

Article

Human embryonic stem cell methyl cycle enzyme expression: modelling epigenetic programming in assisted reproduction?



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Abstract

To investigate a possible mechanism for inducing epigenetic defects in the preimplantation embryo, a human embryonic stem cell model was developed, and gene expression of the key methyl cycle enzymes, *MAT2A*, *MAT2B*, *GNMT*, *SAHH*, *CBS*, *CGL*, *MTR*, *MTRR*, *BHMT*, *BHMT2*, *mSHMT*, *cSHMT* and *MTHFR* was demonstrated, while *MAT1* was barely detectable. Several potential acceptors of cycle-generated methyl groups, the DNA methyltransferases (*DNMT1*, *DNMT3A*, *DNMT3B* and *DNMT3L*), glycine methyltransferase and the polyamine biosynthetic enzymes, *SAM decarboxylase* and *ornithine decarboxylase*, were also expressed. Expression of folate receptor α suggests a propensity for folate metabolism. Methotrexate-induced depletion of folate resulted in elevated intracellular homocysteine concentration after 7 days in culture and a concomitant increase in cysteine and glutathione, indicating clearance of homocysteine through the transsulphuration pathway. These studies indicate that altered methyl group metabolism provides a potential mechanism for inducing epigenetic changes in the preimplantation embryo.

Keywords: DNA methylation, embryo culture, epigenetic defects, folate, human embryonic stem cells, preimplantation embryo

Introduction

The vulnerability of the preimplantation embryo to epigenetic perturbation has now been sufficiently demonstrated in a range of species, including humans, mouse, sheep and cow (reviewed by Gosden *et al.*, 2003; Maher *et al.*, 2003; Allegrucci *et al.*, 2004) to warrant further mechanistic studies. Both embryo culture environments and maternal nutrition appear to influence the DNA methylation status of specific genomic regions in a manner that can have profound or subtle developmental consequences (Young *et al.*, 2001; Mann *et al.*, 2004; Waterland and Jirtle, 2004). The question remains, however, as to how such epigenetic changes are induced in early development and which environmental factors are responsible.

The hypothesis has been developed that altered input to the linked methionine/folate metabolic cycles, either during non-physiological in-vitro embryo culture or through alterations to maternal diet around the time of conception, can modify *S*-adenosyl methionine (SAM)-mediated cellular methylation reactions in the early embryo (Young *et al.*, 2004). Both histone and DNA methylation reactions could be disrupted by this process, since SAM is an essential precursor for establishing de-novo methylation patterns in early developmental lineages as well as for recapitulating methylation patterns at DNA replication. DNA and histone methylation are both crucial regulators of gene expression (Jaenisch and Bird, 2003), although the particularly stable inheritance of aberrant DNA methylation throughout subsequent cell cycles may provide a

more likely potential mechanism to alter development in the longer term. The byproduct of SAM methyl group donation, *S*-adenosyl homocysteine (SAH) also acts as an inhibitor of DNA methyltransferase enzymes, providing another potential route for epigenetic modification via methionine/ folic acid cycle disruption (reviewed by Ross, 2004). Furthermore, the use of SAM in polyamine biosynthesis results in the production of decarboxylated SAM, which is also a competitive inhibitor of DNA methyltransferases.

Since there are potential implications of this type of epigenetic perturbation for assisted reproduction treatment-induced developmental defects and in long-term adult disease programming (Young, 2001; Waterland and Jirtle, 2004; Young *et al.*, 2004), human embryonic stem cell (hESC) cultures have recently been established to model such potential mechanisms in the species of clinical interest, where detailed human embryo studies or nutritional interventions to pregnant women are not possible. In order to investigate the potential of hESC as an epigenetic programming model system, this study initially examined which key enzymes in the folate and methionine cycles (**Figure 1**) are expressed early in development. While production of SAM occurs in all cell types examined thus far, the relative activity of the enzymes in the methionine and linked folate cycles varies considerably, with the liver providing the most active site (Mato *et al.*, 2002). The extent to which metabolism leading to SAM formation occurs in ES cells is unknown and, so far

as is known, these cycles have not been studied in the preimplantation embryo. It was reasoned that the major methylation of the embryonic genome that occurs during preimplantation development (reviewed by Santos and Dean, 2004; Young and Beaujean, 2004) would require high concentrations of SAM, and thus expression of most or all potential components of the methyl regulatory pathways would be evident in human embryonic stem cells. This paper suggests a potential mechanism for epigenetic change in the culture of hESC and the embryos from which they were derived. Since the adaptability of the preimplantation embryo to changes in methyl cycle metabolism *in vitro* is not known, the study also examined the effect of the folate inhibitor, methotrexate, on cell cycle activity, indicated by elevated intracellular homocysteine.

