Obstetrics & Gynaecology Publications

Obstetrics & Gynaecology Department

11-1-2011

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REPRODUCTION

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Abstract

During oogenesis, mammalian oocytes accumulate maternal mRNAs that support the embryo until embryonic genome activation. RNA-binding proteins (RBP) may regulate the stability and turnover of maternal and embryonic mRNAs. We hypothesised that varying embryo culture conditions, such as culture medium, oxygen tension and MAPK inhibition, affects regulation of RBPs and their targets during preimplantation development. STAU1, ELAVL1, KHSRP and ZFP36 proteins and mRNAs were detected throughout mouse preimplantation development, whereas *Elavl2* mRNA decreased after the two-cell stage. Potential target mRNAs of RBP regulation, *Gclc*, *Slc2a1* and *Slc7a1* were detected during mouse preimplantation development. *Gclc* mRNA was significantly elevated in embryos cultured in Whitten's medium compared with embryos cultured in KSOMaa, and *Gclc* mRNA was elevated under high-oxygen conditions. Inhibition of the p38 MAPK pathway reduced *Slc7a1* mRNA expression while inhibition of ERK increased *Slc2a1* mRNA expression. The half-lives of the potential RBP mRNA targets are not regulated in parallel; *Slc2a1* mRNA displayed the longest half-life. Our results indicate that mRNAs and proteins encoding five RBPs are present during preimplantation development and more importantly, demonstrate that expression of RBP target mRNAs are regulated by culture medium, gas atmosphere and MAPK pathways.

Reproduction (2011) **142** 689–698

Introduction

The embryonic culture environment exerts a profound influence on the developmental capacity and gene expression patterns of mammalian preimplantation embryos (Ho *et al*. 1994, Niemann & Wrenzycki 2000, Watson *et al*. 2000). There is increasing use of longer culture intervals for the production of human blastocysts *in vitro* (Jones & Trounson 1999) but there are concerns that assisted reproductive technologies and embryo culture are associated with increased early embryo loss, foetal growth abnormalities, disruptions in imprinting, alterations in behaviour and enhanced susceptibility to disease in later life (Sinclair *et al*. 1999, Fernandez-Gonzalez *et al*. 2004, Watkins *et al*. 2007, Owen & Segars 2009).

The primary mechanisms that are proposed to understand culture-induced changes in embryonic gene expression patterns include alterations in transcription and/or epigenetic reprogramming (Doherty *et al*. 2000, Niemann & Wrenzycki 2000, Rizos *et al*. 2003, Watkins *et al*. 2007). We suggest that at least one additional

regulatory mechanism exists that has not been largely considered to date. While it is known that mRNA deadenylation/polyadenylation occurs during oocyte maturation (Brevini-Gandolfi *et al*. 1999), only a very few studies have begun to examine the influences of varying culture environments on embryo mRNA adenylation and mRNA stability (Ho *et al*. 1994).

RNA-binding proteins (RBP) influence RNA editing, localisation, stability and translation (Saunders & Barber 2003). Regulated mRNA stability occurs in mammalian cells in response to nutrient levels, hormones and environmental stresses such as hypoxia and heat stress (reviewed in Guhaniyogi & Brewer (2001)). There has been little research on the roles of RBPs in mammalian germ cells, oocytes or early embryos. Staufen was first identified in the *Drosophila* oocyte as an RBP involved in establishing anterior–posterior axis specification (St Johnston *et al*. 1991), and has a role in mRNA decay (Kim *et al*. 2005). ELAVL1 protein (HuR) is a widely expressed mammalian RBP (Fan & Steitz 1998) whose localisation is regulated by various stresses

(Yaman *et al.* 2002, Song *et al.* 2005) involving the p38 MAPK pathway (Tran *et al.* 2003, Song *et al.* 2005). ELAVL1 protein binds to AU-rich elements (ARE) and increases the stability (Fan & Steitz 1998) and translation of ARE-containing mRNAs (Peng *et al.* 1998). Similarly, ELAVL2 (HuB) also binds ARE, slows mRNA degradation and may increase protein translation (Jain *et al.* 1997). ZFP36 (tristetraprolin, TTP) is an RBP whose activity is regulated by p38 MAPK (Brook *et al.* 2006). ZFP36 promotes deadenylation of ARE-containing mRNAs and increases their degradation rate (Lai *et al.* 2000). KH-type splicing regulatory protein (KHSRP) is another RBP regulated by MAPK that degrades ARE-containing mRNAs (Briata *et al.* 2005). These studies suggest that several RBPs may mediate environmental influences on transcript stability and translation via the p38 MAPK pathway.

We examined the effects of good and poor culture environments on mRNA abundance and stability. The effects of the culture environment may be communicated to the embryo by the activation of pathways such as the p38 MAPK pathway (Natale *et al.* 2004). Our laboratory has discovered that the p38 MAPK pathway is activated in embryos in response to osmotic stress (Fong *et al.* 2007). In the current studies, we investigated whether culturing embryos in a good medium (KSOMaa, 0.2 mM glucose and amino acids) or poor medium (Whitten's medium containing 5.55 mM glucose and no amino acids) affects RBP expression and the expression of ARE-containing target genes. Development, the number of ICM cells and total cell number in blastocysts is reduced in embryos cultured in Whitten's medium compared with KSOMaa (Rinaudo *et al.* 2006). Our second model of environmental stress examined the effect of incubator oxygen tension on mRNA abundance and stability. Mouse studies have shown for some years (Harlow & Quinn 1979), but more recently human studies have also shown improved developmental and pregnancy rates (Kovacic & Vlaisavljević 2008, Meintjes *et al.* 2009, Waldenström *et al.* 2009) when cultured under a lower oxygen tension (5%) than conventionally used (20%). Furthermore, high oxygen tension greatly increases the number of genes aberrantly expressed in the mouse embryo (Rinaudo *et al.* 2006).

