Essential role of glucose transporter GLUT3 for post-implantation embryonic development

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Abstract

Deletion of glucose transporter gene *Slc2a3* (GLUT3) has previously been reported to result in embryonic lethality. Here, we define the exact time point of growth arrest and subsequent death of the embryo. *Slc2a3*$^{-/-}$ morulae and blastocysts developed normally, implanted *in vivo*, and formed egg-cylinder-stage embryos that appeared normal until day 6·0. At day 6·5, apoptosis was detected in the ectodermal cells of *Slc2a3*$^{-/-}$ embryos resulting in severe disorganization and growth retardation at day 7·5 and complete loss of embryos at day 12·5. GLUT3 was detected in placental cone, in the visceral ectoderm and in the mesoderm of 7·5-day-old wild-type embryos. Our data indicate that GLUT3 is essential for the development of early post-implanted embryos.

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Introduction

The glucose transporter family comprises 14 members, GLUT1–12, GLUT14 and HMIT1 (H$^+$/*myo*-inositol symporter; Joost & Thorens 2001, Scheepers *et al.* 2004). GLUT3 exhibits a high affinity for glucose ($K_m = 1·8$ mM) and was identified as the neuronal glucose transporter primarily located in axons and dendrites (Mantych *et al.* 1992, Nagamatsu *et al.* 1992, McCall *et al.* 1994, Vannucci *et al.* 1997, Simpson *et al.* 2008). Expression of GLUT3 coincides with maturation and regional cerebral glucose utilization (Vannucci *et al.* 1998). However, deletion of one *Slc2a3* allele did not alter maintenance of neuronal energy supply, motor abilities, learning, and memory in mice (Schmidt *et al.* 2008). By contrast, heterozygous disruption of the blood–brain barrier glucose transporter GLUT1 in *Slc2a1*$^{+/-}$ mice resulted in a dramatic phenotype with microencephaly, impaired motor activity, cerebral ataxia, and multiple spontaneous cortical seizures (Wang *et al.* 2006). GLUT3 is also expressed in other cells (sperm, pre- and post-implantation embryo circulating white blood cells, and carcinoma cells) where it triggers the specific requirements for glucose (Simpson *et al.* 2008). Several studies have demonstrated that GLUT3 is crucial for the optimal development of the embryo (Pantaleon *et al.* 1997, Moley *et al.* 1998, Simpson *et al.* 2008, Wyman *et al.* 2008). Until compaction of the morula, the mammalian embryo derives energy from pyruvate and lactate (Barbehenn *et al.* 1974). The switch to metabolize glucose as the primary energy source coincides with the expression of GLUT3 from the late cleavage stages onward (Mantych *et al.* 1992). During early mouse embryogenesis, the expression of seven GLUT isoforms has been described. GLUT1 and GLUT9 were found in all pre-implantation stages, GLUT2 and GLUT3 were detected at the eight–cell stage (Hogan *et al.* 1991, Pantaleon *et al.* 1997), and GLUT4 and GLUT8 at the blastocyst stage and thereafter (Hogan *et al.* 1991, Aghayan *et al.* 1992, Carayanno-poulos *et al.* 2004). By contrast, GLUT12 has only been detected at the eight–cell stage of the pre-implanted embryo (Zhou *et al.* 2004). Thus, the trophectodermic GLUT3 appeared the main glucose transporter responsible for the uptake of maternal glucose into the blastocyst. Its apical localization in the trophectoderm provides the inner cell mass (ICM) with glucose together with the basolateral GLUT1 (Pantaleon *et al.* 1997, Pantaleon & Kaye 1998). Downregulation of GLUT3 expression with antisense oligonucleotides resulted in a marked reduction of glucose uptake and in a 50% decrease in the number of embryos progressing to blastocyst stage. These data indicated that expression of the high-affinity transporter GLUT3 correlates with the switch to a metabolic preference for glucose versus pyruvate (Pantaleon *et al.* 1997). Similar defects in embryo development were observed in mice in which diabetes mellitus was induced by streptozotocin. GLUT3 expression was downregulated in the blastocysts (Moley *et al.* 1998). In addition, *in vitro* culture in high glucose concentration induced a downregulation of GLUT3 and an intrauterine growth retardation after transfer of blastocysts into recipient mice (Wyman *et al.* 2008).

Recently, it was described that disruption of the *Slc2a3* gene in mice leads to embryonic lethality. $Slc2a3^{-/-}$ embryos were detected at the blastocyst stage but displayed increased apoptosis and delayed development. However, despite cell death, $Slc2a3^{-/-}$ embryos implanted but were lost at embryonic day 8·5 (Ganguly *et al*. 2007). Here, we describe the exact time point of embryonic lethality, and identify the defect responsible for death of post-implanted embryos. We show that disruption of GLUT3 expression has no effect on blastocyst development, but arrests embryonic development at day 6·5 correlating with initiation of apoptosis in ectodermic cells.

Materials and Methods

Inactivation of the Slc2a3 gene

For the generation of *Slc2a3* knockout mice, we used the ES cell clone XG611 (Bay Genomics, San Francisco, CA, USA). The clone was tested for a single integration event of the gene trap vector in the *Slc2a3* gene (see below). ES cells of clone XG611 were injected into blastocysts that were implanted into pseudopregnant females. Chimeras were mated with C57BL/6 mice, and F1 progeny carrying the transgene were backcrossed five times onto the C57BL/6 background. Genotyping of blastocysts, embryos, and mice was performed by PCR (for wild-type allele, forward primer: 5′-CCCTG-CATTCACCGTTCC-3′, reverse primer: 5′-GATGACTC-CAGTGTTGTAGC-3′; for knockout allele, forward primer: 5′-GCAGATCGCATCGATAACTTCG-3′, reverse primer: 5′-AGTATCGGCCTCAGGAAGATCG-3′). The animals were housed in air-conditioned rooms (temperature 20 ± 2 °C, relative moisture 50–60%) under a 12 h light:12 h darkness cycle. They were kept in accordance with the UK legal requirements for the care and use of laboratory animals, and all experiments were approved by the ethics committee of the Ministry of Agriculture, Nutrition and Forestry (State of Brandenburg, Germany).

