

# Endogenous folates and single-carbon metabolism in the ovarian follicle, oocyte and pre-implantation embryo

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## Abstract

Maternal B-vitamin status at conception can affect fertility and the health of offspring. This study details transcript expression for genes encoding key enzymes in the linked methionine/folate cycles in the bovine oocyte, somatic cells of the ovarian follicle and pre-implantation embryo. Transcripts for all 12 enzymes that were studied and for the two folate receptors (*FOLR1* and *FOLR2*) and reduced folate carrier (*SLC19A1*) were expressed in liver cells, but transcripts for betaine-homocysteine methyltransferase and methionine adenosyl transferase 1A were absent in all ovarian cells, and transcripts for *FOLR2* were absent in embryonic cells. Transcripts for glycine methyltransferase were also absent/weak in cumulus and granulosa cells. The absence of these enzymes could have a profound effect on single-carbon metabolism within the ovary and pre-implantation embryo. Immunocytochemical analysis revealed *SLC19A1* protein expression on the plasma and basal-lateral membranes of the pre-implantation embryo. The folate antagonist methotrexate (MTX) enters the cell via *SLC19A1*, and in the current study, MTX inclusion in bovine/ovine culture media at either 1 or 10  $\mu\text{M}$  from the 1-cell stage inhibited embryo development beyond the 8-cell stage. Hypoxanthine and thymidine (100  $\mu\text{M}$ ) increased the proportion of embryos that developed to blastocysts, but the cell number was reduced by 20%. The reduced uptake of [ $^{35}\text{S}$ ] methionine into intra-cellular S-adenosylmethionine and S-adenosylhomocysteine pools, together with reduced uptake of glutamate and tryptophan, was consistent with depleted intra-cellular pools of reduced folates. These data provide an insight into the importance of maternal dietary folate/B-vitamin status during the peri-conceptual period.

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## Introduction

Maternal B-vitamin status of intending mothers can have a major bearing on pregnancy establishment and term delivery (Tamura & Picciano 2006, Forges *et al.* 2007). By way of example, whereas poor pre-conception vitamin B<sub>6</sub> status reduced the probability of pregnancy establishment, deficiency of both vitamin B<sub>6</sub> and vitamin B<sub>12</sub> was associated with early pregnancy loss and recurrent miscarriage (Reznikoff-Etiévant *et al.* 2002, Ronnenberg *et al.* 2007). Furthermore, in one recent study, cobalamin (B<sub>12</sub>) deficiency was prevalent in at least one partner in around 40% of couples presenting for unexplained infertility (Pront *et al.* 2009). A significant proportion of women presenting for fertility treatment are known to be polymorphic for genes for at least two key enzymes (i.e. 5,10-methylenetetrahydrofolate reductase (*MTHFR*) and solute carrier family 19, member 1 (*SLC19A1*)) involved in folate metabolism (Altmäe *et al.* 2010). Mild hyperhomocysteinaemia is prevalent in subjects polymorphic for a number of enzymes involved in folate/methionine metabolism (Midttun *et al.* 2007), and total homocysteine

concentrations, measured in follicular fluid, are sensitive to dietary intakes of folic acid (Boxmeer *et al.* 2008) and are associated with pregnancy outcome in clinical IVF cycles (Haggarty *et al.* 2006, Pacchiarotti *et al.* 2007).

A number of putative mechanisms have been proposed to explain these observations, mostly centring on the effects of homocysteine on placental vascular disease (including placental infarction and abruption), which is related to the production of inflammatory cytokines, oxidative stress and apoptosis (discussed by Ebisch *et al.* (2007) and Forges *et al.* (2007)). Although the effects of folate/vitamin B<sub>12</sub> deficiency on the follicle-enclosed oocyte and pre-implantation embryo are generally acknowledged, there has been very little work conducted on these specific cell types. In a sheep model of dietary vitamin B<sub>12</sub> and folate deficiency during the peri-conceptual period (encompassing the periods of oocyte growth and post-fertilisation development to the blastocyst stage), we reported on the extent to which availability of dietary methyl groups can perturb homocysteine metabolism in embryo donor ewes, leading to epigenetic modifications to DNA methylation in offspring that became obese, insulin

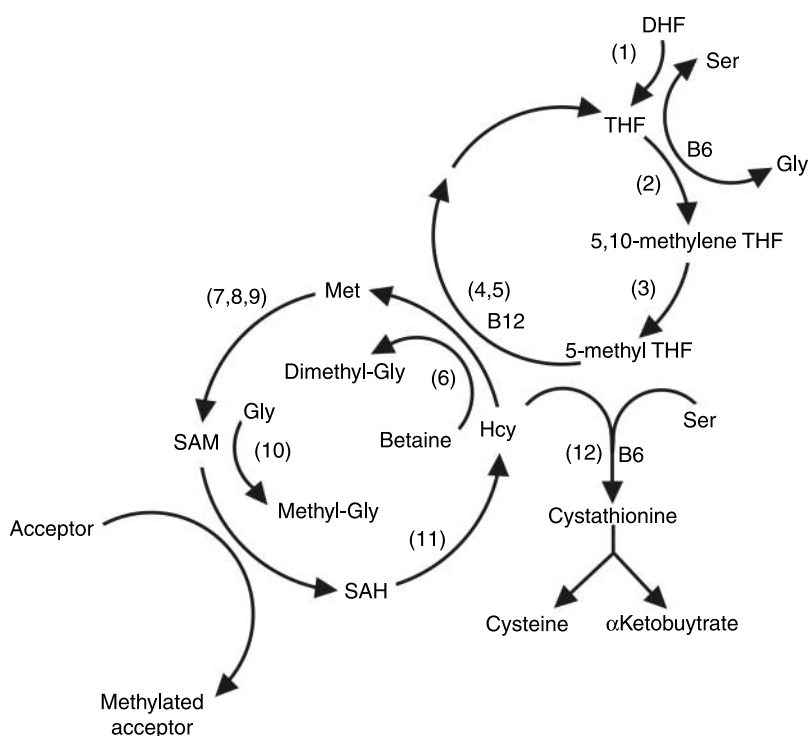
resistant and hypertensive (Sinclair *et al.* 2007). In this model, deranged single-carbon metabolism, leading to elevated intra-cellular homocysteine and reduced *S*-adenosylmethionine (SAM) concentrations, altered the expression of a number of oocyte- and granulosa cell-specific transcripts associated with ovarian follicular development and responsiveness to FSH (Kanakkaparambil *et al.* 2009), effects broadly consistent with those following ovarian stimulation in folate-deficient human IVF cycles (Twig *et al.* 2009).