For these reasons, we have focused our studies on characterising the expression of STAU1, ELAVL1, ELAVL2, KHSRP and ZFP36 RBPs at the mRNA and protein level, during preimplantation development in the mouse. Three RBP target mRNAs were selected, as all are identified as targets of ELAVL1 in other tissues (Yaman *et al.* 2002, Song *et al.* 2005, Gantt *et al.* 2006). γ-Glutamylcysteine ligase catalytic unit (GCLC) is a key enzyme involved in synthesis of glutathione, which reacts to cellular stresses and deactivates destructive reactive oxygen species (Meister 1994). SLC2A1 (GLUT1) is a glucose transporter that is expressed in the preimplantation embryo (Moley *et al.* 1998). SLC7A1

(CAT1) is an arginine/lysine transporter involved in both trophoblast outgrowth and signalling for implantation (Martin *et al.* 2003).

Our results indicate that mRNAs and proteins encoding these five RBPs are present during preimplantation development. Importantly, their target mRNAs are affected by culture environment. These results establish a foundation for investigating the roles of RBPs during early development and more importantly, their collective roles in regulating transcript stability and translation during early embryogenesis.

Results

Protein localisation

ELAVL1 protein was observed in the cytoplasm of oocytes and all embryo stages and was nuclear in some embryos at all stages examined (Fig. 1). ELAVL2 protein immunofluorescence was detected in the cytoplasm of oocytes and embryos at all stages examined (Fig. 1), ELAVL2 was frequently observed in the nuclei but not to the same extent as ELAVL1. KHSRP protein was detected across preimplantation development in both nuclei and cytoplasm (Fig. 1). ZFP36 protein was detected in the cytoplasm and nuclei at all stages throughout preimplantation development (Fig. 1), and included the unique feature of encircling the nucleus in a ring-like fashion. STAU1 protein immunofluorescence was localised to the cytoplasm and in some nuclei (Fig. 1). It was not unusual to also observe punctuate fluorescent foci in the cytoplasm that were suggestive of a non-uniform localisation. GCLC protein was predominantly localised to the cytoplasm at all stages examined (Fig. 2), but interestingly, was also associated with the spindle during mitosis (arrow). GCLC protein fluorescence increased at the blastocyst stage. SLC2A1 protein was quite low in early preimplantation stages but increased markedly at the blastocyst stage (Fig. 2), where it was often localised to cell membranes. SLC7A1 protein was barely detectable in early stages, but was clearly present in the trophectoderm basal membrane at the blastocyst stage (Fig. 2).

Developmental expression

Elavl1 mRNA levels were significantly higher at the blastocyst stage than at earlier developmental stages (Fig. 3A) as were *Gclc* (Fig. 3F), *Slc2a1* (Fig. 3G) and *Slc7a1* mRNAs (Fig. 3H). *Elavl2* mRNA levels were highest at the two-cell stage and were significantly lower at the eight-cell and morulae stages (Fig. 3B). For *Khsrp* (Fig. 3C), *Zfp36* (Fig. 3D) and *Stau1* mRNAs (Fig. 3E) there was no significant variance in mRNA levels across embryonic stages.
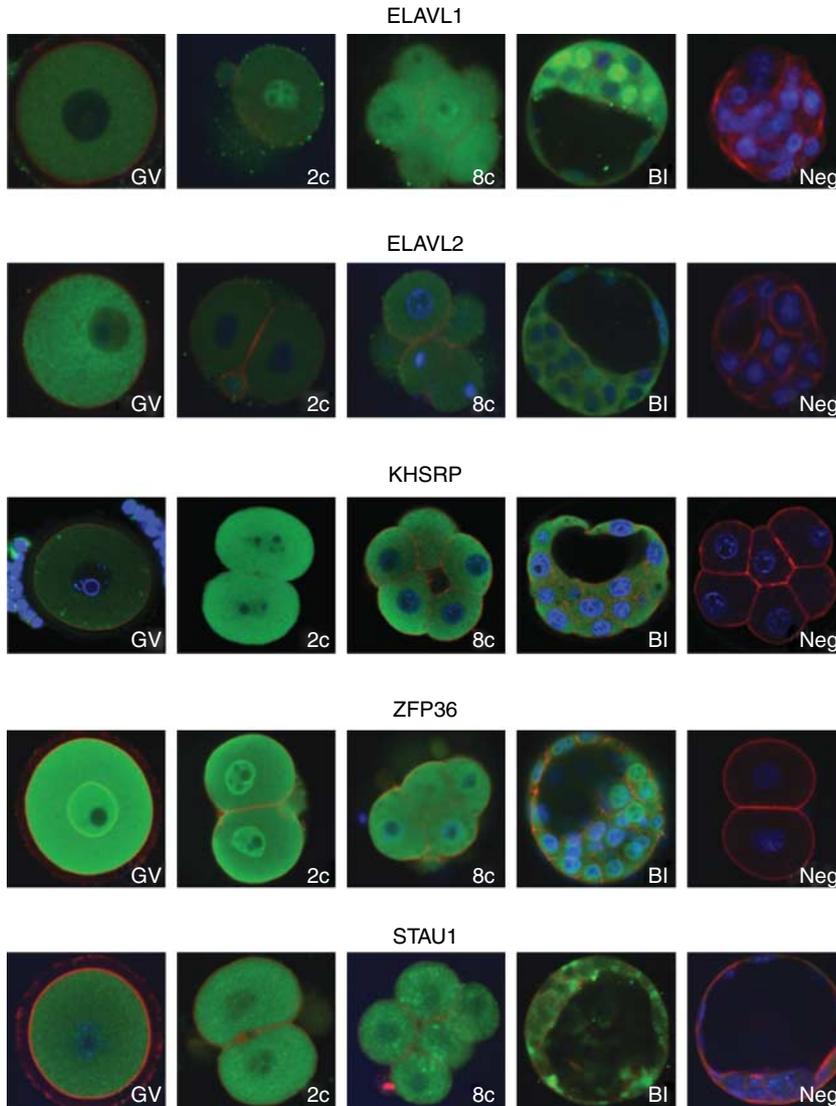
ELAVL1

