

Expression of the gene encoding the high- K_m glucose transporter 2 by the early postimplantation mouse embryo is essential for neural tube defects associated with diabetic embryopathy

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Abstract

Aims/hypothesis Excess glucose transport to embryos during diabetic pregnancy causes congenital malformations. The early postimplantation embryo expresses the gene encoding the high- K_m GLUT2 (also known as SLC2A2) glucose transporter. The hypothesis tested here is that high- K_m glucose transport by GLUT2 causes malformations resulting from maternal hyperglycaemia during diabetic pregnancy.

Materials and methods *Glut2* mRNA was assayed by RT-PCR. The K_m of embryo glucose transport was determined by measuring 0.5–20 mmol/l 2-deoxy[3 H]glucose transport. To test whether the GLUT2 transporter is required for neural tube defects resulting from maternal hyperglycaemia, *Glut2*^{+/-} mice were crossed and transient hyperglycaemia was induced by glucose injection on day 7.5 of pregnancy. Embryos were recovered on day 10.5, and the incidence of neural tube defects in wild-type, *Glut2*^{+/-} and *Glut2*^{-/-} embryos was scored.

Results Early postimplantation embryos expressed *Glut2*, and expression was unaffected by maternal diabetes. Moreover, glucose transport by these embryos showed

Michaelis–Menten kinetics of 16.19 mmol/l, consistent with transport mediated by GLUT2. In pregnancies made hyperglycaemic on day 7.5, neural tube defects were significantly increased in wild-type embryos, but *Glut2*^{+/-} embryos were partially protected from neural tube defects, and *Glut2*^{-/-} embryos were completely protected from these defects. The frequency of occurrence of wild-type, *Glut2*^{+/-} and *Glut2*^{-/-} embryos suggests that the presence of *Glut2* alleles confers a survival advantage in embryos before day 10.5.

Conclusions/interpretations High- K_m glucose transport by the GLUT2 glucose transporter during organogenesis is responsible for the embryopathic effects of maternal diabetes.

Keywords Neural tube · Diabetic pregnancy · Diabetic embryopathy · Embryo · GLUT2 · SLC2A2

Abbreviations

NTD neural tube defect
SLC2 solute carrier 2

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Introduction

Maternal diabetes significantly increases the risk of congenital malformations: while rigorous glycaemic control in the periconception period reduces the incidence of congenital malformations, the incidence remains three to fivefold greater than that for non-diabetic pregnancy [1–5]. These malformations arise during the earliest stages of organogenesis, corresponding to approximately weeks 2–8 in human gestation [6]. Both type 1 and type 2 diabetes increases the risk of congenital malformations [2, 7]. The

metabolic profiles of type 1 and type 2 diabetes are different, except for episodic hyperglycaemia. Numerous studies have shown that the incidences of early miscarriage and congenital malformations are associated with poor glycaemic control [8–10]. These observations suggest that excess glucose, or a metabolic process activated by high glucose concentration in both type 1 and type 2 diabetes, is responsible for the teratogenic effect of diabetic pregnancy.