In spite of these observations, little is known about the uptake and metabolism of folates and other B-vitamins within the ovarian follicle, oocyte and pre-implantation embryo. Indeed, the nature and extent of single-carbon metabolism within these cells are not understood (Sinclair & Singh 2007). Given the importance attached to folic acid in the diet of intending mothers aimed, primarily, at reducing the incidence of neural tube defects (Lucock 2000, Smith *et al.* 2008, Osterhues *et al.* 2009), it seems surprising that so little attention has been directed towards understanding its metabolism and that of related B-vitamins by the follicle-enclosed oocyte and pre-implantation embryo. We have previously shown that human embryonic stem cells express transcripts for most of the methyl cycle enzymes illustrated in Fig. 1 (Steele *et al.* 2005), and at the time of going to press, a report had just emerged to indicate that transcripts for most of these enzymes are also present in human germinal vesicle (GV) oocytes (Benkhalifa *et al.* 2010). However, transcript expression for these enzymes and folate receptors in mammalian

embryos and somatic cells within the ovarian follicle has not been investigated. Furthermore, the functional significance of altering folate metabolism in these cells is poorly understood.

O'Neill (1998) cultured mouse zygotes in the presence of varying doses of methotrexate (MTX), a structural folate analogue and antagonist, either in the presence or in the absence of exogenous folic acid, and reported on the proportion of zygotes that developed to the blastocyst stage. He concluded that the pre-implantation embryo has an absolute requirement for reduced folate, principally for thymidine synthesis, which is met entirely by endogenous sources accumulated, presumably, during the protracted period of oocyte growth that occurs during ovarian folliculogenesis. The study, however, did not go into detail sufficient to provide a clear insight into the underlying mechanisms.

The purpose of the present study, therefore, was twofold. First, to confirm or otherwise the expression of transcripts and proteins for key enzymes and receptors involved in folate and single-carbon metabolism in oocytes, the different somatic cell types within the ovarian follicle, and in blastocysts. Such information would provide a novel and important initial insight into the extent to which these important cycles might function within the ovarian follicle and early embryo, and the degree to which the female gamete and early embryo may be affected directly by maternal diet and/or culture media composition. Secondly, this study sought to extend the observations of O'Neill (1998) by considering the detailed metabolic and developmental



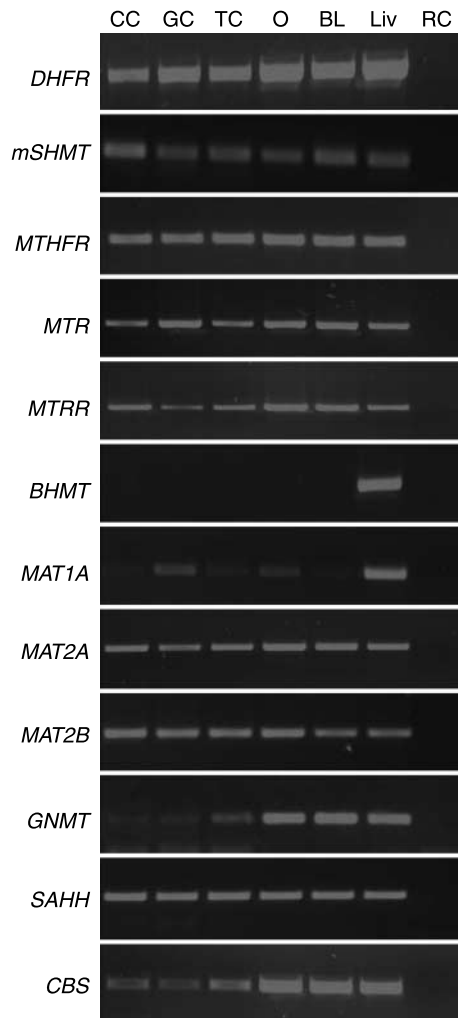
**Figure 1** Combined methionine and folate cycles identifying key metabolites and enzymes investigated in the current study. Metabolites: dihydrofolate (DHF), tetrahydrofolate (THF), serine (Ser), glycine (Gly), pyridoxal phosphate (B6), methylcobalamin (B12), methionine (Met), *S*-adenosylmethionine (SAM), *S*-adenosylhomocysteine (SAH), homocysteine (Hcy). Enzymes: dihydrofolate reductase (DHFR) (1), serine hydroxymethyl transferase (mSHMT) (2), 5,10-methylene THF reductase (MTHFR) (3), methionine synthase (MTR) (4), methionine synthase reductase (MTRR) (5), betaine-homocysteine methyltransferase (BHMT) (6), methionine adenosyl transferase 1A (MAT1A) (7), 2A (MAT2A) (8), 2B (MAT2B) (9), glycine methyltransferase (GNMT) (10), SAH-hydrolase (SAHH) (11), cystathionine  $\beta$ -synthase (CBS) (12).

consequences of disturbing endogenous reduced folate metabolism, through the actions of MTX, for zygotes from two alternative species (i.e. the sheep and cow).