ELAVL2

KHSRP

ZFP36

STAU1

Figure 1 Localisation of STAU1, ELAVL1, ELAVL2, KHSRP and ZFP36 proteins during mouse pre-implantation development. Green is RBP fluorescence. Red is rhodamine-phalloidin staining of F-actin. Blue is DAPI-stained DNA. GV, germinal vesicle stage; 2c, two-cell stage; 8c, eight-cell stage; Bl, blastocyst stage; Neg, negative control.

Influence of culture on RBP and RBP target expression

Culture medium

To test our hypothesis that culture environment would influence RBP expression and thus levels of their target mRNAs, we contrasted mRNA levels between embryos cultured in KSOMaa (good culture medium) and Whitten's medium (substandard medium). As in the developmental series, *Elavl1* mRNA levels were affected by stage ($P<0.001$, Fig. 4A), but there was no effect of medium or interaction. For *Khsrp* mRNA, there were no significant effects of media, stage or interaction (Fig. 4B). There was an effect of stage for *Zfp36* mRNA (Fig. 4C, $P<0.004$), expression was higher at the four-cell stage than the eight-cell and blastocyst stages, but no effect of medium or interaction. For potential RBP target mRNAs, there was a significant effect of medium on *Gclc* (Fig. 4D, $P<0.049$), mRNA levels were higher in

embryos cultured in Whitten's medium than in KSOMaa. For *Slc2a1*, there was a significant effect of stage (Fig. 4E, $P<0.001$), with mRNA levels higher in blastocysts and morulae than in four- and eight-cell stage embryos. For *Slc7a1* mRNA, due to low levels at other stages, we compared only blastocysts and there was no effect of medium (Fig. 4F, $P<0.12$).

Culture atmosphere

Gclc mRNA levels were significantly higher in the high-O_2 atmosphere than the low-oxygen atmosphere (Fig. 5A, $P<0.054$). Neither *Slc2a1* (Fig. 5C, $P<0.324$) nor *Slc7a1* mRNAs (Fig. 5E, $P<0.166$) were affected by gas atmosphere. There were no significant effects of gas atmosphere on any of the RBP mRNAs (data not shown), *Elavl1* ($P<0.742$), *Khsrp* ($P<0.145$) and *Zfp36* ($P<0.313$).
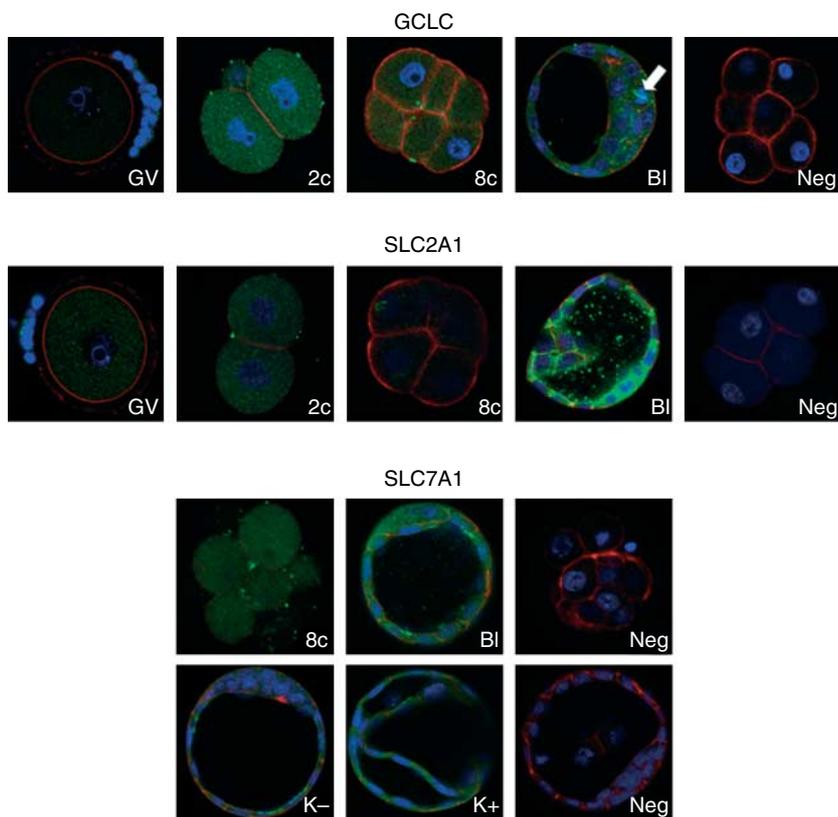
Figure 2 Localisation of GCLC, SLC2A1 and SLC7A1 proteins during mouse preimplantation development. Green is target protein fluorescence. Red is rhodamine-phalloidin stain for F-actin. Blue is DAPI-stained DNA. GV, germinal vesicle stage; 2c, two-cell stage; 8c, eight-cell stage; Bl, blastocyst stage; K−, amino acids withdrawn for 18 h; K+, amino acids present; Neg, negative control. Staining of the spindle in GCLC is indicated by the arrow.