Materials and methods

Culture of BGN01 cells

Culture of human embryonic stem cells BGN01 was carried out under a Material Transfer Agreement from Bresagen (Athens, GA, USA) and with permission from the UK Stem Cell Bank Steering Committee.

BGN01 cells were initially cultured according to the protocol supplied from Bresagen. Briefly, cells were maintained on mitomycin C (10 µg/ml; Sigma, Poole, Dorset, UK)-

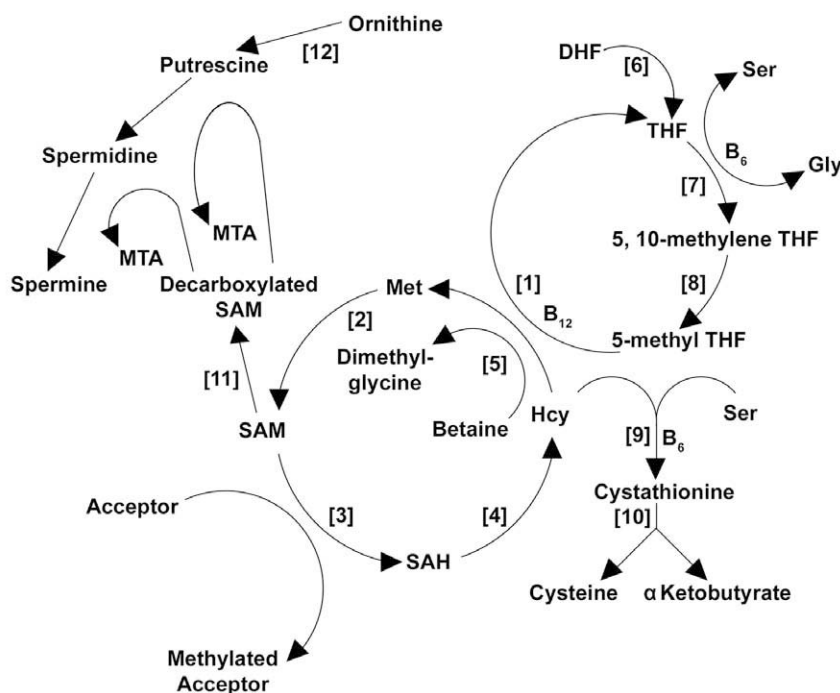


Figure 1. Methylation cycle and polyamine biosynthesis illustrating some of the key intermediary metabolites and enzymes. Metabolites: methionine (Met), *S*-adenosyl methionine (SAM), *S*-adenosyl homocysteine (SAH), homocysteine (Hcy), dihydrofolate (DHF), tetrahydrofolate (THF), serine (Ser), glycine (Gly), methylcobalamin (B12), pyridoxal phosphate (B6), 5-methylthioadenosine (MTA). Enzymes: methionine synthase [1a], methionine synthase reductase [1b], methionine adenosyl transferase [2], SAM-dependent methyltransferases [3], SAH-hydrolase [4], betaine-homocysteine methyltransferase [5], dihydrofolate reductase [6], serine hydroxymethyl transferase [7], 5,10-methylene THF reductase [8], cystathionine β-synthase [9], cystathionine γ-lyase [10], SAM decarboxylase [11], ornithine decarboxylase [12].

inactivated mouse embryonic fibroblasts feeder cells (MEF, from strain CD1, 6×10^4 cells/cm²) in DMEM/F12 (Invitrogen, Gaithersburg, MD, USA) supplemented with fetal bovine serum (FBS, Hyclone, South Logan, UT, USA), 5% knockout serum replacement (KSR, Invitrogen), 1% non-essential amino acids (Invitrogen), 2 mmol/l glutamine (Invitrogen), 0.1 mmol/l β -mercaptoethanol (Sigma), 4 ng/ml basic fibroblast growth factor (bFGF, Sigma) and passaged via dissociation in cell dissociation buffer (CDB, Invitrogen). Cells were seeded at a density of 2.5×10^4 cells/cm².

