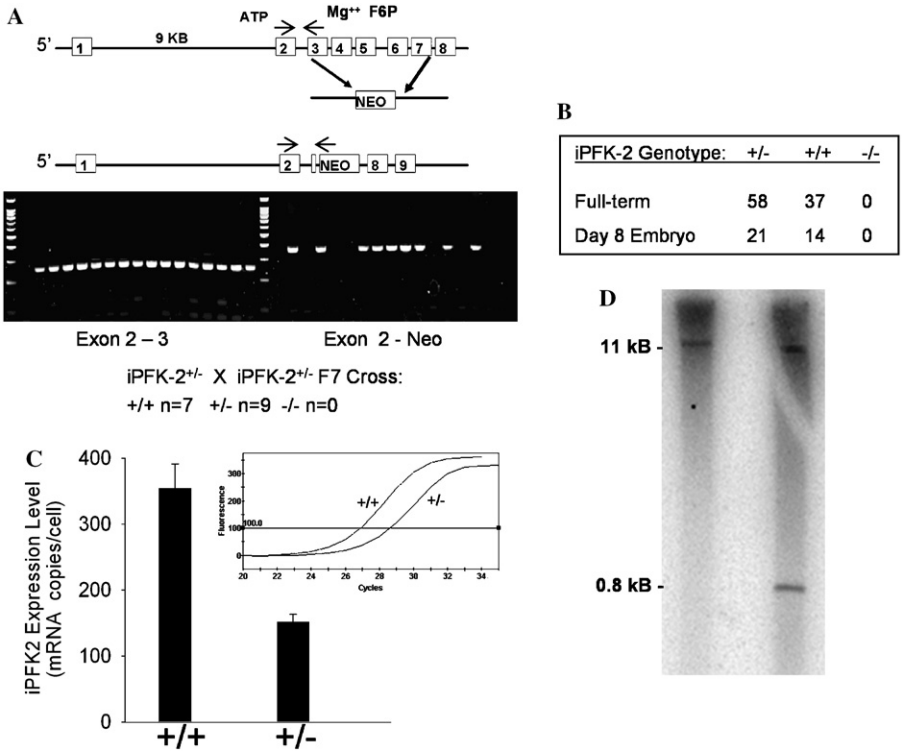

Fig. 5. Targeted disruption of mouse iPFK2 is embryonic lethal. (A) Genomic deletion of iPFK-2 was accomplished with a targeting construct containing the neomycin resistance cassette in substitution for exons 3–7. Resultant offspring from a heterozygote mating were analyzed for the iPFK-2 deletion by PCR using a primer specific for exon 2 with either an exon 3-specific primer (+/+; wild-type) or a neomycin-specific primer (+/−; heterozygote). (B) Thirty-five day 8 embryos and 95 full-term offspring from heterozygote crossings were genotyped using the same PCR method. (C) SYBR green real-time RT-PCR for iPFK-2 mRNA expression in lung tissue. (D) Southern blot of iPFK-$2^{+/+}$ and iPFK-$2^{+/-}$ liver DNA after *sbf*I and *sfi*I digestion using an iPFK-2 specific probe confirming the insertion of the neomycin cassette into exons 3–7 of iPFK-2.

the brain, skeletal muscle, liver, and spleen (data not shown). No gross or histological abnormalities were noted in the brain, heart, liver, kidney, spleen or skeletal muscle of the iPFK$2^{+/-}$ mice. Although routine genotype screening was performed by PCR amplification of the recombined and wild-type alleles, we confirmed that the recombination had occurred by Southern blot analysis of genomic DNA from iPFK$2^{+/+}$ and iPFK$2^{+/-}$ mice after double digestion with the restriction enzymes, *sbf*I and *sfi*I, using a probe that specifically hybridizes to intron 2 of the iPFK2 (PFKFB3) gene just upstream of the neomycin recombination. We observed the predicted wild-type allele 11 kb genomic fragment in the iPFK$2^{+/+}$ mice and iPFK$2^{+/-}$ mice and, in the iPFK$2^{+/-}$ mice, a second 800 bp fragment that is caused by an introduced *sfi*I restriction site within the neomycin cassette (Fig. 5D).

Discussion

We have demonstrated that a mouse gene with 97% predicted amino acid identity to the human iPFK2 protein is highly expressed in cortical neurons, secretory cells of the choroid plexus, adrenal gland and pancreas, epithelial cells, lymphocytes, and megakaryocytes. These data are surprising since a screen for iPFK2 expression in several normal human organs had previously demonstrated that normal constitutive expression was predominant in cells of epithelial origin [3]. However, most iPFK2 expression analyses have been performed on immortalized and transformed cells, and normal and neoplastic brain tissues have not been previously analyzed for in situ iPFK2 expression. Recently, the absent expression of iPFK2 in cultured rat cortical neurons was postulated to explain their relative susceptibility to hypoxia and ATP depletion [22]. Our data suggest that cortical neurons in mice abundantly express iPFK2 in situ and thus do not support this hypothesis. The prior observation that rat cortical neurons do not express iPFK2 in vitro [22] may be related to a species-specific phenomenon or to the effects of the tissue culture environment. Based on the high iPFK2 expression in cortical neurons that we have observed, we intend to examine the relative susceptibility of the iPFK$2^{+/-}$ mice to the effects of cerebral ischemia in vivo in future studies. The finding that iPFK2 is expressed in the adrenal gland and pancreas may indicate that iPFK2 serves certain regulatory roles in the response to stress and changes in glucose homeostasis.