There are 14 known members of the solute carrier 2 (SLC2) family of facilitative hexose and polyol transporters, characterised by 12 transmembrane domains and the presence of several conserved amino acids [11, 12]. The different members of the SLC2 family differ in substrate specificity, affinity for cytochalasin B, inhibition by phloretin or phlorizin, and regulation by insulin [11]. Of the 14 *SLC2* gene family members, expression of *Glut1*, *Glut2*, *Glut3*, *Glut8* and *Glut12* (also known as *Slc2a1*, *Slc2a2*, *Slc2a3*, *Slc2a8* and *Slc2a12*) is detected in preimplantation mouse embryos [13–18]. GLUT12 protein is not detected in early postimplantation embryos, but it is detected later, during fetal development [17, 19]. Only expression of *Glut1*, *Glut2* and *Glut3* has been detected during the stage of postimplantation development that is susceptible to diabetic embryopathy (day 7.5) [12, 13, 20–23]. *Glut1* is expressed primarily in the ectoplacental cone, amnion, chorion and mesodermal layer of the yolk sac, and lower levels are expressed in the embryonic ectoderm. Northern blotting shows that *Glut2* mRNA is expressed at high levels on days 7.5 and 8.5 but is barely detectable on day 9.5; most of the expression is in the endodermal layer of the visceral yolk sac. *Glut3* expression is primarily in the amnion, chorion and endodermal layer of the yolk sac [20]. Both GLUT1 and GLUT3 have affinities that approximate physiological glucose concentrations. GLUT2, however, has a K_m that is two- to threefold higher than physiological glucose concentrations [24]. Postnatally, GLUT2 is produced by pancreatic beta cells, hepatocytes, intestine and kidney—tissues that must transport glucose with high efficiency when its concentration is high, for example after intestinal absorption of nutrients following feeding [11, 25]. GLUT2 is essential for glucose-stimulated insulin release, and also regulates glucose sensing by extrapancreatic tissues [26, 27]. However, the early embryo is not normally exposed to glucose concentrations approaching the K_m of the GLUT2 transporter. Thus, during normal, non-diabetic gestation, glucose transporters with a low K_m (high affinity) would transport glucose into the embryo cells more efficiently than the GLUT2 transporter. There have been some reports that GLUT1 and GLUT3, which are produced in the postimplantation embryo during early organogenesis, are not downregulated during exposure to a hyperglycaemic environment, and so could continue to transport glucose

even during maternal hyperglycaemia [21, 22]. However, the rate of glucose transport by GLUT1 and GLUT3 would saturate during hyperglycaemia. In contrast, transport by the high- K_m GLUT2 transporter would become more efficient during hyperglycaemia and might allow the rate of glucose transport into embryo cells to increase two to threefold.

In the rat, the intracellular glucose concentration in the embryo neuroepithelium is at equilibrium with maternal serum, even when the mother is hyperglycaemic [28]. This indicates that glucose is freely transportable into embryo cells, even when maternal glucose concentrations are above the K_m for low- K_m glucose transporters.

Neural tube defects (NTD), which are among the most common malformations that occur in the offspring of diabetic human mothers [29, 30], are significantly increased in the embryos of diabetic mice and rats [31–33]. That high glucose concentration is responsible for the teratogenic effects of diabetic pregnancy has been demonstrated using three approaches: (1) inducing hyperglycaemia in pregnant non-diabetic mice by glucose injection causes NTD; (2) lowering blood glucose concentrations in pregnant diabetic animals with phlorizin to cause renal glucose clearance reduces NTD; and (3) culturing fragments of neurulating embryos in media containing concentrations of glucose that are equivalent to those in serum during diabetes (15 mmol/l) inhibits expression of *Pax3*, a gene required for neural tube closure, compared with culture in media containing 5 mmol/l glucose [34]. It was noteworthy that in glucose-injected and phlorizin-treated animals, the incidence of NTD was not linearly correlated with maternal blood glucose concentration. Rather, the increase in NTD was not statistically significant until maternal blood glucose concentrations were ≥ 250 mg/dl (13.89 mmol/l) on the day before the onset of *Pax3* expression [34]. This indicates that the threshold of maternal glycaemia above which there is a significant increase in NTD corresponds to the K_m for the GLUT2 glucose transporter. Similarly, in human diabetic pregnancy, the relationship of adverse pregnancy outcome (spontaneous abortion and major malformation) with HbA_{1c} is not linear but sigmoidal [8]. Although high HbA_{1c} levels do not distinguish between episodic glycaemic excursions and chronic hyperglycaemia, they do indicate the steady-state exposure of maternal and embryo tissues to glucose during the preceding several weeks. A sigmoidal, rather than linear, association of poor glycaemic control with adverse pregnancy outcome is consistent with a model in which there is a threshold of maternal glycaemia above which damage to embryo development occurs.

In this study, we tested the hypothesis that the high- K_m GLUT2 glucose transporter is essential for the embryopathic effects of maternal hyperglycaemia.

