Glucose affects monocarboxylate cotransporter (MCT) 1 expression during mouse preimplantation development

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Abstract

Cleavage-stage embryos have an absolute requirement for pyruvate and lactate, but as the morula compacts, it switches to glucose as the preferred energy source to fuel glycolysis. Substrates such as glucose, amino acids, and lactate are moved into and out of cells by facilitated diffusion. In the case of lactate and pyruvate, this occurs via H⁺-monocarboxylate cotransporter (MCT) proteins. To clarify the role of MCT in development, transport characteristics for DL-lactate were examined, as were mRNA expression and protein localisation for MCT1 and MCT3, using confocal laser scanning immunofluorescence in freshly collected and cultured embryos. Blastocysts demonstrated significantly higher affinity for DL-lactate than zygotes (K_m 20 ± 10 vs 87 ± 35 mmol lactate/l; $P = 0.03$ by linear regression) but was similar for all stages. For embryos derived *in vivo* and those cultured with glucose, MCT1 mRNA was present throughout preimplantation development, protein immunoreactivity appearing diffuse throughout the cytoplasm with brightest intensity in the outer cortical region of blastomeres. In expanding blastocysts, MCT1 became more prominent in the cytoplasmic cortex of blastomeres, with brightest intensity in the polar trophectoderm. Without glucose, MCT1 mRNA was not expressed, and immunoreactivity dramatically reduced in intensity as morulae died. MCT3 mRNA and immunoreactivity were not detected in early embryos. The differential expression of MCT1 in the presence or absence of glucose demonstrates that it is important in the critical regulation of pH and monocarboxylate transport during preimplantation development, and implies a role for glucose in the control of MCT1, but not MCT3, expression.

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Introduction

It is well established that glucose cannot support development of the preimplantation mouse embryo prior to compaction (Brinster & Thomson 1966). Freshly collected cleavage stage embryos preferentially consume pyruvate before switching to glucose as the major energy substrate during compaction and blastocyst formation (Leese & Barton 1984). The same phenomenon has been observed in embryos cultured *in vitro* (Gardner & Leese 1986). While preferred, the reliance of morulae on glucose-derived energy is not absolute, as two-cell embryos cultured in the absence of glucose may still form blastocysts by increasing their rate of consumption of pyruvate to meet the energy demands of cavitation (Martin & Leese 1999).

In contrast, freshly fertilised zygotes deprived of glucose from about 16–18 h after administration of human chorionic gonadotrophin (hCG) (i.e. 24 h earlier) cleave to form morulae *in vitro*, but fail to form blastocysts and subsequently degenerate (Chatot *et al.* 1989, Brown & Whittingham 1991, Martin & Leese 1995). These

‘glucose-starved’ embryos do not develop the capacity to utilise glucose preferentially or to increase pyruvate utilisation to compensate (Chatot *et al.* 1989, Brown & Whittingham 1991, Martin & Leese 1995). Glucose exposure during mouse preimplantation development therefore presents an intriguing paradox. While not required as an energy substrate (Leese & Barton 1984), there is an absolute requirement for exposure to glucose prior to the eight-cell stage for blastocyst formation to occur (Chatot *et al.* 1994, Martin & Leese 1995). This exposure facilitates expression of the glucose transporter protein (GLUT3) (Pantaleon *et al.* 2001a), which is essential for blastocyst formation (Pantaleon *et al.* 1997a) and the adaptive responses associated with increased pyruvate utilisation that must occur in the absence of glucose. Therefore, once exposed to glucose, embryos can adjust to its absence (Martin & Leese 1995), suggesting that glucose, rather than as a nutrient per se, may act as a signal during early cleavage that induces metabolic differentiation of the developing embryo. Thus, while not providing a large proportion of ATP to cleavage-stage embryos, glucose may

permit expression of metabolic enzymes and transporters in compacting morulae, capable of generating the energy required for blastocyst formation. Currently, little is known about the expression and function of pyruvate/lactate transporter proteins, or how they may be regulated in early development.