RNA preparation, first-strand cDNA synthesis, and sequencing

ES cells from clone XG611 were harvested for RNA preparation as described (Gawlik *et al*. 2008). Primers specific for GLUT3 or β-geo cassette used in the PCR were as follows: forward primer (f1): 5′-ATGCTTTCGGTGATAGTCCTT-3′; forward primer (f2): 5′-AGGAACACTTGCTGCCGAGA-3′; reverse primer (r1): 5′-AGTATCGGCCTCAGGAAGATCG-3′; and reverse primer (r2): 5′-ATTCAGGCTGCGCAACTG-TTGGG-3′.

Southern blotting

Genomic DNA of ES cell clone XG611 was digested with either BglII or NcoI, separated on a 0·7% agarose gel, and blotted onto a Hybond-N$^+$-nylon membrane (Amersham Pharmacia Biotech). To verify a single recombination of the gene trap vector in the ES cell clone, a 722 bp PCR fragment (forward primer: 5′-TTATCGATGAGCGTGGTGGT-TATGC-3′, reverse primer: 5′-GCGCGTACATCGGG-CAAATAATATC-3′) of the β-geo cassette of the gene trap vector was used as a probe for hybridization after labeling with $[\alpha^{32}P]$dCTP with a random priming kit (Amersham Pharmacia Biotech).

Embryo recovery and culture

Eight- to ten-week-old GLUT3 heterozygous mice were intercrossed overnight. Matings were confirmed by the identification of a vaginal plug in the next morning. For immunohistochemical characterization of blastocysts, the animals were killed on embryonic day 3·5 (E3·5 dpc), and embryos were obtained by flushing the uterine horns and cultured under mineral oil at 37 °C in a humidified atmosphere of 5% CO_2 in DMEM high glucose/Na-pyruvate medium (PAA Laboratories, Linz, Austria) containing 10% FCS (PAN, Aidenbach, Germany). For analysis of outgrowth, blastocysts were cultured in M2 medium (Sigma). For the characterization of the development of morulae to blastocysts, one-cell-stage embryos (E0·5 dpc) were isolated and cultured until day 2·5 or 3·5 dpc (morula or early blastocyst stage, respectively). Thereby one-cell-stage embryos were incubated in M2 medium (Sigma) containing 0·5 mg/ml hyaluronidase (Sigma). In order to remove the cumulus cells, the embryos were washed several times in M2 medium before cultivation in DMEM high glucose/Na-pyruvate medium (PAA Laboratories) containing 10% FCS (PAN).

Immunostaining of blastocysts

Goat anti-GLUT3 antibody (Zhou *et al*. 2002) was obtained from Santa Cruz Biotechnology Inc. (M20; Santa Cruz, CA, USA). Additional stainings of GLUT3 were performed with a polyclonal anti-GLUT3 antibody described by Hellwig *et al*. (1992). The anti-GLUT1 antibody was described previously (Hellwig *et al*. 1992). Results were confirmed with an additional rabbit anti-GLUT1 antibody (Ogawa *et al*. 2007) purchased from Santa Cruz (H43). Blastocysts were fixed in 3% paraformaldehyde, permeabilized with 0·2% saponin (Sigma) in PBS containing 0·1% PVP for 30 min, and blocked with antibody diluent (DakoCytomation, Carpinteria, CA, USA). Blastocysts were incubated with the primary and secondary antibodies. Nuclei and cytoskeleton were counterstained. Fluorescence was detected with laser scanning confocal immunofluorescent microscopy (Leica TCS SP2 system; Leica, Mannheim, Germany). Genotypes of blastocysts were determined by PCR thereafter. Therefore, blastocysts were reincubated with water and proteinase k supplemented PCR buffer and PCR was performed as described above.

Analysis of post-implanted embryos

For histological analysis, uteri were isolated at embryonic days 6·0, 6·5, and 7·5 post-coitum and fixed in 4% paraformaldehyde for 24 h, dehydrated, and embedded in paraffin. Serial sections (2 μm) were generated from the whole embryo (∼16 sections of 6·5-day-old embryo) and stained with hematoxylin and eosin or stained for GLUT3, GLUT1, and activated caspase 3 (Cell Signaling Technology Inc., Beverly, MA, USA), for E-cadherin (clone HECD-1 in a dilution of 1:400) and for N-cadherin (1:500; Zymed, South San Francisco, CA, USA), or MKI67 (1:50; DakoCytomation, Glostrup, Denmark).

Results

Disruption of the Slc2a3 gene

For the generation of conventional GLUT3 knockout mice ($Slc2a3^{-/-}$), we used a gene trap ES cell clone (clone XG611). Insertion of the gene trap vector into intron 1 of $Slc2a3$ (Fig. 1A) resulted in a fusion protein consisting of five amino acids of GLUT3, neomycin phosphotransferase, and β-galactosidase. Correct integration of the gene trap vector into the $Slc2a3$ gene was verified by PCR (Fig. 1B). Sequence analysis of the PCR products indicated correct splicing of exon 1 to the splice acceptor site of the gene trap vector (data not shown). Digestion of genomic DNA from clone XG611 with either BglII or NcoI and hybridization with a β-galactosidase-specific probe resulted in single bands, indicating that the gene trap vector was integrated only once into the ES cell genome (Fig. 1B, right panel). For the generation of $Slc2a3^{-/-}$ mice, ES cells of clone XG611 were injected into blastocysts that were implanted into pseudopregnant females. Chimeras were mated with C57BL/6 mice, and F1 progeny carrying the transgene were backcrossed five times onto the C57BL/6 background.