## Results

### Transcript expression and immunostaining

Transcript expression for each of the enzymes listed in Fig. 1 is presented for bovine cumulus, granulosa and thecal cells, oocytes, day 8 blastocysts and liver in Fig. 2. Of note was the presence of all transcripts in the liver, but the absence of transcripts for betaine-homocysteine methyltransferase (*BHMT*) in all cells of the ovarian follicle, including the oocyte, and in day 8 blastocysts. Also absent, or expressed at very low levels, in these cells were transcripts for the enzyme methionine adenosyl transferase 1A (*MAT1A*). Transcripts for glycine methyltransferase (*GNMT*) were absent/weak in cumulus



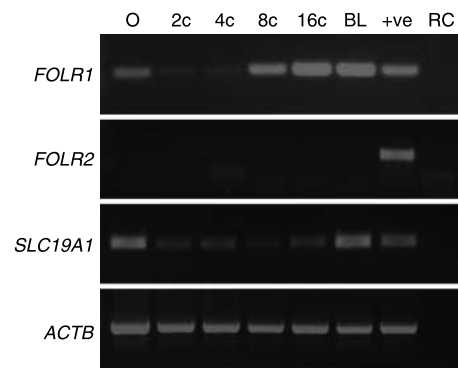
**Figure 2** Transcript expression for each of the methionine/folate cycle enzymes listed in Fig. 1 in bovine ovarian follicular cells, oocytes, day 8 blastocysts and liver. CC, cumulus cells; GC, granulosa cells; TC, thecal cells; O, oocytes; BL, blastocysts; Liv, liver; RC, reagent control.

and granulosa cells, and expressed at very low levels in thecal cells, but were abundantly present in the oocyte and pre-implantation embryo. Similarly, transcripts for *CBS*, the rate-limiting enzyme in the trans-sulphuration pathway, were expressed at very low levels in cumulus, granulosa and thecal cells, but were abundantly expressed in the oocyte and blastocyst. Transcripts for *FOLR1* and *SLC19A1*, but not for *FOLR2*, were expressed in the oocyte, and during the later stages of bovine embryo development up to the blastocyst stage (Fig. 3). Western analysis confirmed the specificity of the antibody used for immunocytochemistry of *SLC19A1* (Fig. 4). A single band was present at 72 kDa, which was absent when the antibody was treated with a blocking peptide. Immunostaining of bovine oocytes and embryos revealed plasma- and basal-lateral-membrane localisation of *SLC19A1* protein at all stages of development studied (Fig. 5).

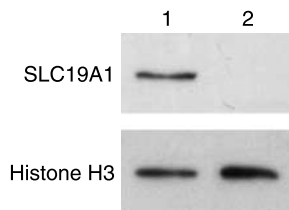
### Developmental and metabolic actions of MTX during embryo culture

In the initial experiment, putative bovine zygotes were transferred to synthetic oviductal fluid (SOF) media containing varying concentrations of MTX 24 h after the onset of fertilisation. Neither the percentage of inseminated oocytes that cleaved following insemination nor the percentage that developed to the 8-cell stage were affected by MTX (Table 1). However, effects of MTX at the higher doses were evident by day 7, and virtually no embryos were present by day 8 when cultured with 10  $\mu$ M MTX. Cell number in day 8 blastocysts was also reduced ( $P=0.022$ ) at 1  $\mu$ M MTX, but the percentage of TUNEL-positive cells was unaltered.

To overcome the toxic effects of 10  $\mu$ M MTX on purine and pyrimidine synthesis, bovine zygotes were subsequently cultured in the presence of hypoxanthine (100  $\mu$ M) and thymidine (100  $\mu$ M). The effect was to rescue a large percentage of embryos, but residual



**Figure 3** Transcript expression for folate receptors 1 and 2 (*FOLR1* and *FOLR2*) and the reduced folate carrier (*SLC19A1*) in bovine oocytes and pre-elongation embryos. O, oocyte; 2c, 2 cell; 4c, 4 cell; 8c, 8 cell; 16c, 16 cell; BL, day 8 blastocyst; +ve, positive control (spleen for *FOLR2* and liver for other transcripts), RC, reagent control.



**Figure 4** Validation of SLC19A1 antibody by western blotting. Proteins extracted from 50 bovine oocytes were separated by SDS-PAGE. PVDF membrane was then incubated with SLC19A1 antibody (lane 1) or SLC19A1 antibody pre-treated with tenfold excess blocking peptide (lane 2). Both blots were re-probed with histone H3 antibody.

effects, in terms of reduced cell number, remained (Table 2). The percentage of TUNEL-positive cells was not affected by treatment.

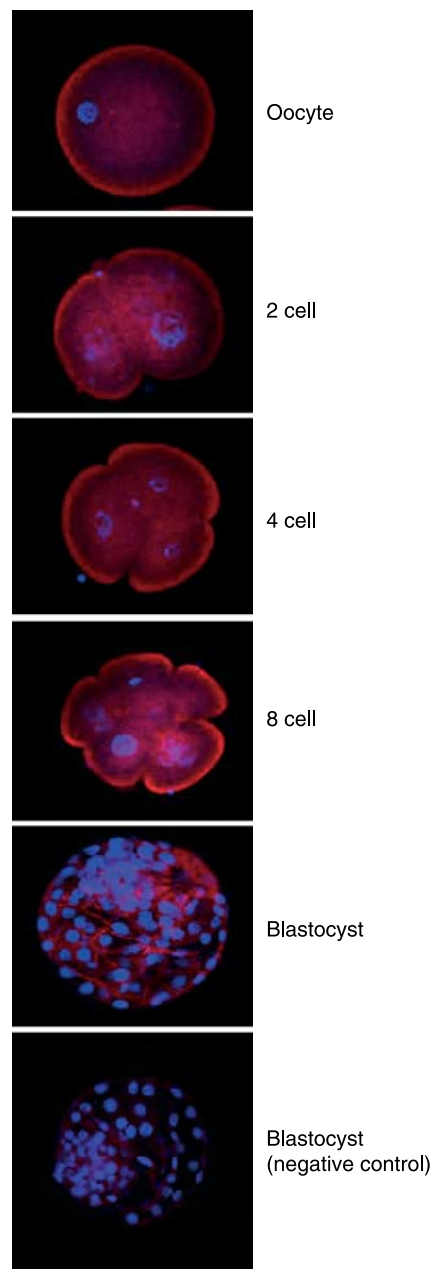
These experiments were extended to consider the culture of ovine zygotes. As was observed with bovine zygotes, culture in the presence of 10  $\mu$ M MTX inhibited development beyond the 8–16-cell stage, but the inclusion of 100  $\mu$ M hypoxanthine and thymidine rescued a large percentage of embryos, which developed to the blastocyst stage by day 7 (Table 3). Once again, however, total cell number was reduced ( $P < 0.001$ ) in MTX-treated embryos. As a third treatment, tetrahydrofolate (THF), at 25  $\mu$ M, was added in the hope of eliminating the residual toxic effects of MTX on embryo development and cell number. In this regard, THF proved ineffective. However, in this experiment, the inclusion of hypoxanthine and thymidine was effective in rescuing the percentage of zygotes that developed to the blastocyst stage, although residual effects on cell number remained (Table 4). The incorporation of [ $^{35}$ S] methionine into intra-cellular SAM and S-adenosylhomocysteine (SAH) pools was also lower ( $P < 0.05$ ) for embryos cultured in the presence of MTX, irrespective of the inclusion of hypoxanthine, thymidine and THF, than for control embryos. Adjusting these values for cell number, however, eliminated these treatment differences.