Human iPFK2 was originally identified as an LPS-inducible mRNA that had an AU-rich instability motif [4]. The stimuli that can induce the iPFK2 mRNA were later broadened to include insulin [23] and hypoxia [3,7,8]. We examined the LPS inducibility of the mouse iPFK2 homolog to confirm this phenomenon across species and to provide evidence that this induction occurs in vivo. We found that the mouse iPFK2 isozyme is induced in both macrophages and cortical neurons by LPS in vivo but not in skeletal muscle, the Kupffer cells of the liver, or splenocytes. The observation that iPFK2 is induced in the cortical neurons of the brain after intraperitoneal injection of LPS suggests that a secondary mediator of LPS, such as macrophage migration inhibitory factor, which promotes glucose utilization during endotoxemia [24], may cause this induction since LPS does not cross the blood–brain barrier.

Null embryos were not recovered after 8 days of embryogenesis, indicating that the loss of iPFK2 activity via targeted disruption of the gene leads to loss of embryonic cell proliferation, differentiation, and/or implantation. Serial analysis of gene expression has indicated that the mouse iPFK2 mRNA becomes detectable during the blastocyst stage of embryogenesis [20]. The dividing embryo relies fully on pyruvate for energy production through the tricarboxylic acid cycle and respiration until the blastocyst stage [17–19]. The development of the blastocyst requires a massive increase in cell proliferation and differentiation, and these processes require glycolysis not only for energy but also to provide an abundant supply of anabolic precursors for amino acid, fatty acid, and nucleic acid synthesis. Accordingly, there is a shift from pyruvate to glucose utilization at the blastocyst stage of embryogenesis. We suspect that this shift may require the synthesis of F2,6BP from iPFK2 for the purpose of activating the rate-limiting step of glycolysis, PFK-1. We had expected that in the absence of iPFK2, one or a combination of the other 3 isozymes of PFK2/FBPase (PFKFB1, 2 or 4) could provide sufficient, compensatory F2,6BP to support embryogenesis, but instead observed a lethal phenotype with the null genotype. These data suggest either that the relatively high kinase:phosphatase ratio of iPFK2 (740:1) [25] is essential for embryogenesis, or that iPFK2 has an alternative function, perhaps via protein–protein interaction, to regulate cell proliferation and/or differentiation. Alternatively, the normal phenotype of the iPFK2$^{+/-}$ mice despite the 50% reduction of iPFK2 expression and activity may indicate that certain metabolic compensation has occurred. In future studies, we intend to examine the functions of iPFK2 in the earliest stages of embryogenesis using in vitro fertilization methods.

In conclusion, we have found that the mouse homolog of iPFK2 is highly expressed in cortical neurons, is induced by LPS in vivo, and is essential for embryogenesis. We predict that the iPFK2$^{+/-}$ mice will facilitate our understanding of the roles of iPFK2 in carcinogenesis, neoplastic growth and survival, embryogenesis, hypoxic challenge, and diabetes mellitus.

Acknowledgments

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References

[1] L. Hue, G.G. Rousseau, Fructose-2,6-bisphosphate and the control of glycolysis by growth factors, tumor promoters and oncogenes, Adv. Enzyme Regul. 33 (1993) 97–110.
[2] E. Van Schaftingen, M.F. Jett, L. Hue, H.G. Hers, Control of liver 6-phosphofructokinase by fructose-2,6-bisphosphate and other effectors, Proc. Natl. Acad. Sci. USA 78 (1981) 3483–3486.
[3] T. Atsumi, J. Chesney, C. Metz, L. Leng, S. Donnelly, Z. Makita, R. Mitchell, R. Bucala, High expression of inducible 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (iPFK-2; PFKFB3) in human cancers, Cancer Res. 62 (2002) 5881–5887.
[4] J. Chesney, R. Mitchell, F. Benigni, M. Bacher, L. Spiegel, Y. Al-Abed, J.H. Han, C. Metz, R. Bucala, An inducible gene product for 6-phosphofructo-2-kinase with an AU-rich instability element: role in tumor cell glycolysis and the Warburg effect, Proc. Natl. Acad. Sci. USA 96 (1999) 3047–3052.
[5] M. Fukasawa, T. Tsuchiya, E. Takayama, N. Shinomiya, K. Uyeda, R. Sakakibara, S. Seki, Identification and characterization of the hypoxia-responsive element of the human placental 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene, J. Biochem. (Tokyo) 136 (2004) 273–277.
[6] U. Mahlknecht, J. Chesney, D. Hoelzer, R. Bucala, Cloning and chromosomal characterization of the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 gene (PFKFB3, iPFK2), Int. J. Oncol. 23 (2003) 883–891.
[7] A. Minchenko, I. Leshchinsky, I. Opentanova, N. Sang, V. Srinivas, V. Armstead, J. Caro, Hypoxia-inducible factor-1-mediated expression of the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3) gene. Its possible role in the Warburg effect, J. Biol. Chem. 277 (2002) 6183–6187.
[8] O. Minchenko, I. Opentanova, D. Minchenko, T. Ogura, H. Esumi, Hypoxia induces transcription of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-4 gene via hypoxia-inducible factor-1alpha activation, FEBS Lett. 576 (2004) 14–20.
[9] D.A. Okar, A.J. Lange, Fructose-2,6-bisphosand and control of carbohydrate metabolism in eukaryotes, Biofactors 10 (1999) 1–14.
[10] D.A. Okar, A. Manzano, A. Navarro-Sabate, L. Riera, R. Bartrons, A.J. Lange, PFK-2/FBPase-2: maker and breaker of the essential biofactor fructose-2,6-bisphosphate, Trends Biochem. Sci. 26 (2001) 30–35.
[11] A. Sakai, M. Kato, M. Fukasawa, M. Ishiguro, E. Furuya, R. Sakakibara, Cloning of cDNA encoding for a novel isozyme of fructose 6-phosphate, 2-kinase/fructose 2,6-bisphosphatase from human placenta, J. Biochem. (Tokyo) 119 (1996) 506–511.
[12] R. Sakakibara, T. Okudaira, K. Fujiwara, M. Kato, T. Hirata, S. Yamanaka, M. Naito, M. Fukasawa, Tissue distribution of