Materials and methods

Animal procedures ICR mice (Taconic, Germantown, NY, USA) were obtained at 5–6 weeks of age and were used to generate embryos for the assay of *Glut2* mRNA and 2-deoxy-[³H]glucose transport. For the assay of *Glut2* mRNA, mice were made diabetic with streptozotocin (STZ) (Sigma Chemicals, St Louis, MO, USA) as described [35]. Briefly, female mice to be made diabetic were injected intraperitoneally with STZ to destroy pancreatic beta cells. Hyperglycaemia before pregnancy was controlled with subcutaneously implanted insulin pellets (Linshin, Inc., Scarborough, ON, Canada). Diabetic and age-matched non-diabetic female mice were mated with non-diabetic ICR male mice about 3 weeks after insertion of insulin pellets and checked daily for copulation plugs. Day 0.5 of pregnancy was determined to be at 12.00 h on the day in which a copulation plug was found. The STZ-diabetic female mice became hyperglycaemic beginning on day 4.5 of pregnancy because of insufficiency of the insulin pellets during pregnancy [35]. Pregnant mice were killed by cervical dislocation to recover embryos on day 7.5 for the assay of *Glut2* mRNA or 2-deoxy-D-[³H]glucose transport.

Mice carrying a *Glut2* knockout allele [26] on a 129/SvJ background were used to generate embryos that were GLUT2-deficient. The *Glut2* knockout strain was originally generated on a mixed C57Bl/6J×129/Sv background. C57Bl/6J embryos are resistant to diabetic embryopathy [36]. Therefore, a congenic strain was generated to place the *Glut2*^{+/-} allele on a 129/SvJ background [37]. Offspring at each generation with the least residual donor background (C57Bl/6J) were identified by marker-assisted gene analysis [38] performed by the Speed Congenics facility of the Jackson Laboratory (Bar Harbor, ME, USA). The strain was considered to be congenic once it retained <0.2% C57Bl/6J DNA [37].

Non-diabetic *Glut2*^{+/-} male and female mice were mated and hyperglycaemia or oxidative stress was induced on day 7.5 as described previously [34, 39]. Briefly, transient hyperglycaemia was induced by injecting 2 ml of 12.5% glucose–PBS solution subcutaneously approximately every 1–2 h between 09.00 and 17.00 h. Blood glucose concentrations were checked hourly, and animals were re-injected as needed to maintain the blood glucose concentration ≥300 mg/dl (16.65 mmol/l). Oxidative stress was induced with a single injection of 3 mg/kg antimycin A (Sigma Chemicals), a mitochondrial complex III inhibitor [40, 41], dissolved in 25% (v/v) propylene glycol. Control mice were injected with PBS or propylene glycol. Embryos were dissected from uteri and removed from surrounding membranes on day 10.5 as described [42] under a Nikon SMZ stereo microscope. Embryos were inspected externally for NTD as defined by an open or malformed cranium or

spinal neural tube. Embryos were photographed with a Spot colour digital camera mounted on the stereo microscope using Spot 3.5 software (Micro Video Instruments, Avon, MA, USA).

All procedures performed using animals followed the National Institutes of Health's *Principles of laboratory animal care* and were approved by the Institutional Animal Care and Use Committee of the Joslin Diabetes Center.

RT-PCR assay of *Glut2* mRNA Day-7.5 embryos from individual diabetic or non-diabetic pregnancies were pooled. RNA was isolated and reverse-transcribed, and serial dilutions of the reverse transcription products were amplified by PCR as described previously [35]. The cDNA products were amplified using primers complementary to the *Glut2* amplicon [43] and the PCR conditions described below, or to constitutively expressed *36B4* (also known as *Arbp*), which was used as a normalisation control, as described [35, 44]. Autoradiographs of the electrophoresed PCR products were scanned, and unsaturated bands were quantified using Adobe Photoshop (version 9.0.1, Adobe Systems, Inc., San Jose, CA). The amount of *Glut2* RT-PCR product was expressed relative to that of the *36B4* RT-PCR product.