Embryonic lethality of Slc2a3$^{-/-}$ mice prior to ED 12·5

Homozygous disruption of the $Slc2a3$ gene resulted in embryonic lethality, since only $Slc2a3^{+/+}$ and $Slc2a3^{+/-}$ but no $Slc2a3^{-/-}$ mice could be genotyped after birth (Table 1). Approximately, twice as many $Slc2a3^{+/-}$ than $Slc2a3^{+/+}$ mice were born, indicating that $Slc2a3^{+/-}$ mice had no disadvantage in embryonic development. In order to narrow down the time point at which disruption of $Slc2a3$ was lethal, embryos were isolated and genotyped at E12·5 dpc. Out of 40 embryos (5 litters) genotyped, no $Slc2a3^{-/-}$ mutants were detected, indicating that $Slc2a3^{-/-}$ mice die early in gestation.

Disruption of Slc2a3 does not interfere with development of blastocysts

In order to determine whether ablation of GLUT3 results in an arrest of growth at the morula or blastocyst stage, 43

one-cell-stage embryos (0·5 dpc) of $Slc2a3^{+/-}$ mice matings (5 litters) were isolated and cultured until day 2·5 or 3·5 pc (morula or early blastocyst stage, respectively). The development of the embryos was documented by light microscopy, and genotypes of embryos were determined by PCR thereafter. Figure 2 illustrates that $Slc2a3^{-/-}$ morulae and blastocysts were indistinguishable from wild-type and $Slc2a3^{+/-}$ genotypes.

Growth of pre-implanted embryos was monitored for 24 h with blastocysts isolated from heterozygous matings at E3·5 dpc and individually cultured *in vitro*. From 10 litters, 25 $Slc2a3^{+/+}$, 20 $Slc2a3^{+/-}$, and 9 $Slc2a3^{-/-}$ blastocysts were obtained. The morphology of $Slc2a3^{-/-}$ blastocysts appeared normal with a proliferating ICM (Fig. 3A). Immunostaining of blastocysts with either of two anti-GLUT3 antibodies (middle panels) confirmed that all blastocysts genotyped as $Slc2a3^{-/-}$ lacked the GLUT3 protein (Fig. 3A). By contrast, $Slc2a3^{+/+}$ and $Slc2a3^{+/-}$ blastocysts showed intense immunoreactivity. Here, GLUT3 was restricted to the apical membranes of the polarized epithelium (trophectoderm). In addition, staining for actin cytoskeleton and nuclei did not show any abnormality of $Slc2a3^{-/-}$ blastocysts (Fig. 3, right panels). In order to test the possibility of a compensatory increase or an altered subcellular distribution of GLUT1 in $Slc2a3^{-/-}$ blastocysts, an additional set of 13 blastocysts (4 litters) was stained with anti-GLUT1 antibody. GLUT1 immunoreactivity was predominantly detected in the basolateral, but also in the apical membranes of the trophectoderm and in the plasma membranes of cells from the ICM. No differences between $Slc2a3^{+/+}$, $Slc2a3^{+/-}$, and $Slc2a3^{-/-}$ blastocysts were detectable (Fig. 3B). The same result was obtained by a second anti-GLUT1 antibody (H43) demonstrating that the signals obtained by immunohistochemistry were specific (data not shown).

In order to test whether extraembryonic components are affected in the absence of $Slc2a3$, we analyzed the trophoblast growth of isolated *in vitro*-cultivated embryos. As shown in Supplementary Fig. 1, see Supplementary data in the online version of the Journal of Endocrinology at http://joe.endocrinology-journals.org/content/vol200/issue1/, no difference in the proliferation capacity of trophectodermal cells was visible between wild-type, heterozygous, and knockout embryos.

Deletion of GLUT3 results in embryonic lethality at the gastrulation stage

In order to determine the exact time point of death of $Slc2a3^{-/-}$ mutants, uteri from heterozygous intercrosses were analyzed at different days of gestation (E6·0, 6·5, and 7·5 dpc). Genotypes were identified by the immunostaining of GLUT3. The distribution of the $Slc2a3$ alleles calculated for all embryos corresponded exactly with the Mendelian segregation, consistent with the conclusion that development and implantation of blastocysts was not affected by disruption of $Slc2a3$ (Table 2).
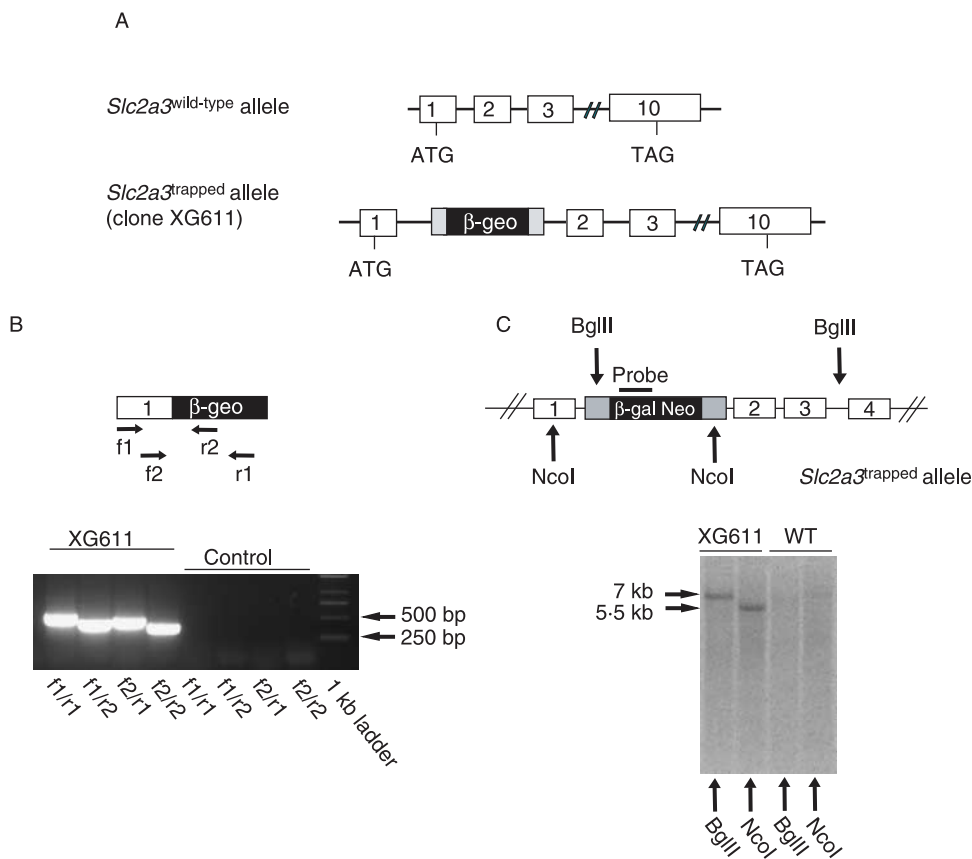
Figure 1 Disruption of the *Slc2a3* gene by gene trapping. (A) Localization of the gene trap between exons 1 and 2 of the *Slc2a3* gene. (B) Integration of the gene trap vector in intron 1 of the *Slc2a3* gene in ES cell clone XG611 results in a fusion transcript containing exon 1 and the β-geo cassette. With the indicated primer pairs, four specific RT-PCR products were detected. (C) Southern blot analysis confirmed the single integration of the gene trap vector into the *Slc2a3* gene of clone XG611. Genomic DNA was digested with BglII or NcoI and analyzed by Southern blotting with the indicated probe. A single 7 and 5·5 kb band respectively was detected in each panel.