This study examines the expression of specific H⁺-monocarboxylate cotransporter (MCT) proteins that facilitate the coupled diffusion of a proton and a monocarboxylate ion (e.g. pyruvate, lactate, acetoacetate or β -hydroxybutyrate). The 14 identified MCT proteins are predicted to have 12 transmembrane α -helical domains with cytoplasmic N- and C-termini (Price *et al.* 1998, Halestrap & Price 1999, Halestrap & Meredith 2004). It is presumed that cellular monocarboxylate homeostasis is regulated by the varying substrate affinities, coupled with distinct cellular and subcellular distributions, in a manner analogous to glucose homeostasis (Halestrap 1976, Gould & Holman 1993, Halestrap & Price 1999). MCT1-4 are the best-characterised isoforms to date. The transport kinetics of MCT, by fluorescence measurement of changing pH, and mRNA expression of these isoforms in mouse embryos, have been examined previously with varying results (Harding *et al.* 1999, Herubel *et al.* 2002).

MCT transport was therefore assessed by radiotracer flux at physiologic pH, and MCT1 and MCT3 were examined for mRNA and protein expression. In retinal pigment epithelium, MCT1 (apical) and MCT3 (basolateral) cooperate to transfer H⁺ and lactate from the highly glycolytic retina to the choroid (Philp *et al.* 1998, 2001, 2003a). Because this reflects the glycolytic activity of morulae and blastocysts, these isoforms were selected to determine whether a similar cooperative mechanism exists for lactate efflux from the blastocoel cavity to the maternal interface. We characterised expression and localisation of MCT1 and MCT3 in mouse embryos developing *in vivo* or with and without glucose *in vitro* to clarify their regulation and function in preimplantation development.

Materials and Methods

Ethics

All experiments on mice were approved by the Animal Ethics and Experimentation Committees of the University of Queensland. These committees are approved by the National Health and Medical Research Council of Australia.

Antisera

Affinity purified anti-MCT1 IgG was raised in goats against a synthetic peptide mapping to the carboxyl terminus of the mouse peptide (Santa Cruz Biotechnology (cat. no. sc-14917), Santa Cruz, CA, USA). Affinity purified anti-MCT3 antiserum (courtesy N Philp, Thomas Jefferson University, Philadelphia, PA, USA) was raised in New Zealand White rabbits against mouse MCT3 (Philp *et al.* 2003a). Second-

ary antibodies included FITC-conjugated rabbit anti-goat IgG (Calbiochem-Novachem, San Diego, USA), Texas Red-conjugated goat antirabbit IgG (Calbiochem), horseradish peroxidase-labelled donkey antirabbit IgG (Amersham) or rabbit anti-goat IgG (Progen Biosciences, Brisbane, Australia).

Embryo collection and culture

Female Quackenbush mice 6–8 weeks of age were injected i.p. with 10 IU pregnant mare serum gonadotrophin (PMSG) (Folligon; Intervet P/L, Bendigo, Australia) followed 48 h later with 10 IU hCG i.p. (Chorulon; Intervet). Mated females were killed by cervical dislocation, and embryos were collected 24, 48, 72 and 96 h after hCG into M2 medium (Fulton & Whittingham 1978) lacking glucose (M2-G) and modified to contain 0.33 mmol sodium pyruvate/l and 4 g BSA/l (Hobbs & Kaye 1985). Cumulus cells were released by incubation with 0.5 g hyaluronidase/l in M2-G followed by washing in fresh M2-G.

In experiments requiring *in vitro* derived embryos, zygotes were collected and denuded at 18 h after hCG for culture in microdroplets at a density of 1 embryo/ μ l under mineral oil at 37 °C in a MINC incubator (Cook, Brisbane, Australia) in 5% O₂, 5% CO₂ and 90% N₂. The culture medium used was KSOM (Lawitts & Biggers 1993) modified to be either glucose free (KSOM-G) or containing 0.6 mmol glucose/l (KSOM). One subset of embryos in KSOM-G received a brief (3 h) exposure to 27 mmol glucose/l at 58–60 h after hCG, referred to in the following text as a glucose 'pulse'. They were then washed three times and returned to culture in KSOM-G for the remaining period. Control cultured embryos (KSOM) were sampled at 48, 72, 90 and 96 h after hCG, and embryos cultured in the KSOM-G and 'pulsed' groups were selected at 90 h after hCG for transport assays and immunohistochemistry. Both KSOM and KSOM-G groups were also sampled 96, 105 and 112 h for immunohistochemical analysis.