Measurement of glucose transport kinetics Uptake of 2-deoxy-D-[³H]glucose by day-7.5 embryos was assayed using a procedure modified after that of Cheatham et al. [45]. Briefly, embryos with surrounding amniotic and yolk sac membranes were dissected in cold DMEM containing 5 mmol/l glucose (Invitrogen, Carlsbad, CA, USA) with 10% FCS. Pooled embryos were washed with KRH buffer [45], and incubated for 5 min with collagenase, while passing up and down with a 1 ml micropipette tip. The embryo fragments were rinsed with KRH buffer, and the equivalent of three or four embryos was distributed into individual tubes. Tissues were incubated for 30 s with 0.5–20 mmol/l 2-deoxy-D-[³H]glucose (2-deoxy-D-[1,2-³H]glucose, specific activity 969 GBq/mmol; Perkin Elmer–New England Nuclear, Boston, MA, USA) at a constant specific activity of 185 Bq/mmol. Samples were incubated without or with cytochalasin B (Sigma), added at 0.4 or 4.0 μmol/l. Samples incubated with 0.5–20 mmol/l L-[³H]glucose (L-[1-³H] glucose; Perkin Elmer–New England Nuclear) were used as controls for non-specific glucose uptake. The specific activity of L-[³H]glucose was comparable with that of 2-deoxy-D-[³H]glucose, and was added to the L-glucose incubation solution to give a constant specific activity of 185 Bq/mmol. Reactions were terminated by adding 1 ml cold PBS, followed by centrifugation and three washes with PBS. Pellets were solubilised with 0.5 ml of 0.05% SDS. Four-hundred microlitres of the solubilised pellet was counted for radioactivity and 100 μl was used for protein

measurement (Bio-Rad, Hercules, CA, USA). The K_m and V_{max} of 2-deoxy-D-glucose uptake were determined using non-linear curve fitting analysis with the Michaelis–Menten equation (Prism, version 4.0; GraphPad Software, Inc., San Diego, CA, USA).

Genotype analysis of *Glut2* allele *Glut2*^{+/-} pups were identified by PCR analysis of tail DNA using the previously reported primer sequences [42, 43] and the following conditions: 94°C for 5 min, followed by 36 cycles of 94°C for 30 s, 53°C for 30 s, 72°C for 30 s and 72°C for 10 min. DNA was prepared using DNazol (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. Genotypes of embryos of *Glut2*^{+/-} × *Glut2*^{+/-} matings were determined similarly using yolk sac DNA.

Statistical analysis χ^2 analyses were performed using Prism (version 4.0) software for Macintosh (GraphPad Software).

Results

Mouse embryos produce a functional high- K_m GLUT2 glucose transporter It had been shown previously that mouse embryos express *Glut2* mRNA from the eight-cell stage until at least day 9.5 [13]. However, expression had not been examined during diabetic pregnancy. Whereas expression of *Glut1* and *Glut3* is not downregulated during maternal hyperglycaemia in the postimplantation embryo [21, 22], expression of *Glut1*, *Glut2* and *Glut3* has been found to be downregulated by high glucose concentration in the preimplantation embryo [46]. *Glut2* mRNA was assayed in embryos recovered on day 7.5, the day of embryonic development that is susceptible to subsequent development of NTD in response to maternal hyperglycaemia [34]. As shown in Fig. 1, *Glut2* mRNA was expressed on day 7.5 by embryos of both diabetic and non-diabetic mice, and there was no effect of maternal diabetes on expression of *Glut2* mRNA.

To determine whether glucose transport by day-7.5 mouse embryos exhibits high- K_m kinetics, the Michaelis–Menten kinetics of glucose transport were assayed. Embryo tissues were incubated with 2-deoxy-D-[³H]glucose in the presence of 0.5–20 mmol/l 2-deoxy-D-glucose. Analysis of the glucose transport curve using the Michaelis–Menten equation demonstrated that the embryo glucose transporter had a K_m of 16.19 mmol/l, which is consistent with the K_m of the GLUT2 glucose transporter (Fig. 2a). Transport of 2-deoxy-D-glucose was not inhibited by 0.4 μ mol/l cytochalasin B (Fig. 2b), a concentration that inhibits glucose