At E6·0 dpc, all embryos appeared normal with a well-organized ectoplacental cone, an extraembryonic and embryonic ectoderm, and a visceral endoderm (Fig. 4A). *Slc2a3*$^{+/+}$ and *Slc2a3*$^{+/-}$ embryos were indistinguishable, with pronounced immunoreactivity of GLUT3 in the ectoplacental cone and visceral endoderm. Adjacent sections were stained for GLUT1, which was detected in all embryonic cells (Fig. 4A, right panels). Again, no compensatory increase in GLUT1 was detected. At E6·5 dpc, *Slc2a3*$^{-/-}$ mutants started to exhibit microscopical abnormalities in that the shape of cells within the ectoderm differed from that of control embryos. The ectodermal cell layer of the *Slc2a3*$^{+/+}$ embryos composed of high columnar epithelial cells, whereas cells of *Slc2a3*$^{-/-}$ appeared round and uniform (see arrows in Fig. 4B). Furthermore, staining of embryos with an antibody against activated caspase 3–detected apoptotic cells in the distal part of the embryonic ectoderm (Fig. 4B). For quantification, we stained sections of six control and five knockout embryos in the area of the amniotic cavity with the anti–caspase 3 antibody and counted the activated caspase 3–positive cells. We detected $10·5 \pm 3·3$ apoptotic cells in 6·5-day-old *Slc2a3*$^{-/-}$ embryos, but no apoptotic cell in control embryos (Mann–Whitney U-test, $P < 0·0043$). In addition, no activated caspase 3–positive cells were obtained in E6·0 dpc embryos of both genotypes (data not shown). The number of caspase-positive cells was markedly increased in *Slc2a3*$^{-/-}$ embryos examined 4–6 h later (Fig. 4B, right panel). At E7·5 dpc, the development of *Slc2a3*$^{-/-}$ embryos was severely

Table 1 Genotype distribution of progeny observed at weaning. Tail biopsies were taken for genotyping by PCR as described in the Materials and Methods section.

|	Number of animals	Distribution (%)	Expected (%)
Genotype			
Slc2a3$^{+/+}$	98	31	25
Slc2a3$^{+/-}$	215	69	50
Slc2a3$^{-/-}$	0	0	25
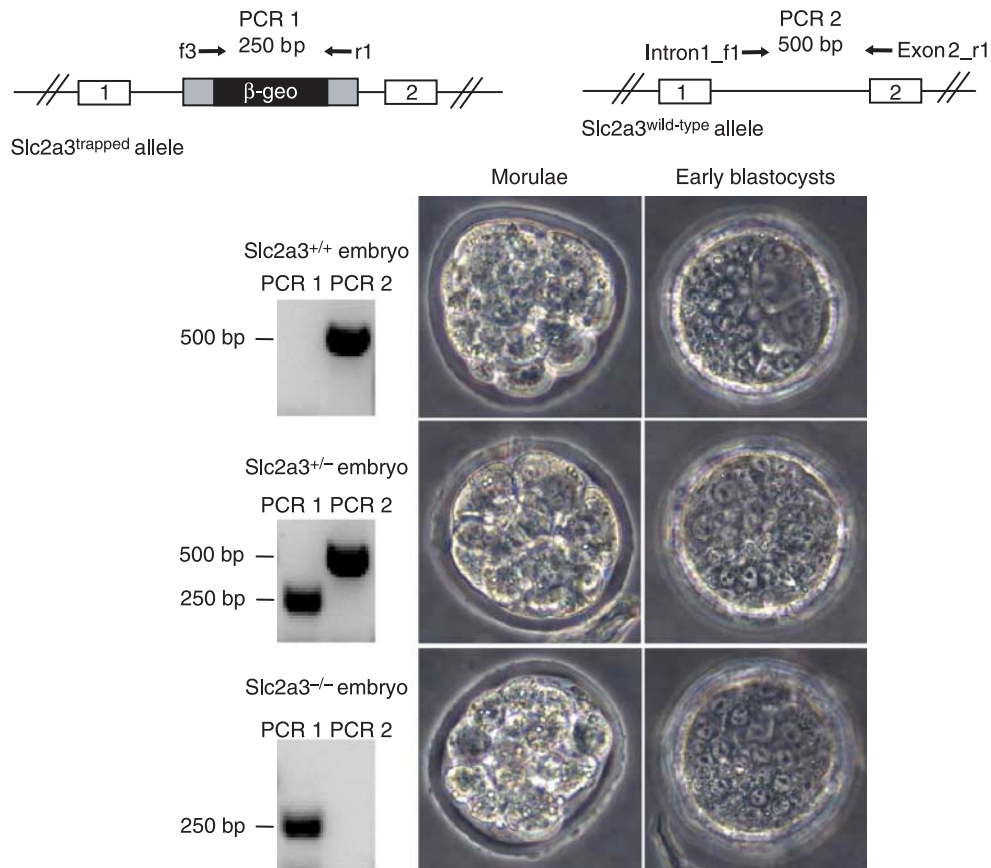
Figure 2 Identification of $Slc2a3^{-/-}$ morulae and early blastocysts. Zygotes were isolated and cultured until the morula or early blastocyst stage, documented, and genotyped by PCR after lysis. PCR 1 generated a product of 250 bp corresponding to the trapped allele (upper left), and PCR 2 generated a 500 bp product of the wild-type allele (upper right). Images of a compacted morula (left) and an early blastocyst (right) of each genotype are shown together with the result of a representative genotyping reaction. Full colour version of this figure available via http://dx.doi.org/10.1677/JOE-08-0262.