In order to establish cell cultures free of feeder cells (which might contaminate expression studies and confound the effects of methotrexate), conditioned medium was first prepared by incubation of MEFs overnight in KSR medium (DMEM/F12 supplemented with 15% KSR (Invitrogen), with 1% non-essential amino acids, 2 mmol/l glutamine, 0.1 mmol/l β -mercaptoethanol and 4 ng/ml bFGF. Conditioned medium was collected every 24 h, filtered, sterilized and then supplemented with an additional 4 ng/ml bFGF. BGN01 cultures were then treated with 0.05% trypsin for 2 min at 37°C and dissociated by gentle pipetting. Cells were seeded at a density of 2.5×10^4 cell/cm² onto Matrigel (Becton Dickinson, Franklin Lakes, NJ, USA)-coated dishes in the presence of MEF-conditioned medium and passaged every 3 days with trypsin.

Methotrexate treatment

BGN01 hES cells cultured in feeder-free conditions were treated for 7 days with 1 μ mol/l methotrexate (MTX, Sigma). In order to support cell growth in the presence of MTX, cells were supplemented with 100 μ mol/l thymidine (Sigma) and 100 μ mol/l hypoxanthine (Sigma). Cell pellets (washed in PBS) and media samples were stored at -80°C until analysis.

Reverse transcriptase-polymerase chain reaction analysis of enzyme expression

RNA was extracted from BGN01 cell pellets (snap frozen in liquid nitrogen and subsequently stored at -80°C) with the RNEasy Mini Kit and QIAshredder kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. cDNA was made from 1 μ g RNA with pd(N)6 primers and the First-Strand Synthesis Kit (Amersham Biosciences, Piscataway, NJ, USA). Polymerase chain reaction (PCR) primer sequences were either obtained from previous publications or designed from the relevant human sequences using Genefisher software (<http://bibiserv.techfak.uni-bielefeld.de/genefisher/>), as indicated in **Table 1**. Reverse transcriptase-PCR (RT-PCR) reactions were carried out in a total volume of 25 μ l containing 2 μ l cDNA, 2.5 μ l 10 \times buffer (Qiagen), 2.5 μ l 10 \times dNTPs (Invitrogen), 2.5 μ l forward and reverse primer mix (5 μ mol/l), 0.125 μ l Hotstar *Taq* (Qiagen) and 1.5–3.5 μ l MgCl₂ (see **Table 1**). After initial denaturation at 95°C for 15 min, 38–40 cycles of PCR (95°C 1 min, 55–60°C (see **Table 1**) 30 s, 72°C 1 min) on a Techne Flexigene thermocycler were followed by 72°C for 5 min. PCR products were analysed on a 1.6% agarose gel containing TBE buffer and ethidium bromide (0.2 μ g/ml). Human adult male liver cDNA (Ambion, Austin, TX, USA) was used as a positive control in all cases. Negative controls containing either no reverse transcriptase or no cDNA were

performed in each case.