[³H]DL-Lactate transport assay

Embryos were washed and preincubated in lactate-free M2-G (pH 7.4) for 30 min at 37 °C. To measure DL-lactate uptake, freshly collected two-cell embryos and blastocysts were incubated at 37 °C or 4 °C under paraffin oil in 10 μ l microdroplets of M2-G containing 37 GBq lactic acid, sodium salt, [²⁻³H]DL-lactate, 0.74 TBq mmol l/l, and 0.1, 5, 25, 40 and 50 mmol DL-lactate/l. Osmolarity was maintained by adjusting NaCl as necessary, and pH was corrected to 7.4. Triplicate samples of three embryos were removed from incubation and washed four times in ice-cold M2, and radioactivity was determined as previously described (Gardner & Kaye 1995).

mRNA analysis

Embryos were collected at 24, 48, 72 and 96 h after hCG, and total RNA was obtained by phenol/chloroform

extraction with the RNeasy Mini Kit (Qiagen). RNA was also extracted from mouse retina, and 1 µg total RNA was used for reverse transcription (RT). RNA was reverse transcribed and amplified by PCR (Arcellana-Panlilio & Schultz 1993) in the presence of specific primers for MCT1 and MCT3. The primer pairs derived from the murine MCT1 and MCT3 cDNA sequences were as follows: MCT1 5' primer, 5'-gcctgagcaagtag-3', and 3' primer, 5'-tca-gacctcgatccagtag-3'; MCT3 5' primer, 5'-catctc-taccttgctgct-3', and 3' primer, 5'-gttagaccctgtcacagt-3'. The 375 bp and 391 bp PCR products for MCT1 and MCT3 respectively were resolved on 2% agarose gels containing 0.5 mg ethidium bromide/l. Genomic contamination of cDNA samples was tested by parallel PCR for mouse β-actin with a primer pair that generates a predicted 243 bp fragment for the cDNA and a 330 bp fragment if contaminating genomic DNA is present (Telford *et al.* 1990).

Immunofluorescence

Embryos were fixed in 2% paraformaldehyde in PBS (pH 7.4) and processed for immunofluorescence as previously described (Pantaleon *et al.* 1997b). Briefly, embryos were incubated overnight at 4°C in primary antibody (2–10 mg/l) and exposed to secondary antisera for 1 h at room temperature prior to mounting in glycerol for examination with a Bio-Rad (Hercules, CA, USA) MRC-2000 confocal laser scanning microscope mounted on a Zeiss (Oberkochen, Germany) Axioskop equipped with a Zeiss Plan-Apochromat × 60 oil-immersion objective. Negative controls for immunofluorescence included normal goat or rabbit serum diluted to the same concentration as primary antibody and omission of primary and secondary antisera.

Western immunoblotting

Tissues were homogenised in ice-cold homogenisation buffer (50 mmol Tris-HCl/l, 150 mmol NaCl/l, 10 mmol NaF/l, 1 mmol Na₃VO₄/l, 1% (v/v) Triton X 100 and one complete protease inhibitor tablet/50 ml (Roche)) and centrifuged at 14 000 g for 15 min at 4°C. Supernatant was removed and mixed with an equal volume of Laemmli sample buffer (10% SDS containing 1% (v/v) glycerol, 0.125 g bromophenol blue/l, 0.125 g xylene cyanol/l, 100 mmol dithiothreitol/l and 125 mmol Tris-HCl/l (pH 6.8) (Laemmli 1970). Samples (10 µg protein/lane as determined by bicinchoninic acid assay) were separated by polyacrylamide electrophoresis and transferred to 0.45 µm Immobilon-P PVDF membrane (Millipore Australia P/L, Sydney, Australia), using Towbin transfer buffer (25 mmol Tris/l, 192 glycine mmol/l, 20% (v/v) methanol (pH 8.3) (Towbin *et al.* 1979). Membranes were incubated for 1 h at room temperature in blocking solution of 5% skim milk (MCT1) or 5% BSA (MCT3) in PBS/0.1% Tween 20. Primary antisera (0.2 mg/l) were applied overnight at 4°C, and membranes were exposed to secondary antibody for 1 h at room

temperature. Proteins were visualised with Supersignal West Pico Enhanced Chemiluminescence Detection Kit (Pierce Biotechnology, Rockford, IL, USA).