affected. Quantification of their mean length indicated that they were smaller than control embryos (mean length of $Slc2a3^{-/-}$ E7·5 dpc embryos, $600 \pm 30·9$ μm; control embryos, $790·0 \pm 51·0$ μm). In addition, the number of proliferating cells in the ectoplacental cone and the embryonic endoderm was reduced in $Slc2a3^{-/-}$ embryos (Supplementary Fig. 2, see Supplementary data in the online version of the Journal of Endocrinology at http://joe.endocrinology-journals.org/content/vol200/issue1/), supporting the finding of a marked growth retardation and a cessation of embryonic development in the absence of GLUT3. In addition, $Slc2a3^{-/-}$ embryos appeared to be disorganized. The higher magnification of HE-stained sections of 7·5-day-old embryos showed a vacuolization of ectodermal cells (see arrows in Fig. 4C). These cells still expressed the adhesion protein E–cadherin, at the cell surface and N–cadherin, a marker for the mesoderm (Hatta & Takeichi 1986). Thus, the $Slc2a3^{-/-}$ embryos started to develop the third germ layer. However, they did not develop the ectoplacental and exocoelomic cavities that were visible in control embryos at

the age of 7·5 days. Instead, a condensed mass of cells was located in the region adjacent to the ectoplacental cone that contained a small amniotic cavity (Fig. 4C). By contrast, wild-type primitive streak-stage embryos exhibited a well-organized ectoderm, endoderm, and mesoderm, and had developed the ectoplacental cone and ectoplacental, exocoelomic, and amniotic cavities. Strikingly, GLUT3 immunoreactivity in primitive streak-stage embryos was restricted to the visceral endoderm and mesoderm, whereas the earliest signs of cell death were detected in the ectodermal cells of $Slc2a3^{-/-}$ embryos (Fig. 4B).

Discussion

The present data indicate that the glucose transporter GLUT3 is essential for substrate supply of the developing embryo, but not for the fertilization of oocytes or growth and implantation of blastocysts. Blastocyst formation was not affected by the absence of GLUT3 because $Slc2a3^{-/-}$ blastocysts were
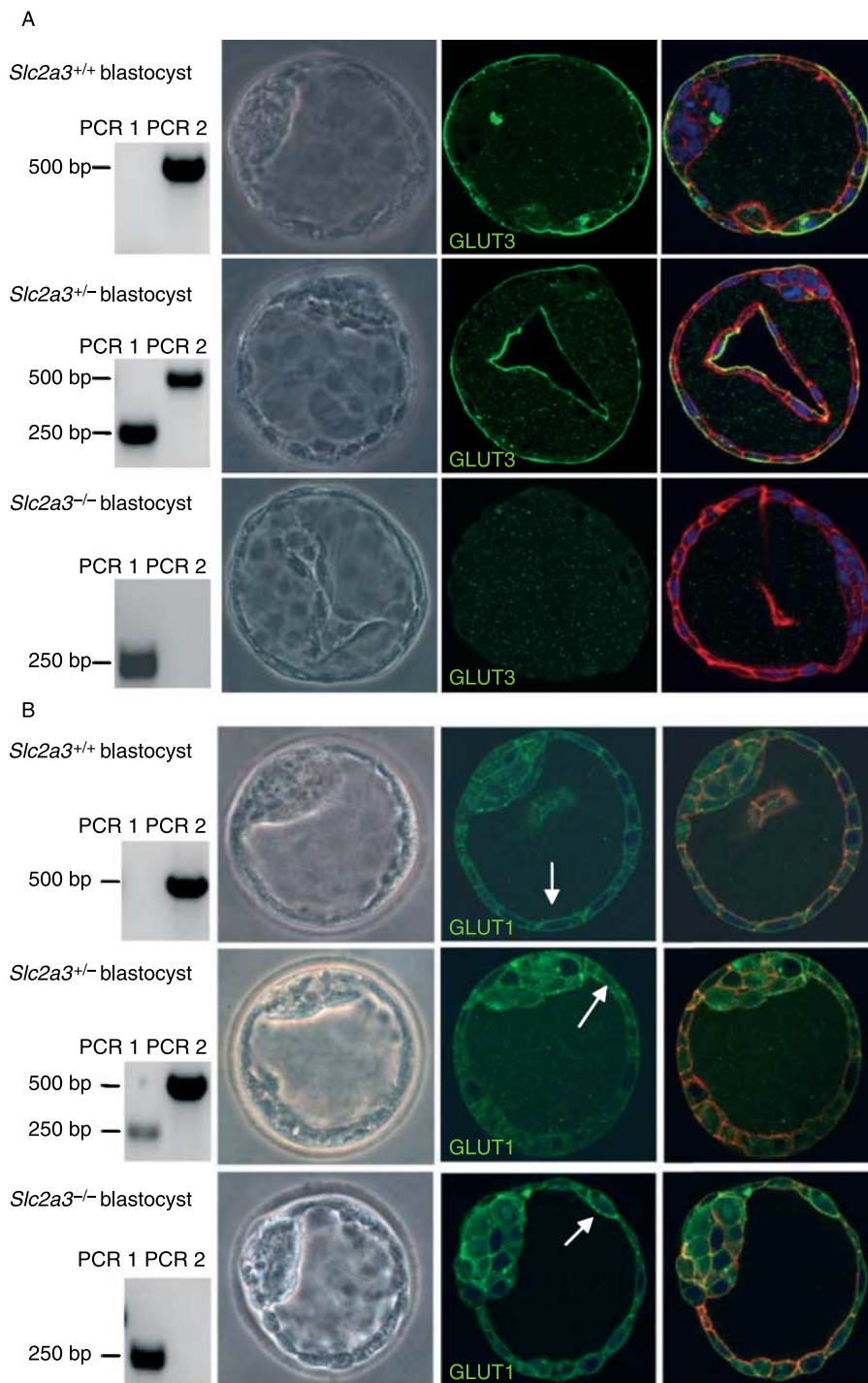
Figure 3 Detection of GLUT3 and GLUT1 in $Slc2a3^{+/+}$, $Slc2a3^{+/-}$, and $Slc2a3^{-/-}$ blastocysts at day 4·5 pc. Blastocysts were isolated and stained for (A) GLUT3 or (B) GLUT1 in combination with an Alexa 488-labeled secondary antibody. Genotyping (left panels) was performed by PCR as described in the legend of Fig. 2. Nuclei (blue) and actin cytoskeleton (red) were counterstained. GLUT1 immunoreactivity is indicated by arrows.