Finally, amino acid turnover in ovine blastocysts was determined by culturing day 6.5 embryos for 12 h in microdrops under oil and by measuring amino acid concentrations in media both before and after culture. Overall, the pattern of amino acid turnover (data not presented) resembled that of previous studies (e.g. Partridge & Leese 1996) with ruminant embryos. Of the 20 amino acids measured, only two were altered ( $P < 0.01$ ) by culture treatment (Fig. 6). The uptake of both glutamate and tryptophan was reduced in the presence of MTX irrespective of the presence of THF.

## Discussion

The current study details, for the first time, transcript expression for genes encoding key enzymes involved in the linked methionine–folate cycles in the bovine

oocyte, and the various somatic cells of the ovarian follicle and pre-implantation bovine embryo. While it is acknowledged that the presence of transcripts for these enzymes does not inform on their functional activity, a number of important conclusions can be drawn from the absence of transcripts for specific enzymes. Of note were the absence of transcripts for the methionine cycle enzyme *BHMT*, and absence or very low expression of *MAT1A* in the bovine ovary, oocyte and pre-implantation embryo. Transcripts for *GNMT* were also absent/weak



**Figure 5** Immunolocalisation of reduced folate carrier (SLC19A1) in oocytes and pre-elongation bovine embryos up to the day 8 blastocyst stage. Blastocyst-negative control with secondary antibody only.



**Table 1** Culture of bovine zygotes in the presence of different concentrations of methotrexate.

Treatment	Control	0.5 (µM)	1.0 (µM)	10.0 (µM)	Significance P
Replicate experiments	4	4	4	4	
Oocytes matured	240	240	240	235	
Cleaved of inseminated, %	82.9±2.7	83.8±2.6	83.4±2.7	84.6±2.6	NS
Percentage of 8 cells <sup>a</sup>	41.9±4.8	42.4±4.8	37.7±4.7	43.2±4.8	NS
Blastocysts of cleaved					
Day 8, %	19.2±4.0	15.9±3.7	15.1±3.7	1.0±1.0	0.004
Blastocyst cell number <sup>b</sup>					
Total	97.0±4.9	91.9±4.8	79.2±4.4	–	0.022
Inner cell mass	46.9±4.9	35.5±4.9	32.5±4.9	–	0.053
Trophectoderm	72.2±6.0	86.3±6.6	66.5±5.8	–	0.066
TUNEL, %	5.3±1.1	6.4±1.3	7.8±1.5	–	NS

<sup>a</sup>Forty-eight hours following initiation of fertilisation. <sup>b</sup>Number of blastomeres in the inner cell mass and trophectoderm does not match total because these cells could not be distinguished in all embryos.

in cumulus and granulosa cells, with low level expression in thecal cells. The absence or low expression of transcripts for these enzymes may have profound implications for how single-carbon metabolism operates within the ovary and pre-implantation embryo. The bovine oocyte and pre-implantation embryo were, however, found to express transcripts for *FOLR1* and *SLC19A1*, but not for *FOLR2*. We previously reported transcripts for *FOLR1* but not for *FOLR2* in human ES cells (Steele *et al.* 2005). Immunocytochemical analysis revealed that SLC19A1 protein was expressed on the plasma and basal-lateral membranes of the pre-implantation embryo at all stages of development up to and including the blastocyst stage. The structural folate analogue and antagonist MTX is known to enter the cell via SLC19A1 (Bosson 2003). In the current study, MTX inclusion in culture media at either 1 or 10 µM from the 1-cell stage inhibited embryo development beyond but not prior to the 8-cell stage, indicating that there were sufficient intra-cellular reserves of reduced folates, presumably acquired during oocyte growth, to sustain initial cell-cycle progression following fertilisation. The inclusion of thymidine and hypoxanthine significantly increased the proportion of MTX-treated embryos that developed beyond eight cells. However, blastomere number was reduced by around 20% in these embryos compared with the controls, although the proportion of apoptotic (specifically TUNEL-positive) cells was unaltered. As the decrease in cell number was proportionately similar for the inner cell mass (ICM) and trophectoderm (TE), these results point to a general slowing down of cell division rather than to programmed cell death as the cause. The reduced uptake of the amino acids glutamate and tryptophan by day 8 blastocysts in the presence of MTX is consistent with there being a depleted intra-cellular pool of reduced folates in these embryos. In contrast, the reduced uptake of [<sup>35</sup>S] methionine into intra-cellular SAM and SAH pools is more likely to be related to the reduction in blastomere number.

### Methionine cycle enzymes

The general absence of transcripts for *BHMT*, *MAT1* and *GNMT* in somatic cells of the bovine follicle, and for *BHMT* and *MAT1* in the oocyte and blastocyst, is significant for it indicates that under physiological conditions, these cells are not best equipped to metabolise high concentrations of methionine. However, supra-physiological concentrations of methionine are present in many commercially available cell and embryo-culture media formulations (Steele *et al.* 2005), including that (SOFBSAaa) used in the current study. At present, the consequences of this, in terms of effects on intra-cellular transmethylation and epigenetic programming, are not known, but are the subject of ongoing investigations at our laboratory. BHMT (EC 2.1.1.5) is a zinc metalloenzyme which uses preformed methyl groups from dietary betaine, or betaine derived from choline, for the methylation of homocysteine to methionine (Fig. 1). Activity and expression measurements indicate that BHMT is mainly located in the liver where, in the rat, it can process up to 25% of

**Table 2** Culture of bovine zygotes in the presence of 10 µM methotrexate (MTX) with 100 µM hypoxanthine (H) and 100 µM thymidine (T).