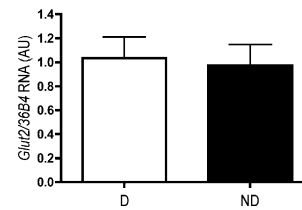


Fig. 1 RT-PCR of *Glut2* and *36B4* RNA from day 7.5 embryos of diabetic and non-diabetic mice. Embryos from individual diabetic (D) or non-diabetic (ND) pregnancies were pooled and assayed for *Glut2* or *36B4* RNA as described in **Materials and methods**. The abundance of the *Glut2* PCR product is expressed relative to the abundance of the *36B4* PCR product. The data are the results of three diabetic and three non-diabetic pregnancies (mean \pm SEM). There is no significant difference between the abundance of *Glut2* RNA relative to that of *36B4* ($p=0.74$). AU Arbitrary units

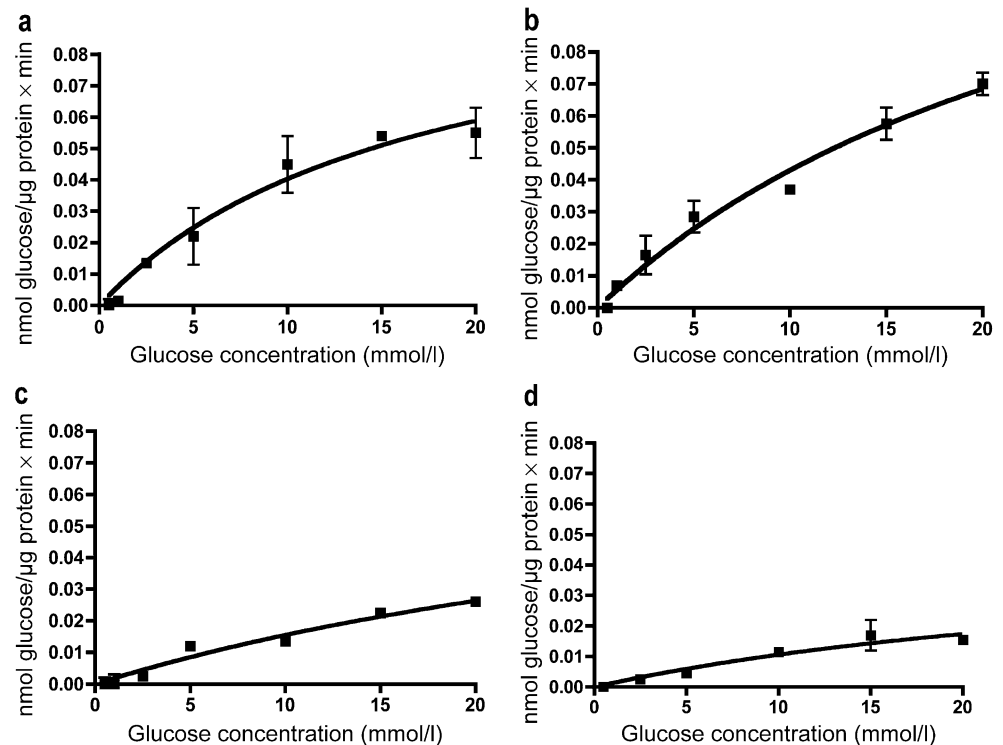
transport by GLUT1 and GLUT3, but 2-deoxy-D-glucose transport was inhibited by 4.0 μ mol/l cytochalasin B (Fig. 2c), a concentration that inhibits glucose transport by GLUT2. The embryo glucose transporter was specific for the D-glucose isoform, as specific, saturable transport of L-glucose did not occur (Fig. 2d).

Mouse embryos lacking a functional GLUT2 glucose transporter are resistant to hyperglycaemia-induced NTD If glucose uptake by the GLUT2 glucose transporter is essential for diabetic embryopathy, then embryos in which only GLUT2-mediated glucose uptake is non-functional should be resistant to the teratogenic effects of hyperglycaemia. It is possible to test this using *Glut2*^{-/-} embryos.