Table 2 Genotype distribution of all embryos observed between embryonic days 6·0 and 7·5

|	$Slc2a3^{+/+}$ and $Slc2a3^{+/-}$ embryos	$Slc2a3^{-/-}$ embryos	Total number of embryos
Embryonic day			
6·0	6	3	9
6·5	17	6	23
7·5	24	8	32
Sum 6·0–7·5	47	17	64
Expected distribution (6·0–7·5)	48	16	64

viable, capable to grow *in vitro*, and to implant *in vivo*. In wild-type blastocysts, GLUT3 was detected at the apical membrane of the trophectoderm (Fig. 3), as already described earlier (Pantaleon *et al*. 1997, Pantaleon & Kaye 1998), whereas GLUT1 was predominantly present in the basolateral and, to a smaller extent, in the apical membranes of the trophecto-derm, and at the surface of cells from the ICM. This observation is in contrast to the results described by Pantaleon
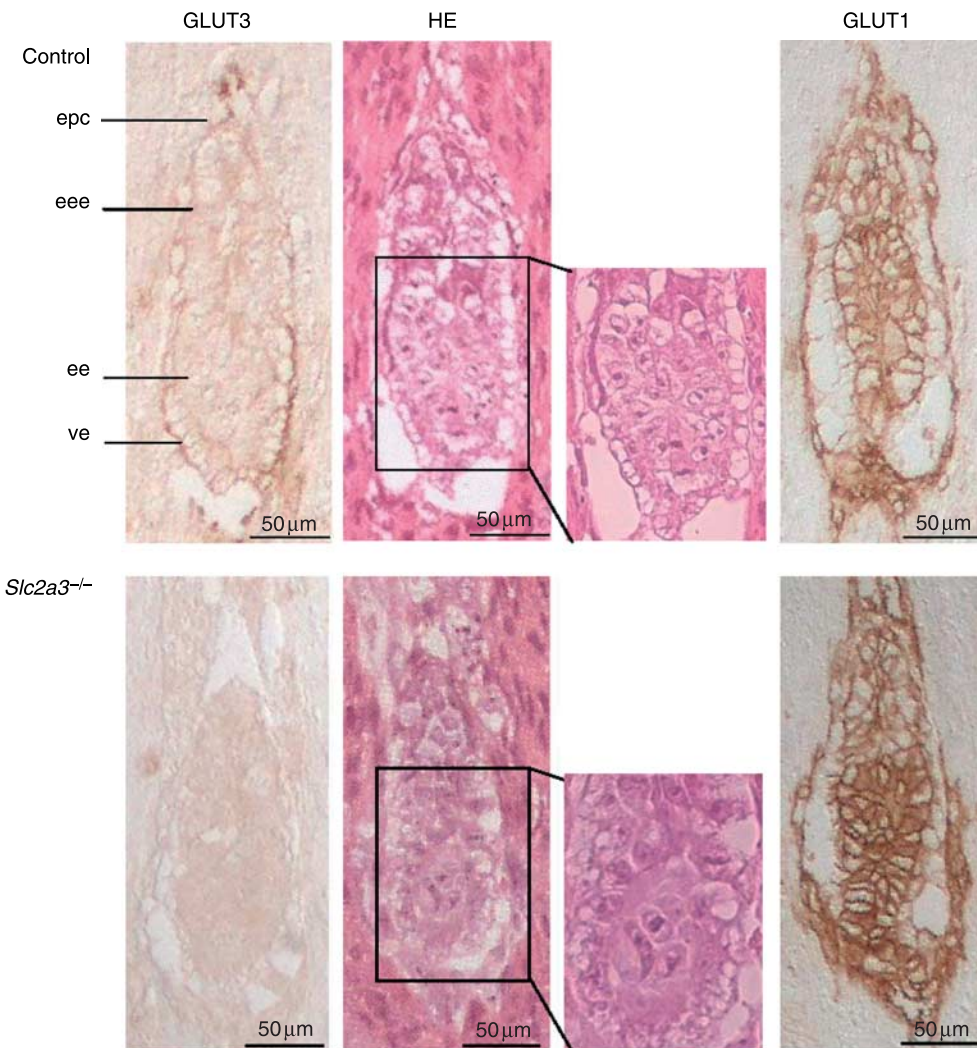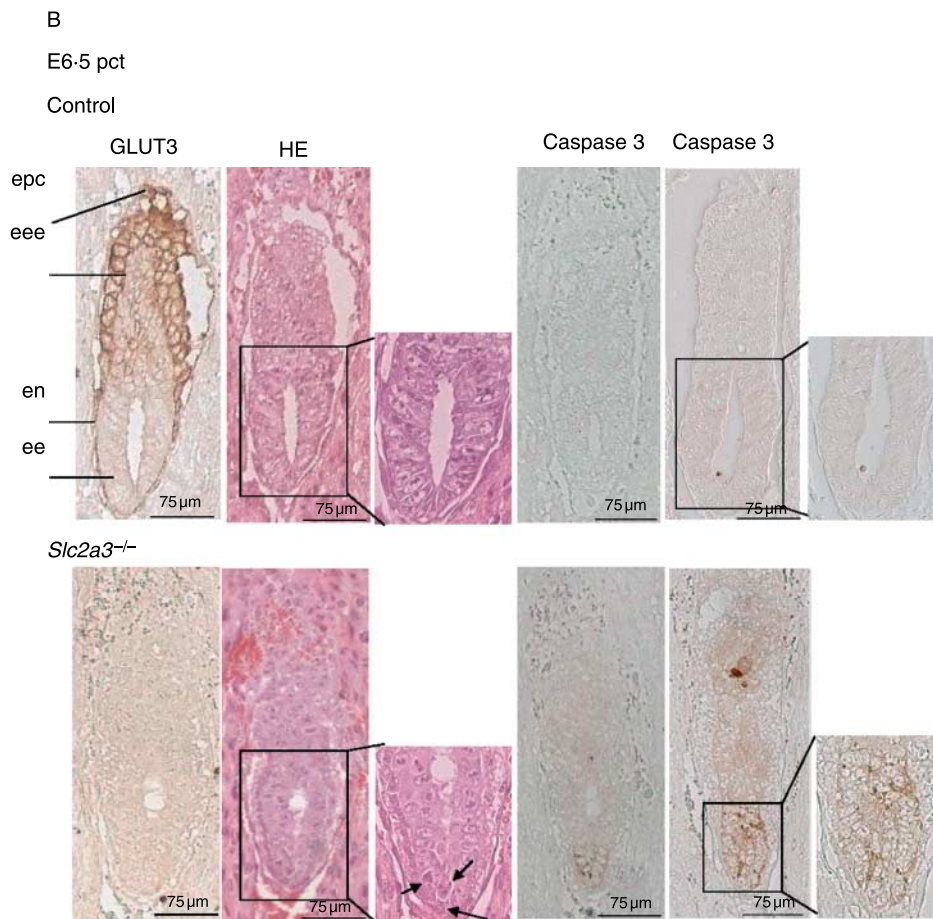