Treatment	Control	MTX+H+T	Significance P
Replicate experiments	9	9	
Oocytes matured	493	504	
Cleaved of inseminated, %	78.7±1.8	81.0±1.8	NS
Blastocysts of cleaved			
Day 8, %	23.2±2.1	14.0±1.7	<0.001
Blastocyst cell number <sup>a</sup>			
Total	115.2±2.8	85.5±1.9	<0.001
Inner cell mass	45.2±2.2	37.7±1.7	<0.001
Trophectoderm	71.3±2.8	61.6±2.2	0.006
TUNEL, %	3.4±0.4	3.3±0.4	NS

<sup>a</sup>Number of blastomeres in the inner cell mass and trophectoderm does not match total because these cells could not be distinguished in all embryos.

**Table 3** Culture of ovine zygotes in the presence of 10 µM methotrexate (MTX) alone or with 100 µM hypoxanthine (H) and 100 µM thymidine (T).

Treatment	Control	MTX	MTX+H+T	Significance <i>P</i>
Replicate experiments	5	5	5	
Oocytes matured	871	871	871	
Cleaved of inseminated, %	87.8±3.3	86.9±3.4	85.7±3.5	NS
Blastocysts of cleaved				
Day 7, %	59.1±3.2	0.0	48.4±3.3	<0.001
Blastocyst cell number				
Total	119.1±3.3	–	89.8±2.9	<0.001

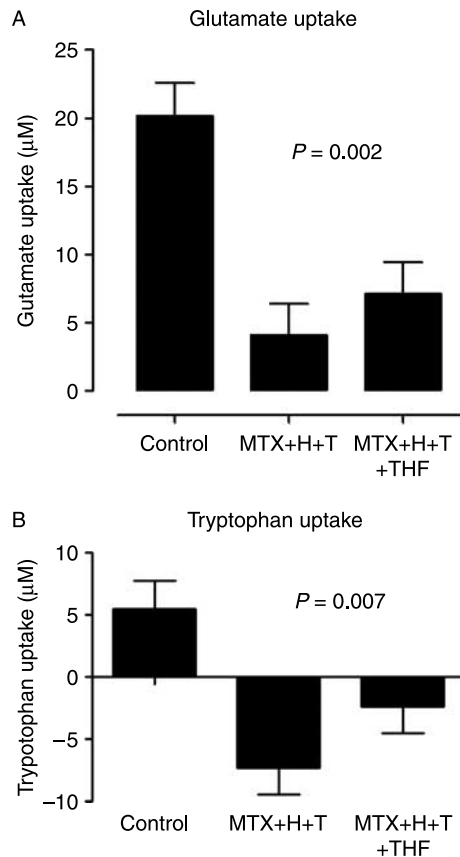
cellular homocysteine (Pajares & Pérez-Salab 2006). The absence of *BHMT* transcript expression in the bovine oocyte and pre-implantation embryo is somewhat at odds with a recent report in the human oocyte (Benkhalifa *et al.* 2010), mouse blastocyst (Lee *et al.* 2009) and our previous findings in human ES cells (Steele *et al.* 2005), and may reflect species-specific differences in the importance of this enzyme. It is known, for example, that the specific activity of hepatic BHMT is several fold greater in the rat than in the sheep, and this, together with the relatively low specific activity of hepatic GNMT (EC 2.1.1.20), is thought to be the principal means by which ruminants have adapted to methyl-poor diets (Snoswell & Xue 1987). GNMT has a high  $K_m$  for SAM and so serves as a benign high-capacity methyltransferase that regulates intra-cellular transmethylation and *de novo* methyl group synthesis by facilitating the methylation of glycine to sarcosine (Stipanuk 2004). The absence or very low expression of transcripts for *GNMT* in somatic cells of the bovine follicle, but their presence in both the oocyte and blastocyst, raises intriguing questions concerning the nature and extent of SAM-mediated transmethylation reactions in these different cells, and merits further investigation.

The enzyme MAT (EC 2.5.1.6) catalyses the activation of methionine by ATP to form SAM (Fig. 1). The isoenzyme encoded by *MAT1A* exists as two proteins (MAT1 and MAT3) with moderate and high  $K_m$  values respectively for methionine (Stipanuk 2004). In contrast, the inter-related products of *MAT2A* and *MAT2B* have a very low  $K_m$  for methionine. In the current study, transcripts for *MAT1A* were absent or very poorly expressed in all the cells studied, apart from the liver, and this is in agreement with the observations of Steele *et al.* (2005) for human ES cells. In contrast, transcripts for *MAT2A* and *MAT2B* were expressed in all cell types, consistent with ovarian cells and embryos encountering physiological concentrations (14–50 µM) of methionine in follicular and uterine fluids (Casslen 1987, Tay *et al.* 1997). In the current study, day 7 ovine blastocysts were capable of metabolising radio-labelled methionine to SAM and SAH (Table 4), confirming the activity of this enzyme system in ruminant embryos. However, low-level transcript expression of *CBS* in cumulus and granulosa cells, relative to oocytes and thecal cells, is consistent with low intra-follicular methionine concentrations, and further indicates regional intra-follicular variation in components of the methionine cycle. Cystathionine β-synthase (*CBS*: EC 4.2.1.22) is the

**Table 4** Culture of ovine zygotes in the presence of 10 µM methotrexate (MTX) alone or with combinations of 100 µM hypoxanthine (H), 100 µM thymidine (T) and 25 µM tetrahydrofolate (THF).

Treatment	Control	MTX+H+T	MTX+H+T+THF	Significance <i>P</i>
(A) Embryo development				
Replicate experiments	8	8	8	
Oocytes matured	1239	1239	1239	
Cleaved of inseminated, %	89.3±3.8	87.5±4.0	88.0±4.0	NS
Blastocysts of cleaved				
Day 7, %	46.4±3.6	42.2±3.6	39.1±3.5	NS
Replicate experiments	4	4	4	
Blastocyst cell number				
Total	106.4±5.1	80.8±4.5	91.1±4.7	0.001
Inner cell mass	45.9±3.4	34.5±2.9	37.2±3.1	0.028
Trophectoderm	78.4±4.4	67.4±4.1	72.8±4.3	NS
TUNEL, %	5.0±1.0	7.2±1.4	5.9±1.2	NS
(B) [ <sup>35</sup> S] methionine incorporation <sup>a</sup>				
Replicate experiments	3	3	3	
SAM	3014±235	2356±234	2213±234	0.049
SAH	75.92±6.5	50.6±6.5	50.7±6.5	0.013
SAM:SAH	44.1±3.6	49.3±3.6	49.8±3.5	NS

<sup>a</sup>Incorporation of [<sup>35</sup>S] methionine into *S*-adenosylmethionine (SAM) and *S*-adenosylhomocysteine (SAH) pools in groups of four day 7 blastocysts during a 2-h incubation.