Statistics

Kinetic parameters of [³H]DL-lactate transport were determined by non-linear regression with Prism (GraphPad Software, Inc., CA, USA). Values presented are means ± S.E.M. for a series of three experiments per embryo stage or culture type. Since each data point was produced from up to nine embryos, the values were averaged to provide results for each experiment. Statistical testing of differences between *K_m* and *V_{max}* was by Student's *t*-test with Welch's correction for unequal variance.

Results

[³H]DL-Lactate uptake in preimplantation embryos

Uptake of DL-lactate by two-cell embryos and blastocysts was determined over 25 min (Fig. 1). Radioactivity was detected just above background levels at 0.1 mmol DL-lactate/l. Transport equilibrium was apparent by 12 min with 5 and 25 mmol DL-lactate/l in two-cell embryos and 5 mmol DL-lactate/l in blastocysts, but may not have been

Figure 1 DL-Lactate uptake in mouse embryos. Uptake of DL-lactate by two-cell mouse embryos and blastocysts. Lactate concentrations: (solid diamond) 0.1, (solid inverted triangle) 5, (solid square) 25 and (solid triangle) 50 mmol/l. Each point represents mean ± S.E.M. for four separate experiments; *n* = 32 observations per data point.

complete by 25 min with 50 mmol DL-lactate/l. The initial rate was approximately constant up to 5 min in all concentrations over 0.1 mmol DL-lactate/l. This uptake time was used for subsequent kinetic studies.

At each stage, the initial rate of DL-lactate uptake showed a relationship with DL-lactate concentration best described by the Michaelis–Menten equation (Fig. 2). Zygote uptake showed a K_m almost fourfold higher than blastocysts (Table 1) ($P = 0.03$ by linear regression). This trend of increased affinity with development was apparent in morulae, but was not significant ($P = 0.06$ by linear regression). V_{max} did not change during preimplantation development (Table 1). Furthermore, there was no difference in transport characteristics between embryos developing *in vivo* or *in vitro*, or between embryos cultured with and without glucose or receiving a glucose pulse (results not shown).

MCT1 and MCT3 expression

MCT1 protein (Fig. 3A–D) showed diffuse staining throughout the cytoplasm at all preimplantation stages, with apparently brighter intensity in the plasma membrane and outer cortical region of zygotes and two-cell embryos. In blastocysts, the cytoplasm of both inner cell mass (ICM) and trophectoderm (TE) were richly stained for MCT1. Normal goat serum controls for MCT1 (Fig. 3K and L) revealed no specific autofluorescence or immunofluorescence. Immunoreactivity for MCT3 (Fig. 3E–H) was not different from normal rabbit serum-treated control embryos (Fig. 3I and J), suggesting that MCT3 is not expressed during preimplantation development. In agreement with protein expression data, MCT1 mRNA was detected in zygotes, two-cell embryos, morulae and blastocysts (Fig. 4A), while MCT3 mRNA was not detected in embryos (Fig. 4B).

Western blotting demonstrated that both MCT antibodies were monospecific with molecular mass of

Table 1 V_{max} and K_m of DL-lactate transport in embryos.

	Zygote	Two-cell embryo	Morula	Blastocyst
V_{max} (pmol/embryo/5 min)	4.0 ± 1.1	2.4 ± 0.9	2.1 ± 0.3	2.8 ± 0.6
K_m (mmol/l)	87 ± 35*	47 ± 30	30 ± 10	20 ± 10*

* $P = 0.03$; data calculated from Fig. 2.

approximately 45 kDa for MCT1 (Fig. 5A) and 51 kDa for MCT3 (Fig. 5B).