Figure 4 (*continued*)

Figure 4 (*continued*)

et al. (1997) and Pantaleon & Kaye (1998), who confined basolateral localization of GLUT1 in both morulae and blastocysts. One reason for this discrepancy might be the difference in the sensitivity of the anti-GLUT1 antibodies. A second reason for the discrepancy in GLUT1 distribution could be a difference in culture conditions, e.g., the glucose concentration. We cultivated blastocysts in the presence of 4·5 g glucose/l, which might alter the rate of glycolysis and thereby GLUT1 expression and/or localization. Several parameters, for example, oxygen concentration (Harvey et al. 2004), modify GLUT1 expression in blastocysts; also, glucose concentration itself was described to change GLUT1 protein levels in parallel with the transport activity (von der Crone et al. 2000) in other systems such as 3T3-L1 adipocytes.

Since ablation of GLUT1 (Wang et al. 2006) and also of GLUT8 (Membrez et al. 2006, Gawlik et al. 2008) did not affect implantation, blastocysts appear to be able to compensate for a reduction in the glucose transport capacity. This conclusion is in contrast to previous reports describing that *in vitro* cultivation of zygotes or two-cell embryos in the absence of glucose led to inhibition of blastocyst formation

(Brown & Whittingham 1992) or a reduced number of ICM and trophectoderm cells (Brown & Whittingham 1991, Luisier et al. 2001). However, consistent with our results, Martin & Leese (1995) described that blastocysts developed *in vitro* from two-cell embryos in the complete absence of glucose, suggesting that pyruvate uptake can fully sustain the energy supply for blastocyst development.