mRNA half-life

Target gene mRNAs were investigated to determine whether the mRNAs decayed with similar kinetics under high- or low-oxygen conditions. For the *Gclc* regression line, the significance for the overall analysis was $P<0.001$, and the adjusted R^2 value was 0.558. For treatment (high versus low oxygen) the P value was 0.163, for hour was $P<0.001$, while the replicate was $P<0.001$ (Fig. 5B). *Gclc* mRNA half-life was ∼7 h. For the *Slc2a1* mRNA regression, there was an overall $P<0.001$, and the adjusted R^2 value was 0.410 (Fig. 5D). The P value for treatment was 0.734, for hour $P<0.001$ and for replicate, $P<0.001$. The half-life of *Slc2a1* mRNA was ∼10.5 h. For *Slc7a1* mRNA (Fig. 5F), there was an overall $P<0.001$ for the regression, and the adjusted R^2 value was 0.576. The P value for treatment was 0.079, for hour was $P<0.001$, and for replicate was $P<0.001$. The half-life for *Slc7a1* mRNA was ∼8.5 h.

Regulation by MAPKs

For the target genes, p38 MAPK inhibition with SB220025 significantly reduced *Slc7a1* mRNA (Fig. 6C, $P<0.001$). We also observed a trend towards decreased *Slc2a1* mRNA levels (Fig. 6B, $P<0.09$). P38 MAPK inhibition did not affect *Gclc* mRNA. When ERK1/2 MAPK activation was inhibited by UO126, *Slc2a1* mRNA levels significantly increased (Fig. 6E,

$P<0.04$). We observed no effect of the SP600225 drug (JNK/SAPK inhibition) on mRNA levels. Inhibition of the various MAPKs also did not significantly affect RBP mRNA levels (data not shown).

Discussion

We have discovered that mRNAs and proteins encoding five RBPs (STAU1, ELAVL1, ELAVL2, KHSRP and ZFP36) are present during mouse preimplantation development. *Elavl1* mRNA increased to the blastocyst stage, which agrees with qRT-PCR and microarray studies (Evsikov *et al*. 2004, Zeng *et al*. 2004). We report both cytoplasmic and nuclear protein expression in mouse embryos here that agrees with previous mouse and bovine studies (Evsikov *et al*. 2004, Calder *et al*. 2008). Although ELAVL1 protein localisation has been reported as mostly nuclear in several cell types (Fan & Steitz 1998, Peng *et al*. 1998) when activated by stress ELAVL1 moves to the cytoplasm (Peng *et al*. 1998) where it protects ARE-containing mRNAs from degradation (Fan & Steitz 1998). *Elavl2* mRNA decreased after the two-cell stage by qRT-PCR analysis, evidence of maternal expression, which agrees with qRT-PCR and microarray studies of preimplantation embryos (Evsikov *et al*. 2004, Zeng *et al*. 2004). ELAVL2 protein was expressed at all stages in the nuclear and cytoplasmic compartments, similar to what was been observed in bovine embryos (Calder *et al*. 2008). However, ELAVL2 protein apparently decreased
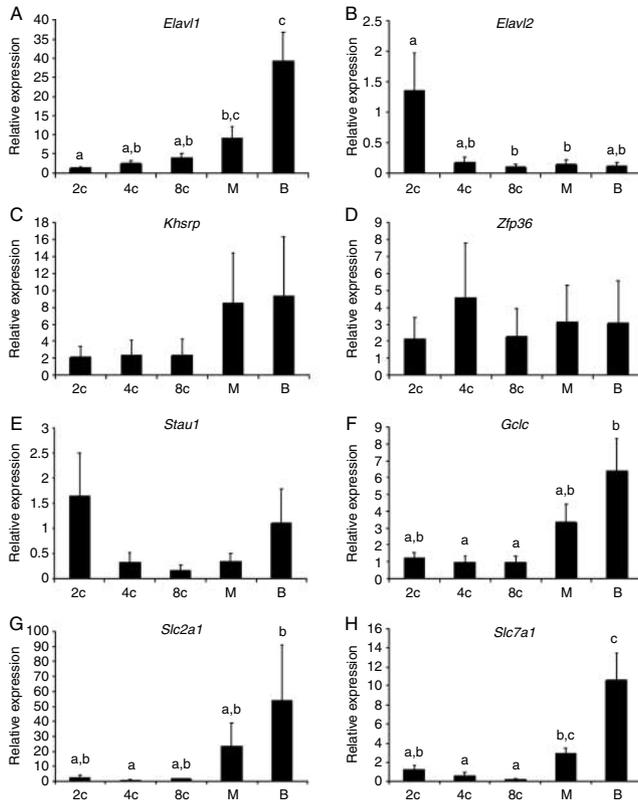
Figure 3 Expression of RBP mRNAs and potential target gene mRNAs by real-time qRT-PCR during mouse preimplantation development. A, *Elavl1*; B, *Elavl2*; C, *Khsrp*; D, Zfp36; E, *Stau1*; F, *Gclc*; G, *Slc2a1*; H, *Slc7a1*. 2c, Two-cell stage; 4c, four-cell stage; 8c, eight-cell stage; M, morulae stage; B, blastocyst stage. [a,b,c]Bars with no letters in common are significantly different. There were four independent replicates for each gene.

mRNA and protein only at the mouse blastocyst stage (Stover *et al.* 2000). The increase in *Slc2a1* mRNA (Zeng *et al.* 2004) and protein, especially at the cell membrane, at the blastocyst stage is consistent with earlier studies (Pantaleon *et al.* 2001) and with the timing of the change in embryo metabolism from lactate/pyruvate to glucose utilisation (Gardner & Leese 1986). *Slc7a1* is involved in both trophoblast outgrowth and in synthesis of nitric oxide for implantation (van Winkle 2001, Martin *et al.* 2003). Although the mRNA was reported to be detectable from two-cell to blastocyst stage previously (van Winkle 2001), here mRNA expression was observed to increase at the blastocyst and the protein began to be expressed at the basal membrane surrounding the blastocyst cavity.