Effect of culture on MCT expression

Embryos cultured from 18 h after hCG were selected at 48, 72 and 96 h after hCG and pooled for RNA extraction and RT–PCR, or fixed for immunolocalisation of MCT1 and MCT3. Protein localisation was not changed by 30 and 54 h of culture (Fig. 6A–C). After 78 h, cultured blastocysts demonstrated a more diffuse distribution of MCT1 in the cytoplasm of TE and ICM cells. However, staining was present in nuclei in direct contrast to cleavage-stage embryos, and blastocysts that had developed *in vivo* (compare Fig. 3D with Fig. 6C). MCT1 mRNA was still detected in embryos after 30, 50 and 78 h of culture (Fig. 6D). MCT3 protein was not present in cultured embryos, and mRNA was not detected at any stage (results not shown).

Effect of glucose on MCT expression in vitro

Zygotes (18 h after hCG) were randomly assigned for culture in KSOM or KSOM–G. Half of the KSOM–G embryos received a glucose ‘pulse’ at 58–60 h after hCG (four-cell stage). Morulae (90 h after hCG) were selected and either fixed and stained for MCT1 and MCT3, or pooled in groups of 100 embryos for RNA extraction and RT–PCR. MCT1 protein appeared to be expressed at similar intensity in all three culture groups at 90 h after hCG (Fig. 7A–D). However, in the complete absence of glucose, mRNA for MCT1 was not detected in the morulae. This loss of MCT1 mRNA expression in the absence of glucose was rescued by a glucose ‘pulse’ (Fig. 7E). MCT3 protein was absent from embryos in all three culture conditions, and MCT3 mRNA was not expressed (results not shown).

The discrepancy in protein and mRNA expression for MCT1 in the absence of glucose prompted follow-on culture experiments with and without glucose to investigate whether MCT1 protein persisted in the absence of glucose and RNA message. Immunoreactivity for MCT1 diminished in the absence of glucose at 105 and 112 h after hCG (Fig. 8E–H) coincident with death of the morulae (Fig. 8H). Interestingly, as blastocysts expanded and hatched in the presence of glucose, MCT1 expression became more intense in the polar TE (Fig. 8B–D). Nuclear MCT1, usually detected 96 h after hCG, was absent by 112 h (Fig. 8D).

Figure 2 DL-Lactate uptake kinetics in mouse embryos. At each stage, the initial rate of DL-lactate uptake (over 5 min) as a function of DL-lactate concentration was fitted by the least-squares analysis to the Michaelis–Menten equation for oocytes (solid square), two-cell embryos (solid triangle), morulae (inverted solid triangle) and blastocysts (open triangle). Each point represents mean ± S.E.M. for 12 experiments; $n = 27$ observations per data point.

Figure 3 MCT protein expression in freshly collected mouse embryos. Freshly collected embryos (24, 48, 72 and 96 h after hCG) treated with goat antimouse MCT1 (A–D) or with rabbit antimouse MCT3 (E–H) antisera followed with appropriate secondary antibodies. Immunoreactivity for MCT1 is diffuse in the cytoplasm throughout preimplantation development (A–D) but more intense in the outer cortex of zygotes (A) and two-cell embryos (B). MCT3 (E–H) demonstrates a pattern and intensity of immunoreactivity that is similar to normal serum controls, suggesting that protein may not be present in the embryo. No background immunofluorescence or autofluorescence is evident in normal rabbit serum (I and J) or normal goat serum (K and L) controls. Magnification $\sim \times 200$.

Figure 4 MCT mRNA expression in freshly collected mouse embryos. Freshly collected embryos (24, 48, 72 and 96 h after hCG) were pooled in groups of 100 for RT–PCR. MCT1 mRNA was detected throughout development (A), consistent with protein expression data, while MCT3 mRNA could not be detected (B).

Figure 5 Western blots of MCT antisera. Western blots for both MCT antisera on mouse tissues. Antisera demonstrated monospecificity, with single bands corresponding to molecular mass around 45 kDa for MCT1 (A) and 51 kDa for MCT3 (B).

Figure 6 Effect of embryo culture on MCT expression. Zygotes (18 h after hCG) were cultured in KSOM. Two-cell embryos, morulae and blastocysts were sampled at 48, 72 and 96 h after hCG respectively for immunohistochemical analysis with MCT1 antisera (A–C). Magnification $\sim \times 300$. MCT1 was found in the nucleus of cells in the TE and ICM of blastocysts (C), a phenomenon not observed for blastocysts derived *in vivo*. No other changes in protein expression were apparent between *in vitro* and *in vivo* derived embryos for MCT1. Some cultured embryos were pooled in groups of 100 for RT–PCR. MCT1 mRNA was expressed throughout *in vitro* preimplantation development (D).