placenta-type 6-phosphofructo- 2-kinase/fructose-2,6-bisphosphatase, Biochem. Biophys. Res. Commun. 257 (1999) 177–181.
[13] R. Kessler, K. Eschrich, Splice isoforms of ubiquitous 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase in human brain, Brain Res. Mol. Brain Res. 87 (2001) 190–195.
[14] A. Manzano, J.L. Rosa, F. Ventura, J.X. Perez, M. Nadal, X. Estivill, S. Ambrosio, J. Gil, R. Bartrons, Molecular cloning, expression, and chromosomal localization of a ubiquitously expressed human 6-phosphofructo-2-kinase/ fructose-2, 6-bisphosphatase gene (PFKFB3), Cytogenet. Cell Genet. 83 (1998) 214–217.
[15] A. Navarro-Sabate, A. Manzano, L. Riera, J.L. Rosa, F. Ventura, R. Bartrons, The human ubiquitous 6-phosphofructo-2-kinase/ fructose-2,6-bisphosphatase gene (PFKFB3): promoter characterization and genomic structure, Gene 264 (2001) 131–138.
[16] J.A. Hamilton, M.J. Callaghan, R.L. Sutherland, C.K. Watts, Identification of PRG1, a novel progestin-responsive gene with sequence homology to 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, Mol. Endocrinol. 11 (1997) 490–502.
[17] M.M. Chi, A. Hoehn, K.H. Moley, Metabolic changes in the glucose-induced apoptotic blastocyst suggest alterations in mitochondrial physiology, Am. J. Physiol. Endocrinol. Metab. 283 (2002) E226–E232.
[18] D.K. Gardner, H.J. Leese, The role of glucose and pyruvate transport in regulating nutrient utilization by preimplantation mouse embryos, Development 104 (1988) 423–429.
[19] K.L. Martin, H.J. Leese, Role of glucose in mouse preimplantation embryo development, Mol. Reprod. Dev. 40 (1995) 436–443.
[20] R. Edgar, M. Domrachev, A.E. Lash, Gene expression Omnibus: NCBI gene expression and hybridization array data repository, Nucleic Acids Res. 30 (2002) 207–210.
[21] E. Van Schaftingen, B. Lederer, R. Bartrons, H.G. Hers, A kinetic study of pyrophosphate: fructose-6-phosphate phosphotransferase from potato tubers. Application to a microassay of fructose-2,6-bisphosphate, Eur. J. Biochem. 129 (1982) 191–195.
[22] A. Almeida, S. Moncada, J.P. Bolanos, Nitric oxide switches on glycolysis through the AMP protein kinase and 6-phosphofructo-2-kinase pathway, Nat. Cell Biol. 6 (2004) 45–51.
[23] L. Riera, A. Manzano, A. Navarro-Sabate, J.C. Perales, R. Bartrons, Insulin induces PFKFB3 gene expression in HT29 human colon adenocarcinoma cells, Biochim. Biophys. Acta 1589 (2002) 89–92.
[24] F. Benigni, T. Atsumi, T. Calandra, C. Metz, B. Echtenacher, T. Peng, R. Bucala, The proinflammatory mediator macrophage migration inhibitory factor induces glucose catabolism in muscle, J. Clin. Invest. 106 (2000) 1291–1300.
[25] R. Sakakibara, M. Kato, N. Okamura, T. Nakagawa, Y. Komada, N. Tominaga, M. Shimojo, M. Fukasawa, Characterization of a human placental fructose-6-phosphate, 2-kinase/ fructose-2,6-bisphosphatase, J. Biochem. (Tokyo) 122 (1997) 122–128.