Female and male *Glut2*^{+/-} mice were crossed to generate wild-type, *Glut2*^{+/-} and *Glut2*^{-/-} embryos. On day 7.5 of pregnancy, female mice were made hyperglycaemic by subcutaneous glucose injection at approximately hourly intervals. This is sufficient to induce NTD associated with diabetic embryopathy [34]. Control pregnancies were injected with PBS. The mean blood glucose concentration was 18.91 \pm 0.54 mmol/l, compared with 6.88 \pm 0.18 mmol/l in PBS-injected controls.

Embryos were recovered on day 10.5 of gestation and scored for NTD. Embryo genotype was determined as described in **Materials and methods**. In the PBS-injected pregnancies, all embryos of all genotypes were normal, except for one heterozygous embryo, which displayed a mild defect (a closed neural tube with a slightly under-expanded IVth ventricle, resembling holoprosencephaly) (Fig. 3a). However, in glucose-injected pregnancies there was a significant effect of embryo genotype on the incidence of NTD (Fig. 3b). Thirty-seven per cent of wild-type embryos displayed NTD (severe, open NTD, primarily exencephaly, as shown in Fig. 4), whereas less than half as many (16%) of haploinsufficient *Glut2*^{+/-}

Fig. 2 Kinetics of glucose uptake by day 7.5 embryos. **a** Transport of 0.5–20 mmol/l 2-deoxy-D-[³H]glucose. V_{\max} =0.109 mmol/l, K_m =16.91 mmol/l. **b** Transport of 0.5–20 mmol/l 2-deoxy-D-[³H]glucose in the presence of 0.4 μ mol/l cytochalasin B. **c** Transport of 0.5–20 mmol/l 2-deoxy-D-[³H]glucose in the presence of 4.0 μ mol/l cytochalasin B. **d** Transport of 0.5–20 mmol/l L-[³H]glucose



embryos displayed NTD, and none of the GLUT2-deficient *Glut2*^{-/-} embryos were affected by NTD.

To determine whether GLUT2-deficient embryos were susceptible to NTD caused by biochemical events distal to glucose transport, pregnant mice were given a single injection of antimycin A on day 7.5. Antimycin A is a mitochondrial complex III inhibitor that stimulates superoxide production [40, 41] and mimics the effects of maternal diabetes on *Pax3* expression and NTD [39, 47]. As shown in Fig. 3c, antimycin A induced NTD in embryos of all genotypes. While the incidence of NTD in the *Glut2*^{-/-} embryos was less than in *Glut2*^{+/+} and *Glut2*^{+/-} embryos (15 vs 44%), there was no significant difference in the incidence of NTD of embryos of any genotype.

It was noteworthy that, whereas the average number of embryos recovered per pregnancy from each treatment

group did not differ (6.7, 6.3 and 7 from PBS-, glucose- and antimycin A-injected pregnancies, respectively), the genotypes of embryos did not occur with the expected Mendelian frequency of one-fourth wild-type, one-half heterozygous and one-fourth homozygous *Glut2*-deficient (Table 1). Indeed, the percentages of the three genotypes were not 25:50:25 but 52:29:17. χ^2 analyses showed that the variance with regard to the expected Mendelian frequency of each genotype was statistically significant, and that the incidence of each genotype was not different in any of the treatment groups. This suggests that the presence of two wild-type *Glut2* alleles confers a survival advantage before day 10.5 of embryonic development, and that the presence of one wild-type *Glut2* allele is advantageous over total *Glut2* deficiency, at least on a 129Sv/J background.