Figure 7 Effect of glucose on MCT1 expression in cultured mouse morulae. Zygotes (18 h after hCG) were cultured in KSOM or KSOM-G, or received a glucose 'pulse' at 58–60 h after hCG. Compact morulae were selected at 90 h after hCG for immunohistochemical analysis of MCT1 (A–C) or normal serum control (D), or were pooled in groups of 100 for RT-PCR to detect MCT1 (E). Without glucose, MCT1 protein appears to be expressed at similar intensity at 90 h after hCG (B) in embryos cultured with glucose (A), or receiving a glucose 'pulse' (C). Magnification $\sim \times 300$. MCT1 mRNA was not detected in morulae cultured in the absence of glucose, but was present in morulae exposed to glucose either continuously or via a brief glucose pulse (E).

Discussion

Expression of MCT during preimplantation development

We have used RT-PCR to demonstrate, for the first time, MCT protein expression and localisation in embryos. While MCT1 mRNA and protein are expressed from the zygote to the blastocyst stage, MCT3 could not be detected as mRNA or protein. Expression of MCT1, MCT2 and MCT4 mRNA has previously been demonstrated throughout preimplantation development in the mouse (Harding *et al.* 1999, Herubel *et al.* 2002). However, there is conflict over the expression of MCT3 mRNA in developing mouse embryos. While Harding *et al.* (1999) report mRNA expression for MCT3, our results confirm those of Herubel *et al.* (2002), who found that MCT3 is absent during this period of development.

MCT1 is ubiquitously distributed in tissues (Garcia *et al.* 1994, Jackson *et al.* 1997, Wilson *et al.* 1998). It can catalyse the exchange of one monocarboxylate for another, or net transport of a monocarboxylate along with a proton in either direction across the cell membrane (Halestrap & Meredith 2004). This suggests that MCT1 plays an important role in regulating intracellular lactate and pyruvate to

balance nutrient, pH and redox status. Pyruvate is required for the first cleavage division in mouse embryos (Biggers *et al.* 1967, Leese & Barton 1984), so the appearance of MCT1 in early preimplantation development was not surprising.

While MCT1 is generally localised to the plasma membrane (Philp *et al.* 1998, 2003a, 2003b, Bergersen *et al.* 1999, Fanelli *et al.* 2003, Clamp *et al.* 2004), tissue fractionation has revealed MCT1 in sarcoplasmic reticulum, T-tubules, triads, and intracellular membranes of rat heart and skeletal muscle (Bonen *et al.* 2000) and in rat liver peroxisomes (McClelland *et al.* 2003). These data are consistent with our finding that MCT1 immunoreactivity, while it appears brighter in the cytoplasmic cortex of cleavage-stage embryos, persists diffusely in the cytoplasm of morulae and early blastocysts. This suggests that MCT1 may reside in a cytoplasmic vesicular pool, possibly in mitochondria or peroxisomes.

Cytoplasmic staining of MCT1 appears to be less diffuse in expanding blastocysts, with clear localisation to the cytoplasmic cortex. This suggests that MCT1 becomes more important for rapid monocarboxylate exchange at this stage of development. While present throughout ICM and TE cells, the highest intensity of MCT1

Figure 8 Effect of glucose on MCT1 expression in cultured mouse embryos. Zygotes (18 h after hCG) were cultured in KSOM (A–D) or KSOM–G (E–H). Embryos were collected at 90, 98, 105 and 112 h after hCG for immunohistochemical analysis of MCT1. Culture in the presence of glucose at 90 (A), 98 (B), 105 (C) and 112 h after hCG (D) demonstrates that immunoreactivity to MCT1 protein becomes more intense at the polar TE in expanding and hatching blastocysts (arrows: B–D) with loss of nuclear localisation (D). Without glucose, MCT1 immunoreactivity diminishes from 90 (E) and 98 h after hCG (F) to barely detectable levels at 105 (G) and 112 h after hCG (H), concurrent with a characteristic failure to develop to a blastocyst (G) and subsequent degeneration (H). Magnification $\sim \times 300$.