Importantly, we discovered that culture conditions and MAPKs have a significant impact on the mRNA levels of these RBP targets. *Gclc* mRNA was significantly elevated in embryos cultured under high oxygen tension. Increased reactive oxygen species have been previously detected in embryos cultured under high-oxygen conditions (Goto *et al.* 1993). Expression of *Gclc* mRNA was also higher in Whitten's medium compared with embryos cultured in KSOMaa. Differences in *Gclc* mRNA expression may be linked to changes in metabolism leading to the generation of reactive oxygen species. *Slc2a1* mRNA was not significantly lower in
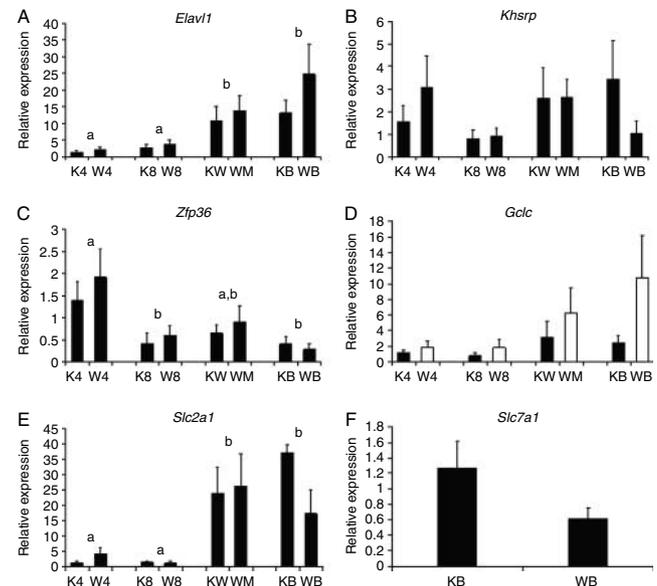
after the zygote stage in a previous study (Evsikov *et al.* 2004). As *Elavl2* mRNA decreases after the two-cell stage, it is likely that some maternal protein persists to the blastocyst stage. It has been suggested that these two ELAV proteins may control translation of maternal mRNAs in the mouse embryo (Evsikov *et al.* 2004).

Expression of KHSRP protein was detected here in mouse and bovine embryos (Calder *et al.* 2008) in both the cytoplasmic and nuclear compartments. ZFP36 protein was localised to the cytoplasm, nucleus and nuclear envelope of mouse embryos and had previously been reported to shuttle between the nucleus and cytoplasm in other tissues (Phillips *et al.* 2002). STAU1 protein is both nuclear and cytoplasmic here in mouse embryos and in bovine embryos (Calder *et al.* 2008). The presence of these three RBPs in the embryo suggests roles in mRNA decay as reported in other tissues (Lai *et al.* 2000, Briata *et al.* 2005, Kim *et al.* 2005).

RNAs of three known targets of ELAVL1 regulation, *Slc7a1*, *Gclc* and *Slc2a1* (Yaman *et al.* 2002, Song *et al.* 2005, Gantt *et al.* 2006), which contain 3′ UTR-like ARE sequences, all increased in expression at the blastocyst stage. Our data are consistent with detection of *Gclc*

Figure 4 Influence of a poor or more optimised culture medium on RBP and target mRNA expression by real-time qRT-PCR during mouse preimplantation development. A, *Elavl1*; B, *Khsrp*; C, *Zfp36*; D, *Gclc*; E, *Slc2a1*; F, *Slc7a1*. K, KSOMaa; W, Whitten's medium. 4c, Four-cell stage; 8c, eight-cell stage; M, morulae stage; B, blastocyst stage. Different coloured bars indicate a significant effect of medium. [a,b]A significant difference between stages is indicated by bars without a letter in common (P<0.05). There were four replicates for except *Slc2a1*, where there were three replicates. For *Slc7a1*, only blastocysts are compared, there were five replicates.
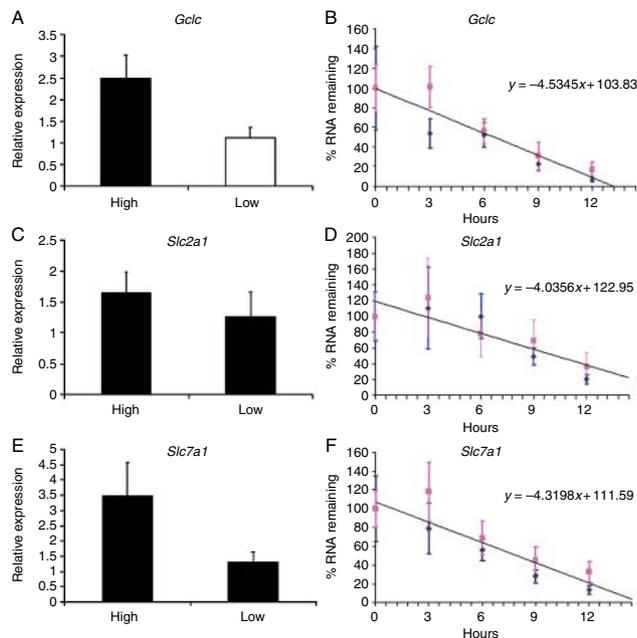
Figure 5 Influence of culture under a high- (H, 20%) or low-oxygen (L, 5%) atmosphere on target mRNA expression and decay using real-time qRT-PCR. mRNA abundance A, *Gclc*; C, *Slc2a1*; E, *Slc7a1*. There were six independent replicates, except for *Slc7a1* mRNA where $N=7$. Different coloured bars indicate a significant effect of oxygen, $P<0.054$. mRNA decay B, *Gclc*; D, *Slc2a1*; F, *Slc7a1*. There were five replicates. The pink square symbols are low oxygen and the blue diamond symbols are high oxygen mean and standard error. The average decay rate is indicated by the black line and the calculation for slope is indicated.