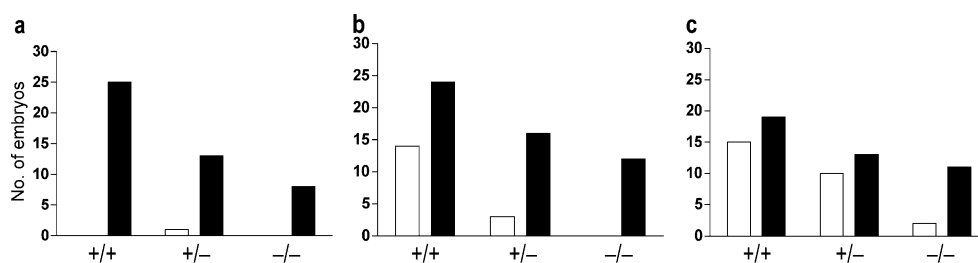


Fig. 3 Incidences of malformed (open bars) and normal (solid bars) neural tube development in wild-type, *Glut2*^{+/-} and *Glut2*^{-/-} embryos in pregnancies treated with **a** PBS ($n=7$ pregnancies), **b** glucose ($n=11$ pregnancies) or **c** antimycin A ($n=10$ pregnancies) on day 7.5.

There was a significant difference in the incidence of malformation in embryos of different genotypes by χ^2 analysis only in (b) ($p<0.02$). +/+, *Glut2* wild type; +/-, *Glut2*^{+/-}; -/-, *Glut2*^{-/-}

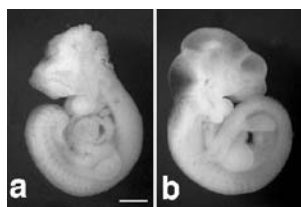


Fig. 4 Examples of malformed and normal embryos on day 10.5. **a** Embryo with exencephaly affecting the midbrain and hindbrain. **b** Normal embryo. Scale bar=1 mm in **(a)** and **(b)**

Discussion

The data reported here confirm that the early post-implantation embryo, at the stage of development that is susceptible to NTDs induced by diabetic pregnancy, expresses the gene encoding the high K_m GLUT2 glucose transporter. Expression of *Glut1*, *Glut2* and *Glut3* is suppressed in preimplantation embryos of diabetic mice [46], and expression of *Glut1* has been found to be both unchanged or suppressed by maternal diabetes or high-glucose culture in postimplantation embryos [21, 22]. However, in the present study, expression of *Glut2* was unaffected by maternal diabetes. Furthermore, the early embryo produces a functional high- K_m transporter, as the K_m of glucose uptake is 16.19 mmol/l, and it was not inhibited by 0.4 $\mu\text{mol/l}$ cytochalasin B but it was inhibited by 4.0 $\mu\text{mol/l}$ cytochalasin B. GLUT2-deficient embryos of hyperglycaemic mothers were resistant to hyperglycaemia-induced NTD, indicating that glucose transported into embryo cells by GLUT2, but not other glucose transporters, mediates the embryopathic effects of maternal hyperglycaemia.

It is interesting to note that in patients with diabetes, the tissues that suffer from the adverse effects of hyperglycaemia during the pathogenesis of diabetic complica-

tions, such as the vasculature and nerves, do not express GLUT2. However, if the rate of glucose phosphorylation by these tissues is sufficiently high, a concentration gradient driving sustained glucose uptake can be maintained. On the other hand, it may take years for the accumulated damage from hyperglycaemia to be clinically detectable, whereas the adverse effects of maternal hyperglycaemia on the embryo are apparent within hours. Therefore, the rate of glucose metabolism and ensuing adverse effects on embryo physiology may be far in excess of that which occurs in tissues producing only low- K_m glucose transporters.

During non-diabetic pregnancy, the embryo or fetus would not be exposed to glucose at concentrations approaching the K_m of the GLUT2 transporter. Therefore, there does not appear to be an occasion when this transporter would mediate glucose uptake. Furthermore, since embryos also produce GLUT1 and GLUT3, which mediate low- K_m (high-affinity) glucose transporters at normal, non-diabetic, circulating glucose concentrations, the need for a high- K_m (low-affinity) transporter is not obvious.