**Figure 6** Net uptake of glutamate (A) and tryptophan (B) by day 6.5 ovine blastocysts following 12-h incubation. Blastocysts produced *in vitro* in the absence (Control) or presence of methotrexate with hypoxanthine and thymidine (MTX+H+T) or methotrexate with hypoxanthine, thymidine and tetrahydrofolate (MTX+H+T+THF). Values (µM) are adjusted to an average of 14.3 embryos by covariance analysis where number of embryos per drop formed the covariate.

rate-limiting enzyme in the trans-sulphuration pathway, and has a high  $K_m$  for homocysteine (Stipanuk 2004). Its expression was thought to be restricted to certain tissues such as the liver and kidney, but *Cbs* transcripts have recently been shown to be expressed in mouse cumulus cells, and functional suppression by RNA interference results in a significant increase in the number of GV-arrested oocytes (Liang *et al.* 2007).

### Folate metabolism

The enzyme primarily responsible for the methylation of homocysteine to methionine in the ruminant liver is methionine synthase (EC 2.1.1.13), where the methyl donor is 5-methyl THF (Fig. 1). Specific activity of this enzyme in sheep hepatocytes is fourfold greater than in the rat (Snoswell & Xue 1987). Transcripts for this vitamin B<sub>12</sub>-dependent enzyme were expressed in all cell types studied in the current experiment, as were transcripts for all the other major folate cycle enzymes including those for dihydrofolate reductase

(DHFR; Fig. 2). DHFR (EC 1.5.1.3) catalyses THF regeneration by reduction of dihydrofolate using NADPH as a cofactor, and is a target for the antifolate chemotherapeutic drug MTX. MTX enters the cell via the reduced folate carrier (SLC19A1; Bosson 2003). Now, we have shown, for the first time, that SLC19A1 is expressed at all stages of mammalian embryo development up to the blastocyst stage. MTX is immediately polyglutamated by folylpolyglutamyl synthetase upon entry into the cell (Bosson 2003). Polyglutamation enhances cellular retention and increases the affinity of MTX for DHFR and three other folate-dependent enzymes involved in pyrimidine (thymidylate synthase) and purine (glycineamide ribonucleotide transformylase and aminoimidazolecarboximide ribonucleotide transformylase) synthesis (Chabner *et al.* 1985).

The inhibitory effects of 10 µM MTX on mouse embryo development in the study of O'Neill (1998) were completely reversed by the inclusion of 100 µM thymidine. This is curious because mouse embryos are known to possess functional *de novo* purine-synthesis and purine-salvage pathways, and to be sensitive to the inclusion of purine synthesis inhibitors in culture media (Alexiou & Lesse 1992, 1994). What is more, we found, at least in HT29 colon cancer cells, that one of the primary effects of MTX was to reduce the synthesis of purines such as adenine (Singh *et al.* 2006). Consequently, we elected to include the purine hypoxanthine together with the pyrimidine thymidine in our culture media. However, unlike O'Neill (1998), we were unable to completely reverse the inhibitory effects of MTX on embryo development, as evidenced by the reduced proportion of zygotes that developed to the blastocyst stage, the reduced number of blastomeres in day 8 bovine embryos and day 7 ovine embryos and the reduced uptake of glutamate and tryptophan by day 6.5 ovine embryos. Furthermore, the addition of THF to culture media was without effect. It is possible that the residual impairment in embryo development was due to the timing and level of hypoxanthine used in the current study (100 µM from the 1-cell stage), for Alexiou & Leese (1992) reported that hypoxanthine when added at 50 µM from the 1-cell stage as opposed to the 2-cell stage reduced the proportion of mouse zygotes that developed to the blastocyst stage. The mechanism underlying this effect is not known. The reduced uptake of glutamate and tryptophan (the latter amino acid donates a formate group in the synthesis of 10-formyl THF from THF) by sheep embryos, however, is indicative of depleted intracellular pools of reduced and polyglutamated folates, which cannot be corrected by exogenous THF. The kinetics of SLC19A1-mediated transport of MTX and THF into the cell cannot explain this effect (Matherly & Goldman 2003). Instead, the effect is more likely due to the high affinity of MTX for DHFR, which is around 1000-fold that of folate (Schweitzer *et al.* 1990).

**Table 5** Primers used for RT-PCR analysis of transcript expression in bovine ovarian somatic cells, oocytes and embryos.