embryos cultured in Whitten's medium containing higher glucose than KSOMaa. Earlier studies demonstrated lower expression of *Slc2a1* mRNA and protein in blastocysts developing in diabetic compared with normoglycaemic mice (Moley *et al.* 1998). There were no effects of oxygen tension on *Slc2a1* mRNA, similar to previous results in mouse embryos (Kind *et al.* 2004). Surprisingly, *Slc7a1* mRNA was not expressed at different levels between embryos cultured in KSOM that contains amino acids, compared with Whitten's medium that is not supplemented with amino acids. Amino acids are necessary at ~120 h post human chorionic gonadotrophin (hCG) for blastocyst trophoblast outgrowth to occur (Martin *et al.* 2003).

Target gene mRNAs were examined to determine whether the decay kinetics were similar under high- or low-oxygen conditions. Pipetting embryos can increase MAPK activation (Xie *et al.* 2007), and α-amanitin may not completely block mRNA transcription until 2 h of treatment (Kidder *et al.* 1985), which may explain why some transcript levels are higher at the 3 h timepoint than at 0 h. Nonetheless, *Slc7a1* mRNA tended to decay faster in the higher oxygen environment. Our calculations for mRNA half-lives for *Gclc*, *Slc2a1* and *Slc7a1* were all longer than those calculated from a microarray

study of mouse ES cells (Sharova *et al.* 2009). However, the half-life for *Gclc* mRNA in our study is similar to that in colorectal cells of 6.3–6.8 h (Song *et al.* 2005). The half-life for *Slc2a1* mRNA in embryos was twice as long as for ES cells.

Our laboratory has shown that treatment of early embryos with p38 MAPK inhibitors resulted in a blockade of early development (Natale *et al.* 2004), in addition, we demonstrated that p38 MAPK regulates the embryo's response to osmotic stress (Fong *et al.* 2007). Furthermore, ELAVL1, KHSRP and ZFP36 are all regulated by the p38 MAPK pathway (Brook *et al.* 2006, Tran *et al.* 2003, Briata *et al.* 2005). Therefore, we tested the effects of inhibitors of three different MAPK pathways on RBP and target mRNA expression. MAPK inhibitors had no effects at the mRNA level of these RBPs, however, RBPs are usually regulated at the protein level by phosphorylation or changing the nuclear/cytoplasmic localisation (Brook *et al.* 2006, Tran *et al.* 2003, Briata *et al.* 2005). *Slc7a1* mRNA was significantly reduced by p38 MAPK inhibitor but not by the other MAPK inhibitors. This observation is in agreement with earlier research demonstrating that induction of *Slc7a1* mRNA was reduced by inhibiting the p38 pathway (Baydoun *et al.* 1999). *Slc2a1* mRNA expression increased when the ERK MAPK pathway was inhibited.

In conclusion, mRNAs and proteins encoding STAU1, ELAVL1, ELAVL2, KHSRP and ZFP36 are expressed during preimplantation development. These RBPs regulate mRNAs via effects on stability, translation or repression. Several studies that contrasted mRNA levels in embryos cultured under different conditions have suggested that transcriptional activity largely governs changes in mRNA abundance (Niemann & Wrenzycki 2000, Rizos *et al.* 2003). However, mRNA levels are a balance between rate of transcription and the rate of degradation (Guhaniyogi & Brewer 2001). Our results have initiated studies directed towards an eventual understanding of the role RBPs play in controlling mRNA turnover and stability during mammalian oocyte maturation and early embryogenesis.