Before we began this study, we considered that, because *Glut2*^{-/-} pups are viable until weaning [26], GLUT2 is not necessary for embryonic or fetal development. Therefore, as explained above, its presence or absence would appear to have little consequence during non-diabetic pregnancy, and so the expression of *Glut2* may be vestigial. For example, embryos of oviparous animals derive glucose that is stored as glycogen in egg yolk at quantities sufficient to provide energy until hatching. The concentration of glucose that is transported to the embryo from the egg yolk might approach the K_m of GLUT2. Indeed, the concentration of glucose in the yolk of a neurulating chick embryo is approximately 11 mmol/l (Fine and Loeken, unpublished data). Thus, there might be no selective pressure to retain expression of *Glut2*, but also no selective pressure against its expression during normal mammalian gestation.

However, the frequency of occurrence of each *Glut2* genotype in embryos recovered from *Glut2*^{+/-} × *Glut2*^{+/-} matings suggests that, although the expression of *Glut2* is not absolutely required for embryonic life, GLUT2 sufficiency confers a significant survival advantage before embryonic day 10.5 over GLUT2 deficiency. It is not known when, before day 10.5, the GLUT2-deficient embryos are lost, but because there were no implantation sites containing reabsorbing embryos, and implantation sites of embryos that die at day 7.5 or later were still detectable on day 10.5, the embryos must have died before day 7.5. *Glut2* mRNA is detected beginning at the eight-cell stage [13], so the embryos were probably lost between the eight-cell stage and very early postimplantation development.

The non-Mendelian occurrence of *Glut2*^{+/-} and *Glut2*^{-/-} embryos was not expected, because when the *Glut2* knockout line was first generated the occurrence of each of

Table 1 Total number of embryos of each *Glut2* genotype in each treatment group from matings of *Glut2*^{+/-} with *Glut2*^{+/-} mice

Genotype	PBS*	Glucose**	Antimycin A***
+/+	25	38	34
+/-	14	19	23
-/-	8	12	13

Data from Fig. 3

χ^2 analysis was performed comparing the actual number of embryos of each genotype with the expected Mendelian frequency of 1/4 *Glut2*^{+/+}, 1/2 *Glut2*^{+/-} and 1/4 *Glut2*^{-/-}, and with the occurrence of each genotype in the other treatment groups

* $p=0.0034$ vs expected Mendelian frequency

** $p=0.003$ vs expected Mendelian frequency

*** $p=0.0062$ vs expected Mendelian frequency

the genotypes from *Glut2*^{+/-} parents was Mendelian [26]. However, the original *Glut2* knockout line was generated on a mixed C57Bl/6J × 129/Sv background, whereas the mice used here were on a pure 129Sv/J background. This suggests that there are polymorphic modifier genes that can complement the function of GLUT2 on some strain backgrounds but not on others. It is possible that GLUT2 transports a solute other than glucose during normal embryogenesis. It was recently recognised that GLUT2 is a high-affinity (low- K_m) transporter of glucosamine [48]. Glucosamine can be a substrate for the intermediates of post-translational glycosylation. Carbohydrate modification of proteins is essential for the production of extracellular matrix, as well as secreted and intracellular glycoproteins. The principal site of serum glucosamine synthesis is the liver [49]. Although glucosamine-6-phosphate is synthesised from the glycolytic intermediate, fructose-6-phosphate, and glutamine, it can also be generated by phosphorylation of glucosamine transported from extracellular sources. Because the early embryo derives its energy primarily through glycolytic (anaerobic) glucose metabolism [50], it is possible that in 129Sv/J embryos the rate of conversion of fructose-6-phosphate to fructose-1,6-biphosphate leaves a suboptimal concentration of fructose 6-phosphate to serve as a substrate for hexosamine biosynthesis, thereby increasing the need to derive glucosamine from maternal circulation.

In conclusion, production of a functional, high K_m GLUT2 glucose transporter confers sensitivity of the early postimplantation embryo to hyperglycaemia-induced malformations. Embryos lacking GLUT2 glucose transporters are structurally normal, although GLUT2-haploinsufficient and -deficient embryos are less viable than embryos that express two wild-type *Glut2* alleles. However, when maternal glucose concentrations approach the K_m of GLUT2, excessive glucose transport into embryo cells will affect embryonic development adversely.

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Duality of interest The authors do not have any duality of interest.

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