immunoreactivity is in the polar TE. Glucose utilisation via glycolysis in blastocysts results in lactic acid accumulation in cells and in the blastocoel cavity (Brison *et al.* 1993). In the mouse, the polar TE contacts the uterine wall before further differentiation of the TE into cytotrophoblast and invading syncytiotrophoblast. Polarisation of MCT1 to this region around the time of implantation may optimise rapid exchange of pyruvate/lactate with the developing fetal-maternal circulation. Indeed, MCT1 has been localised to the basement membrane of human term placenta, and is thus well positioned for a role in fetal-maternal exchange (Settle *et al.* 2004). Efflux of H^+ would also enable pH homeostasis for continued development, and prevent inhibition of glycolytic enzymes (e.g. 6-phosphofructokinase) (Barbehenn *et al.* 1974, Kusen *et al.* 1975), thereby allowing glycolysis to continue.

MCT3 transports lactate with higher affinity than pyruvate and is considered to be important in pH, nutrient and fluid homeostasis in retinal pigment epithelium (Yoon *et al.* 1997, Philp *et al.* 1998, 2001, 2003a). The retina, like blastocysts, predominantly metabolises glucose via glycolysis and achieves the vectorial transport of fluid and metabolites by development of tight junctions between epithelial cells, and polarised distribution of transport proteins (Philp *et al.* 1998, 2003a). MCT3 exists on the basolateral membrane, and MCT1 on the apical membrane of retinal epithelial cells for the cooperative, unidirectional transport of excess lactate from the subretinal space to the systemic circulation (Philp *et al.* 1998). We proposed that

blastocysts might have a similar polarised distribution of MCT1 and MCT3 to export lactate from the blastocoel cavity and ICM to the external environment. MCT3 has not been located outside the retina, except for reports of mRNA expression in preimplantation embryos (Harding *et al.* 1999).

Immunohistochemical analysis of MCT3 revealed immunoreactivity at a level similar to normal rabbit serum controls, indicating that this protein isoform may not be present in early mouse embryos, or that it is expressed at much lower levels than MCT1. This finding is supported by the absence of MCT3 mRNA and is consistent with the findings of Herubel *et al.* (2002).

Lactate uptake in preimplantation embryos

Lactate transport via MCT involves the initial binding of a proton followed by the lactate anion, which are then translocated across the membrane and subsequently released. This process is freely reversible (Halestrap & Price 1999). In these experiments, lactate uptake reached a plateau as quickly as 12 min in some embryos, at concentrations of 5 and 25 mmol DL-lactate/l. This is probably due to bidirectional transport via the MCT according to the following formula: $[\text{lactate}]_{\text{in}}/[\text{lactate}]_{\text{out}} = [H^+]_{\text{out}}/[H^+]_{\text{in}}$. It is also possible that a low pH_i or increasing intracellular lactate generated by H^+ /lactate influx decreased the rate of DL-lactate uptake to effect a steady state by this time.

The results show that MCT transport in preimplantation embryos is concentration dependent and follows Michaelis–Menten kinetics. The decrease in K_m with constant V_{max} during development contrasts with an earlier study which used fluorescent pH fluctuations as a measure of lactate transport (Harding *et al.* 1999). MCT kinetics in mouse embryos were reported to demonstrate a constant K_m with an increase in V_{ma} during development that was significant on regression analysis ($P < 0.01$) (Harding *et al.* 1999). One explanation for this difference is that the previous study used a medium in which all anions had been replaced with the impermeant anion gluconate, causing H^+ efflux and a subsequent increase in pH_i. It is known that creating high pH_i with respect to the extracellular environment increases the V_{max} of MCT transport (Halestrap & Price 1999), and this may have amplified any minor developmental changes in V_{ma} to show a significant effect.