Materials and Methods

Mouse embryos and developmental series

Four-week-old female MF-1 mice and adult male CD-1 mice were obtained from Charles River Laboratories International, Inc. (Wilmington, MA, USA; CD-1) or Harlan Laboratories, Inc. (Indianapolis, IN, USA; MF-1). Females were injected with 7.5 IU pregnant mare serum gonadotrophin (PMSG; Folligon, Intervet, Whitby, ON, Canada) to stimulate follicular development, followed by 7.5 IU hCG (Chorulon, Intervet) 46–48 h after PMSG and placed with a male for mating, day of copulation plug is considered D1. Germinal vesicle (GV) stage oocytes were collected 48 h after PMSG. Ovaries were removed and placed into M2 medium (Sigma). Large follicles were punctured with 30 g needles and large oocytes with
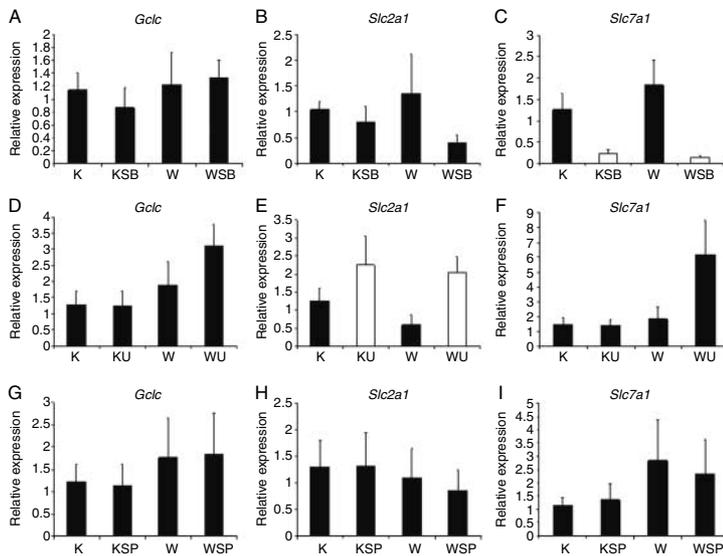
Figure 6 Influence of culture in KSOMaa (K) or Whitten's medium (W) and MAPK inhibitors on target mRNA expression measured by qRT-PCR. (A–C) DMSO control or p38 inhibitor SB220025 (SB) A, *Gclc*; B, *Slc2a1*; C, *Slc7a1*. (D–F) DMSO control or inhibitor of ERK1/2 activation, UO126 (U) D, *Gclc*; E, *Slc2a1*; F, *Slc7a1*. (G–I) DMSO control or JNK/SAPK inhibitor SP600225 (SP) G, *Gclc*; H, *Slc2a1*; I, *Slc7a1*. K, KSOMaa, W, Whitten's medium. Different coloured bars indicate a significant effect of the MAPK inhibitor. There were five replicates for all except *Slc2a1* with the p38 inhibitor, where there were four replicates.

compact GV were collected, vortexed to remove cumulus and washed extensively. Later developmental stages were collected by flushing two-cell embryos from oviducts with M2 on D2 and placing embryos into culture in KSOMaa (Millipore, Phillipsburg, NJ, USA) at 37 °C in 5% O_2, 5% CO_2 and 90% N_2 atmosphere. Two-cell embryos were collected at 46–48 h post hCG, four-cell embryos at 66 h, eight-cell embryos at 72 h, morulae at about 90 h and blastocysts at 96–100 h. Animal care and handling was according to the guidelines of the University of Western Ontario Animal Care Committee approved by the Canadian Council on Animal Care.

Whole-mount immunofluorescent confocal microcopy

For immunofluorescent microscopy, pools of embryos at the different stages were fixed in 2% paraformaldehyde in PBS for 20 min at room temperature and stored in PHEM (60 mM PIPES, 25 mM HEPES, 10 mM EGTA and 1 mM $MgCl_2$-$6H_2O$) buffer at 4 °C. For immunostaining, fixed embryos were permeabilised and blocked in $1\times$ PBS $+5\%$ goat or donkey serum (Cedarlane, Burlington, ON, Canada) 0.01% Triton X-100 for 1 h at room temperature (Madan *et al*. 2007). Rabbit polyclonal anti-human STAU1 (1:100), ELAVL1 (1:50) and ELAVL2 (1:100) antibodies were obtained from Chemicon (Temecula, CA, USA). Anti-KHSRP (1:100) was obtained from Cedarlane and anti-ZFP36 (1:100), anti-SLC2A1 (1:100) and anti-GCLC (1:50) antibodies were obtained from Abcam (Cambridge, MA, USA). Two anti-SLC7A1 (CAT1) antibodies were assayed, the first was a rabbit anti-mouse polyclonal used at 1:50 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; sc-66825). The second was rabbit anti-human polyclonal antibody used at 1:50, obtained from Proteintech (Chicago, IL, USA, 14195-1-AP). Embryos were counterstained with rhodamine-phalloidin to stain actin and DAPI to stain DNA (Madan *et al*. 2007). Negative control embryos were not exposed to primary antibody. Embryos were examined by confocal laser scanning microscopy (Olympus FV1000, Olympus Canada, Inc., Markham, ON, Canada).

Quantitative PCR-RNA extraction and RT

Total RNA was extracted from pools of 50 frozen embryos (two-, four- and eight-cells, morulae and blastocyst), using a Picopure kit (Arcturus, Mountain View, CA, USA). There were four distinct replicates to demonstrate mRNA expression across preimplantation development. Samples were spiked with exogenous control Luciferase mRNA (Promega Corporation) at 0.025 pg/embryo before extraction (Fong *et al*. 2007). Removal of genomic DNA was performed with a DNAse digestion step (Qiagen). Following extraction, the volume was 12 µl. RNA samples were RT with Sensiscript (Qiagen) in a mix containing 2 µl $10\times$ buffer, 2 µl 5 mM dNTPs, 2 µl 10 µM oligodT$_{(20)}$ (Applied Biosystems, Foster City, CA, USA), 1 µl 10 U/µl RNAse inhibitor (Invitrogen) and 1 µl Sensiscript in a volume of 20 µl.