In the present study, the absence of GLUT3 failed to alter expression or distribution of GLUT1 (Fig. 3B). By contrast, Ganguly et al. (2007) detected GLUT1 exclusively at the basolateral surface of wild-type blastocysts, at apical and basolateral membranes of heterozygous blastocysts, and in a scattered cytoplasmic distribution in $Slc2a3^{-/-}$ blastocysts. In addition, they detected Tunnel-positive, apoptotic cells in $Slc2a3^{-/-}$ blastocysts, whereas $Slc2a3^{-/-}$ blastocysts appeared healthy, and were able to grow *in vitro* and to implant into the uterus in the present study. It is unclear whether differences in the genetic background or in the conditions of blastocyst culture can account for these discrepant results. It should be noted, however, that Ganguly et al. (2007) distinguished the wild-type and $Slc2a3^{+/-}$ blastocysts not by genotyping but through the
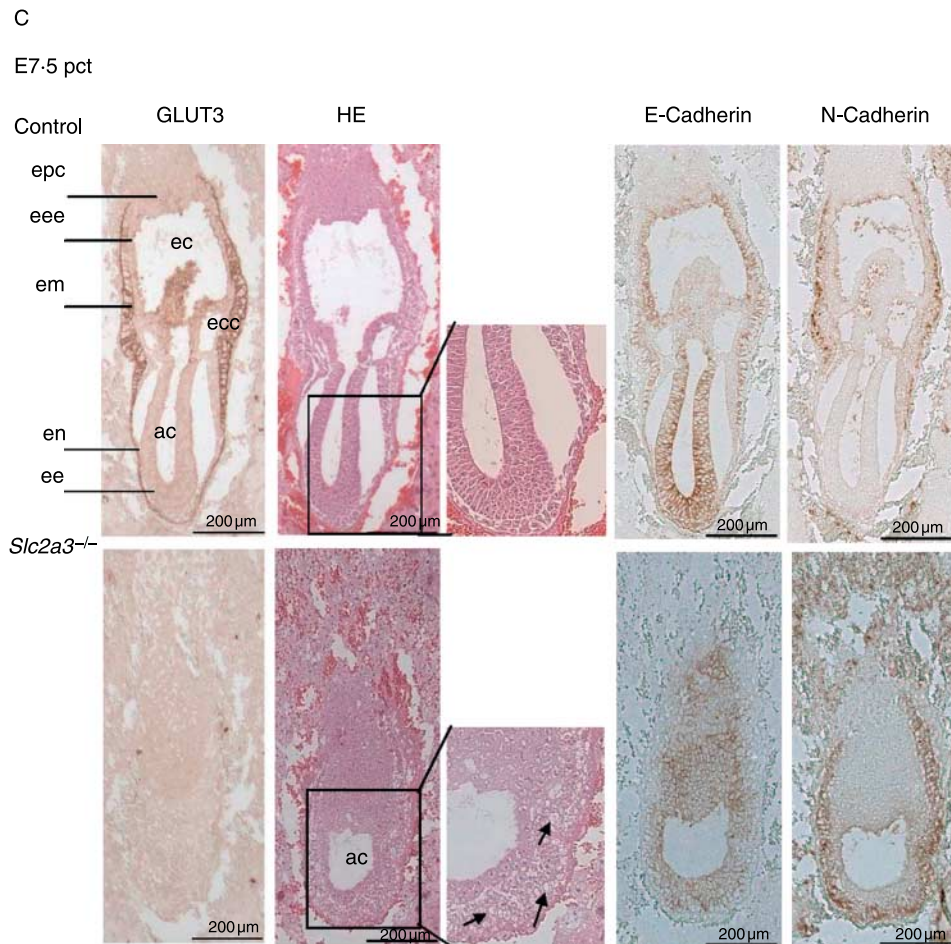
Figure 4 Defective development and apoptotic cell death in *Slc2a3* mutant embryos. Histological analysis of *Slc2a3*$^{-/-}$ and control embryos (either *Slc2a3*$^{+/+}$ or *Slc2a3*$^{+/-}$). Serial sagittal sections of uteri from heterozygous matings at (A) day 6·0, (B) 6·5, and (C) 7·5 pc were stained with hematoxylin and eosin (HE), or used for staining of GLUT3, GLUT1, activated caspase 3, E-cadherin, or N-cadherin. ac, amniotic cavity; ec, ectoplacental cavity; ecc, exocoelomic cavity; ee, embryonic ectoderm; eee extraembryonic ectoderm; em, embryonic mesoderm; en, endoderm; epc, ectoplacental cone. Microscopical abnormalities in ectodermal cells of mutant embryos are indicated by arrowheads.

localization of GLUT3 (apical versus basolateral surface of the trophectoderm). Thus, the study lacks unambiguous proof that the observed differences between wild-type and heterozygote blastocysts are associated with the respective genotype.

The present data precisely identified the developmental stage and time point at which disruption of *Slc2a3* is lethal. *Slc2a3*$^{-/-}$ embryos appeared normal until day 6·0, exhibited distinct morphological changes including apoptotic cells at day 6·5, were arrested in growth at day 7·5, and were completely lost at day 12·5 post-coitum. Ganguly *et al.* (2007) had indirect evidence that failure of *Slc2a3*$^{-/-}$ embryos occurred at neurulation, which begins with neural tube closure at embryonic day 8·5. They detected all three genotypes (wild-type, heterozygous, and knockouts) at embryonic day 8·5, but failed to find knockout embryos at day 9·5. According to our results *Slc2a3*$^{-/-}$, embryos at day 8·5 were probably partially degraded. We discovered

events of apoptosis starting at E6·5 dpc and a marked growth retardation and a cessation of development in the absence of GLUT3 at E7·5 dpc. This is similar to other knockout mice, e.g., the *ZO2*$^{-/-}$ mutant (Xu *et al.* 2008) or *Arfrp1*$^{-/-}$ mice (Mueller *et al.* 2002), in which apoptotic events during gastrulation resulted in a loss of the embryos at early time points.

Thus, lethality of *Slc2a3*$^{-/-}$ embryos started at day 6·5 by apoptosis of ectodermal cells. Two alternative hypotheses might explain why ectodermal cells that do not express GLUT3 undergo apoptosis. i) Defective placental development of *Slc2a3*$^{-/-}$ mice might be responsible for lethality of the embryos. ii) Reduced substrate supply via visceral endoderm that lack GLUT3 might arrest embryonic development. The finding that *in vitro*-cultivated *Slc2a3*$^{-/-}$ embryos did not show defects in trophectoderm development (Supplementary Fig. 1) might indicate that GLUT3 is a

crucial transporter in a placenta-like function of the visceral endoderm, and that its disruption causes a deficiency in ectodermal cells.

Several studies have linked a reduced glucose transport to the initiation of apoptosis (Kan *et al.* 1994, Berridge *et al.* 1996). In the models of neuronal development and trophic factor deprivation, a decrease in glucose uptake is one of the earliest changes observed in the cascade of apoptosis (Johnson *et al.* 1996).

In summary, deletion of GLUT3 in mice arrests early embryonic development due to apoptosis of ectodermal cells shortly after implantation (E6·5 dpc). By contrast, the development of pre-implantation embryos is independent of GLUT3.

Declaration of interest

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