Gene	Accession number	Primer sequence (5'-3')	Product length	Annealing temperature (°C)
<i>MAT1A</i>	BC105410	GCC GAG CTA ACT TTC ACA GG TCC CTT TGG TTC TGA CTT GG	265	58
<i>MAT2A</i>	BT030708	TGG CTT GTG AAA CTG TTG CT TGT GTG GAC TCT GAT GGG AA	446	58
<i>MAT2B</i>	BC114151	ATG ATT TTC AGC CCC ATG TC AAG GTG GGT TTG TTC CAT CA	192	58
<i>MTR</i>	DQ084519	AAA CTG GGC TTC AAT CCA AA CCT GAG TCT GGG CGT AGT GT	385	58
<i>MTRR</i>	DQ084520	GCA GAC ACG AGG AAG AAA GG GAA GAT GTT CGA GCA GGA GG	298	56
<i>MTHFR</i>	AY854631	GAG GCT GAT CTG AAG CAC CT GGA GTG GTA GCC CTG AAT TG	171	58
<i>mSHMT</i>	BC103242	AGA ACG GGT GTT GGA ACT TG TCA TCT TCA AGG AAC CCT CG	132	58
<i>CBS</i>	BC151539	TCA ACA AGA TCG GGA GGA AC CGC AAG ACA TCC ACC TTC TC	271	58
<i>GNMT</i>	XM_618409	CCT GGC TTG AGT AAG TTC CG ACG TGG ATG AAA TAG CAG GG	164	58
<i>BHMT</i>	AY854632	AAT ATC CGG GCA GAA AGT CA GGT TTC CCT GAT GCT TTC AA	261	58
<i>SAHH</i>	BT030537	GGC CAC AGA TGT CAT GAT TG GGT GAC AAA GAT GTT GCC CT	208	58
<i>DHFR</i>	BC114162	TTC CAG AAT ATC CAG GTG TTC C GGA CTC TCC CAC TTT GGG TT	430	58
<i>FOLR1</i>	XM_589186	GCG GCC TGC TCT TAT TGT TA TTC AAA CAG TTT CCA CCA AGG	121	57
<i>FOLR2</i>	NM_001075325	CAC TCT GGC TCC ATG ACT GA GCT GTC AGA GAA ACC CCA AG	167	63.5
<i>SLC19A1</i>	BC118431	GCA GCC TCT TCT TCA ACC AC GGA GTT GAA GAC CCA CCA GA	221	57
<i>ACTB</i>	BT030480	ACT GGG ACG ACA TGG AGA AGA T TGC TCG AAG TCC AGG GCC ACG T	442	57

## Conclusions

The current study has identified, for the first time, the presence of transcripts for most, but not for all, of the key enzymes involved in the linked methionine/folate cycles in the oocyte, ovarian follicle and pre-implantation bovine embryo. Of note was the absence of transcripts for certain enzymes involved in methionine metabolism, which may have profound implications for our understanding of how this cycle operates within the ovary and early embryo with implications for numerous intra-cellular transmethylation reactions including DNA methylation. While species-specific differences in transcript expression may account for the cellular distribution of some enzymes (e.g. BHMT), they do not appear to account for the distribution of others (e.g. MAT1A); embryonic cells in both the cow and human are devoid of transcripts for this enzyme. A comprehensive comparative analysis of transcript/protein expression for enzymes of the linked methionine/folate cycles in ovarian cells and embryos from a range of rodent and large animal species is currently underway at our laboratory and will inform on this. The bovine embryo, however, expresses transcripts for *FOLR1* and *SLC19A1*, and *SLC19A1* protein is expressed on the plasma and basal-lateral membranes throughout development up to the blastocyst stage. The mammalian

pre-implantation embryo, therefore, expresses at least two important transporters to facilitate the uptake of folate. The functionality of these transporters, however, awaits further investigation. Nevertheless, the structural folate analogue and antagonist MTX is known to enter mammalian cells via *SLC19A1*, and here, we have demonstrated that MTX added to culture media disrupts intra-cellular folate metabolism in embryos. Indeed, the present data indicate that the pre-implantation embryo relies, to a large extent, on re-cycling endogenous pools of reduced folates, acquired during oocyte growth, primarily for the synthesis of purines and pyrimidines. These data provide an insight into the importance of maternal dietary folate status during the peri-conceptual period.

## Materials and Methods

### Transcript analysis in bovine follicular cells and blastocysts

Transcript expression for the enzymes described in Fig. 1 was determined by RT-PCR from RNA extracted from bovine oocytes, cumulus cells, granulosa cells, thecal cells and day 7 blastocysts. Total RNA was extracted from cumulus cells, granulosa cells and thecal cells using RNeasy Mini Kit (Qiagen Ltd). Then, 900 ng of RNA were treated with RQ1 DNase



(Promega) before being RT using Omniscript RT (Qiagen Ltd) in a 20- $\mu$ l reaction mixture. PCR was performed in a 25- $\mu$ l reaction mixture containing 1 $\times$  ImmoMix Red (Bioline Ltd, London, UK), 20 pmol/ $\mu$ l of each primer and 0.5  $\mu$ l of cDNA. The PCR program consisted of a preheating step at 95 °C for 10 min followed by 40 cycles of 94 °C for 15 s, primer-specific annealing temperature (Table 5) for 15 s and 72 °C for 30 s and a final extension at 72 °C for 7 min.

For transcript expression analysis in oocytes and embryos, poly A<sup>+</sup> RNA was extracted from pooled oocytes/embryos using Dynabeads mRNA DIRECT kit (Invitrogen Ltd). Poly A<sup>+</sup> RNA was RT into cDNA using QuantiTect RT kit (Qiagen Ltd) according to the manufacturer's instruction. Following DNase treatment using the kit, 1  $\mu$ l was removed for -RT reaction before RT. cDNA equivalent to two (for methyl/folate cycle enzymes) or three (for folate transporters) oocytes/embryos was used in the PCR in a total volume of 25- $\mu$ l reaction mix using ImmoMix Red and program as described above. PCR products (10  $\mu$ l) were run on agarose gels and visualised under u.v. All PCR products were sequenced to confirm primer specificity.

### Western blotting and immunostaining of bovine oocytes and embryos

Goat anti-human reduced folate carrier antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used at 1:400 for western blotting and at 1:100 for immunocytochemistry. Rabbit anti-human histone H3 antibody (Abcam, Cambridge, UK) was used at 1:20 000 for western blotting.

Denuded bovine oocytes (GV stage) in PBS/PVA were mixed with sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue and 0.175 mM dithiothreitol), and were boiled for 5 min. Protein was separated on 12% SDS-PAGE, followed by transfer to PVDF membranes for 2 h at 80 V in 25 mM Tris, 192 mM glycine and 20% methanol. After transfer, membranes were blocked for 1 h with PBS/0.1% (v/v) Tween 20 containing 5% milk (for reduced folate carrier antibody and histone H3) prior to incubation with the primary antibody (reduced folate carrier (RFC-1 (N-13)), Santa Cruz, Cat. no. sc-47 362) overnight at 4 °C. Membranes were then washed five times in PBS/0.1% Tween 20, followed by 1 h incubation with secondary antibodies (1:10 000 bovine anti-goat (Jackson ImmunoResearch Europe Ltd, Newmarket, UK), 1:20 000 goat anti-rabbit (Abcam) conjugated with HRP). Membranes were washed five times with PBS/0.1% Tween 20 before protein bands were visualised using ECL Plus (Amersham-Pharmacia Biotech). The specificity of the reduced folate carrier antibody was confirmed by treating the antibody with tenfold excess antigenic peptide (Santa Cruz, Cat. no. sc-47362 P) prior to western blot analysis.