For real-time qRT-PCR, we used the external (Luciferase) control for quantification. PCR was performed in an ABI PRISM 7900HT sequence detection system (Applied Biosystems) using TaqMan Gene Expression Assays (Applied Biosystems). A custom TaqMan primer and probe set for Luciferase were designed using the Applied Biosystems Assays-by-Design File Builder programme (Fong *et al*. 2007). In addition, commercially available TaqMan Gene Expression Assays for *Elavl1* (Assay ID: Mm00516011_m1), *Elavl2* (Assay ID: Mm00516015_m1), *Khsrp* (Assay ID: Mm01232838_g1), *Zfp36* (Assay ID: Mm00457144_m1), *Stau1* (Assay ID: Mm00488462_m1) and *Gclc* (Assay ID: Mm00802655_m1) were also used. For *Slc2a1* (Assay ID: Mm00441473_m1, exons 1–2 boundary) was used for the developmental series and culture medium experiments but for later experiments Assay Mm01192272_g1 (exons 9–10 boundary) was used because it was closer to the 3' end of the mRNA, amplified better with oligodT and had greater sensitivity. Because the 3' UTR of *Slc7a1* is very long, predesigned primers did not amplify well after RT. Therefore, primers and a fluorescent probe were designed using the Assays-by-Design File Builder programme within the mouse *Slc7a1* 3' UTR region, ordered

from Applied Biosystems and validated. Standard thermal cycling conditions were applied as in previous experiments (Fong *et al.* 2007) for 60 cycles. However, for *Slc7a1*, the PCR mixture contained 1.875 μl of each primer, 0.0867 μl of the fluorescent probe and 0.33 μl water per 25 μl reaction. Each sample was measured in triplicate.

Influence of culture on RBP and RBP target expression

Culture medium

Mice were superovulated as described earlier. Two-cell embryos were flushed from oviducts with M2 on D2 and embryos were washed with KSOMaa or Whitten's medium (Whitten & Biggers 1968) and placed into culture at 37 °C in 5% CO_2, 5% O_2 and 90% N_2 atmosphere. Paired Whitten's and KSOMaa samples were collected at each timepoint, but since development was slower in Whitten's medium, more embryos were cultured in Whitten's and blastocysts were collected later, at 114 h, to allow for blastocyst formation to occur. RNA was extracted as above for quantitative qRT-PCR. Due to our interest in genes regulated by p38 MAPK, we assayed for *Elavl1*, *Khsrp*, *Zfp36*, *Slc2a1*, *Gclc* and *Slc7a1* mRNAs in addition to the control gene, luciferase. There were four replicates, except for *Slc2a1* of which there were three.

Culture atmosphere

Embryos were cultured in KSOMaa medium in either a high- (5% CO_2, balance room air ($\sim$20% O_2)) or low-oxygen (5% O_2, 5% CO_2 and 90% N_2) atmosphere from the two-cell stage to the blastocyst stage (96 h post hCG) and frozen for RNA extraction and real-time qRT-PCR as described earlier. There were six replicates for all genes except *Slc7a1* mRNA, for which there were seven.

mRNA half-life

Target gene mRNAs were examined to determine whether they decayed with similar kinetics under high- or low-oxygen conditions. Embryos were cultured in KSOMaa from the two-cell stage until 96 h under 5 or 20% oxygen, there were five replicates. Twenty blastocysts were then collected, washed three times and cultured in KSOMaa containing 50 μg/ml α-amanitin to inhibit transcription and placed back into either high- or low-oxygen conditions. Embryos were collected at 0, 3, 6, 9 and 12 h. The amount of mRNA (%) remaining at each timepoint was compared with 0 h.

MAPK regulation

Embryos were cultured in KSOMaa or Whitten's from the two-cell to 96 h blastocyst stage under low-oxygen conditions as above. Embryos were then washed three times and placed into the same medium with either DMSO (vehicle control) or active MAPK inhibitor for 18 h and collected for qRT-PCR, as described earlier. The inhibitors were obtained from EMD Chemicals (Newark, NJ USA) and were diluted stored as 20 mM stock concentrations in DMSO. SB220025 was used at final concentration of 20 μM and used to inhibit p38 MAPK. U0126 was used at 20 μM as an inhibitor of ERK1/ERK2, and

SP600225 was used at 20 μM as an inhibitor of JNK/SAPK. There were five replicates of each, except *Slc2a1* with the p38 inhibitor, where *N*=4.

Statistical analysis

For all samples, quantification was normalised to the exogenous control luciferase RNA levels. Expression levels were calculated according to the method of Pfaffl (2001), where expression level is calculated as the ratio between $E\text{target}^{\Delta C_T(\text{target gene})}/E\text{Luc}^{\Delta C_T(\text{Luc})}$. E=Efficiency of the primer set, which was calculated by the slope of tenfold dilutions of a standard sample according to the formula of $E=10^{(-1/\text{slope})}$. The ΔC_T value=C_T(avg of control)−C_T(each sample). Statistical analysis was performed by SigmaStat 3.5 (Jandel Scientific Software, San Rafael, CA, USA) software package. Real-Time qRT-PCR results are presented as the mean±S.E.M.

Real-time qRT-PCR data were log transformed before ANOVA was performed, followed by a Tukey's test for multiple comparisons. In addition, ANOVA was performed on the untransformed values, followed by a Holm–Sidak multiple comparison test. For the developmental experiment, comparisons were made among stages. In the culture medium experiment, the data were analysed by two-way ANOVA after transformation, with the effects of medium, stage and interaction examined, followed by Holm–Sidak multiple comparison tests. For *Slc7a1*, due to low expression at other stages, only blastocysts were compared by a *t*-test after log transformation. There were five replicates. For the culture gas experiment, *t*-tests were performed. Analysis of mRNA decay was performed with multilinear regression using treatment, hour and replicate as factors after setting the time 0 values to (100%), data were left untransformed. In the MAPK experiments, the control was KSOMaa treated with DMSO (vehicle). The data were analysed by two-way ANOVA, with the effects of medium, MAPK inhibitor and interaction examined, followed by Holm–Sidak multiple comparison tests. For all data analyses, $P \leq 0.05$ was considered statistically significant.

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