With the [3H]DL-lactate uptake assay at physiological pH (7.4) and in the presence of 25 mmol/l pyruvate, blastocysts demonstrated a significantly lower K_m than earlier preimplantation stages. At physiological concentrations of around 5 mmol lactate/l (Gardner & Leese 1990) and 10 mmol lactate/l in standard KSOM (Lawitts & Biggers 1993), blastocysts would be almost twice as active in lactate uptake as cleavage-stage embryos. There is evidence that increasing lactate concentrations in culture causes a decrease in V_{ma} for pyruvate uptake at all preimplantation stages, and this has been attributed to competition by both substrates for MCT (Lane & Gardner 2000). The effect was more marked in the blastocyst (Lane & Gardner 2000). This observation, and the reduced K_m of blastocysts indicating increased affinity for lactate, may reflect the concentration of MCT1 to the polar TE of expanding and hatching blastocysts. However, changes in the affinity or K_m , but not V_{max} , as the embryos develop, is more likely to suggest a variation in the expression of other MCT protein isoforms at later preimplantation stages, although this has not yet been reported, and no evidence for MCT3 was obtained. Both PCR and immunohistochemical data support the observation that there are no differences in the molecular characteristics of MCT transport between embryos developing *in vivo* and *in vitro*.

Effect of glucose on MCT expression

The persistent uptake of DL-lactate demonstrates net activity of MCT throughout preimplantation development. These data complement observations that lactate and pyruvate may sustain blastocyst formation when embryos are cultured from the two-cell stage in the absence of glucose (Martin & Leese 1995). However, while culture in glucose-free medium from the eight-cell stage did not appear to affect the activity of MCT (Harding *et al.* 1999), previous studies demonstrated earlier effects of glucose on pyruvate metabolism and preimplantation embryo physiology (Chatot *et al.* 1989, Brown & Whittingham 1991,

Martin & Leese 1999, Pantaleon *et al.* 2001a, 2001b). In this investigation, culture in the absence of glucose from the zygote stage reduced MCT1 mRNA expression by 90 h after hCG, with a subsequent decrease in MCT1 protein levels. Embryos receiving a brief exposure to glucose around the four-cell stage of development, however, retained their ability to express MCT1 in a subsequent glucose-free environment. Unfortunately, these observations were not supported by a significant difference in lactate transport under varying conditions of glucose exposure, probably because of a lack of sensitivity in this approach.

The DL-lactate uptake assay did not detect a change in net lactate uptake rates, probably because of the high variance of the V_{max} estimates. Mixed D- and L-isomers were used in the assay, and this may have contributed to the difficulty in detecting small differences in uptake rates. MCT1 is stereospecific for lactate, and K_m for the L-isomer is an order of magnitude lower than for the D-isomer (Broer *et al.* 1998, Manning Fox *et al.* 2000). Of the available lactate in the media, 50% was D-lactate, possibly masking any measurable changes in K_m . The co-expression of other MCT isoforms cannot be excluded, and this may also have affected net lactate uptake. The transport assay employed could not resolve the contribution of multiple MCT isoforms.

Nevertheless, results are consistent with metabolic studies reporting that embryos deprived of glucose from the zygote stage demonstrate a continual decline in both glucose and pyruvate utilisation from around 90 h after hCG until they eventually degenerate (Martin & Leese 1995, 1999). The data also complement studies that demonstrate a similar glucose-activated expression of GLUT3, a high-affinity glucose transporter. Taken together, these results support the hypothesis that glucose acts as a metabolic signal to cleavage-stage embryos, permitting the expression of metabolic enzymes required for blastocyst formation (Chatot *et al.* 1994, Pantaleon *et al.* 1997a, Pantaleon & Kaye 1998).

Conclusion

This investigation confirms the presence of MCT1 and absence of MCT3 mRNA during preimplantation development, and demonstrates MCT1 (but not MCT3) protein localisation for the first time in embryos. Cleavage-stage exposure to glucose is required for persistent MCT1 expression. Culture without glucose from the zygote stage leads to complete absence of MCT1 mRNA and a gradual decline in protein coincident with degeneration. Embryos cultured with glucose, or briefly exposed to glucose at the four-cell stage, retain MCT1 mRNA and protein expression. These data are consistent with previous observations of transporter expression and pyruvate utilisation in the presence and absence of glucose, and support the hypothesis that glucose is important as a signalling molecule for metabolic differentiation in the preimplantation embryo.

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