After removal of zona pellucida in 10% acetic acid in PBS/0.1% (w/v) PVA, embryos were washed three times in PBS/PVA before fixing in 2.5% paraformaldehyde (PFA) at room temperature for 10 min. PFA-fixed embryos were permeabilised in 0.25% Triton X-100 for 15 min at 39 °C. Fixed embryos were blocked in PBS/0.1% PVA containing 3% BSA and 0.1% Tween 20 for 30 min before incubating overnight at 4 °C with primary antibodies prepared in PBS/0.1% PVA/0.1% Tween 20. On the following morning, embryos were washed three times

in PBS/0.1% PVA before incubating with bovine anti-goat IgG conjugated with DyLight 549 fluorescent dye (1:400; Stratech, Suffolk, UK) for 1 h. Embryos were washed three times in PBS/0.1% PVA before mounting in Vectashield+ DAPI (Vector Laboratories, Peterborough, UK). A negative control with the secondary antibody only was used at the same time. Samples were viewed under a Nikon confocal microscope.

### In vitro embryo production and assessment

Full details of these procedures for bovine and ovine embryos were given previously (Adamiak *et al.* 2006, Wonnacott *et al.* 2010), and only a brief summary is provided here. All reagents and media used in the experiment were obtained from Sigma unless otherwise stated. Cumulus-oocyte complexes (COCs) were graded on a four-point scale by the number of compact cumulus cell layers and granulation of the oocyte cytoplasm as described previously (Wonnacott *et al.* 2010), and grades 1 and 2 COCs were matured in groups of up to 60 in 500  $\mu$ l of bicarbonated-buffered TCM199 with supplements. For fertilisation, 1 $\times$ 10<sup>6</sup> motile spermatozoa from a single bull or ram were added per millilitre of media containing matured oocytes (up to 60 per well), and incubated for 22 h at 38.8 °C in a humidified atmosphere of 5% CO<sub>2</sub> in air. Putative zygotes were cultured at 39 °C in a humidified atmosphere of 5% O<sub>2</sub>, 5% CO<sub>2</sub> and 90% N<sub>2</sub> in groups of up to 60 in 400  $\mu$ l of SOF medium with 0.3% w/v fatty acid-free BSA, 2% v/v essential and 1% v/v non-essential amino acids (aa), and antibiotics alone (control), or with additional various combinations and levels (0.5–10  $\mu$ M) of MTX with or without hypoxanthine (100  $\mu$ M), thymidine (100  $\mu$ M) and THF (25  $\mu$ M).

Day 7 sheep and day 8 cattle blastocysts were assessed using a differential staining technique combined with TUNEL labelling, which discriminates blastomeres from the ICM from those of the TE (Fouladi-Nashta *et al.* 2005). Briefly, following permeabilisation, TE cells were stained with 30  $\mu$ g/ml propidium iodide for 5 min, and fixed in 4% PFA with 10  $\mu$ g/ml bisbenzimidazole (Hoechst 33342) for 20 min prior to incubation in small droplets of *In situ* Cell Death Detection Kit solution (Roche) for 45 min at 37 °C. Following washing, embryos were mounted in small droplets of Vectashield Mounting medium (Vector Laboratories Ltd) on glass slides and examined under a Leica epifluorescent microscope (Leica, Germany).

### HPLC analysis of amino acids, SAM and SAH

For amino acid analysis, day 6.5 sheep blastocysts were cultured for 12 h in microdrops, and following incubation, spent media were mixed with norleucine internal standard and 30 mg of 5-sulphosalicylic acid and were allowed to stand at 4 °C for 30 min. After centrifugation at 16 000 g for 10 min at 4 °C, the supernatant was passed through a PVDF Millipore syringe-driven filter unit with 0.22  $\mu$ m pore size, and amino acids were measured using Biochrom 20 amino acid analyser (Pharmacia LKB, Biochrom Ltd, Cambridge, UK) with ninhydrin detection (Steele *et al.* 2005, Singh *et al.* 2006). Peak integration was performed using EZChrom Elite Software (Scientific Software International Ltd, Duston, Northampton, UK).

For analyses of SAM and SAH, groups of four day 7 sheep blastocysts were incubated for 2 h in SOF/PVA without essential amino acids but with [<sup>35</sup>S] methionine (74 kBq/nmol; Amersham, AG1594), and were then extracted with 50 µl of 0.4 M perchloric acid according to the method of Sinclair *et al.* (2007). Briefly, following centrifugation at 10 000 g for 15 min at 4 °C, a 25-µl aliquot of the acid extract was directly applied onto HPLC column (Zorbax, C18 column, 250×4.6-mm inner diameter, 5 µm particle size). Separation was achieved using an Agilent 1100 HPLC system and u.v. detection at 254 nm. The mobile phase consisted of two solvents: solvent A, 8 mM octanesulphonic acid and 50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 3.0 and solvent B, 100% HPLC-grade methanol. The column was equilibrated at 80% solvent A and 20% solvent B before use. Following sample injection, the conditions were held at 20% solvent B for 8 min. Then, solvent B was increased to 40% over 0.5 min and held there for 12.5 min before the initial conditions were re-established. Cold SAM and SAH standards were used to trace the radio-labelled peaks, and specific fractions were transferred to vials containing scintillation fluid and counted.

### Statistical analyses

All statistical analyses were conducted using Genstat release 11.1 (Genstat 2008). The proportions of inseminated oocytes that cleaved and zygotes that developed into blastocysts were analysed by analysis of deviance using Generalised Linear Models (GLMs) assuming binomial errors and with logit link functions. Data are presented as predicted back-transformed means ± s.e.m. Embryo cell number was analysed using a GLM assuming a Poisson error distribution and with a logit link function. Amino acid turnover during short-term embryo culture in microdrops was analysed by covariance analysis where embryo number per drop formed the covariate. Predicted means ± s.e.m are presented.

### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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