Western blot analysis

Cell pellets were mixed with SDS-PAGE sample buffer [62.5 mmol/l Tris (pH 6.8) containing 2% SDS, 10% glycerol, 5% mercaptoethanol, and 0.001% bromophenol blue] and boiled for 5 min. The polypeptides were then separated on an 8% SDS-polyacrylamide gel and electrotransferred to nitrocellulose membrane for 1 h at 100 V in 25 mmol/l Tris, 192 mmol/l glycine, and 20% methanol. After transfer, the membranes were blocked with PBS containing 5% Marvel, and 0.1% Tween-20 for 1 h and then incubated with primary antibody for 1 h (anti-methionine synthase; 2 μ g/ml or *S*-adenosyl homocysteine hydrolase, 1 μ g/ml; Abcam, Cambridge, UK) at room temperature. The membranes were then washed five times with PBS containing 0.1% Tween-20, followed by incubation with appropriate peroxidase-conjugated secondary antibody (peroxidase-conjugated anti-goat antibody for methionine synthase (1 in 3000; Sigma, UK) and peroxidase-conjugated anti-rabbit antibody for *S*-adenosyl homocysteine hydrolase (1 in 2000; Pierce, Cramlington, UK) for 1 h at room temperature. The membranes were washed again and the bound antibody was detected using an enhanced chemiluminescence kit (Amersham-Pharmacia Biotech, Piscataway, NJ, USA), according to the manufacturer's directions.

Determination of thiols, amino acids and total protein

Cell pellets were suspended in 700 μ l phosphate-buffered saline containing a mixture of protease inhibitor cocktail (Sigma) and the suspensions sonicated on ice using three 20-s pulses to lyse the cells, followed by centrifugation at 18,000 *g* for 15 min. The supernatant fraction was then used to measure intracellular thiols and amino acids according to the methods of Pfeiffer *et al.* (1999) and Jauniaux *et al.* (1999) respectively. The method for thiol analysis was modified to reduce the pH of the mobile phase in order to facilitate better separation of cysteinyl glycine and glutathione. For amino acid analysis, the cytosol preparation (490 μ l) was mixed with 10 μ l norleucine internal standard and 30 mg 5-sulphosalicylic acid and was 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 μ m pore size and amino acids were measured using Biochrom 20 amino acid analyser with ninhydrin detection (Pharmacia LKB, Biochrom Ltd, Cambridge, UK). Peak integration was performed using EZChrom Elite Software (Scientific Software International Ltd, Duston, Northampton, UK). Total protein content was measured using the method of Bradford (1976) with bovine serum albumin as the standard protein. All metabolic determinations were normalized to the determined protein content.

Table 1. Primers for enzyme expression analysis.

Gene	Accession number	Primer sequence	Primer position		Product length	Optimum Mg ²⁺ concentration (mmol/l)
			F	R		
<i>MAT1A</i>	X69078	5'-GTT CAC ATC GGA GTC TG-3' ^a 5'-TCT CCT CCA GCG TGA TG-3'	57	652	596	2.5
<i>MAT2A</i>	NM_005911	5'-CTG TGC AGT ATA TGC AGG A-3' 5'-CTC TCT CTC ACT CTT CTG A-3'	554	1032	479	2.5
<i>MAT2B</i>	AF182814	5'-AAT CCA CCT TAC AGA GAG GA-3' 5'-GGT TGA AGG CAT CTG CAA-3'	430	811	382	2.5
<i>GNMT</i>	AF101477	5'-AAG AGG GCT TCA GTG TGA CG-3' ^a 5'-TCC TGG GCG ACT TCA AGC CT-3'	230	825	596	1.5
<i>SAHH</i>	NM_000687	5'-CTA TGG TGA TGT GGG CAA-3' 5'-CTT GTC TGG ATG GGT CCA-3'	660	1134	475	2.5
<i>CBS</i>	BC011381	5'-ACA TGA CCA AGT TCC TGA GC-3' ^a 5'-GCC ACG AAG TTC AGC AAG TC-3'	1142	1631	490 + 532	1.5
<i>CGL</i>	NM_153742	5'-GCA AGT GGC ATC TGA ATT TG-3' ^b	366	536	171	2.5
	NM_001902	5'-CCC ATT ACA ACA TCA CTG TGG-3'	366	668	303	
<i>MTR</i>	HSU73338	5'-ATG TCA CCC GCG CTC CAA GAC-3' ^a 5'-TCC AGA AGT CCT TTG GCC TGC-3'	1	554	554	2.5
<i>MTRR</i>	AF025794	5'-CCC TTG TGG ACT ATA CCA-3' 5'-CTG TCT TCA TGG GAT GCA-3'	1160	1536	376	3.5
<i>BHMT</i>	HSU50929	5'-GTC ATG CAG ACC TTC ACC TTC TA-3' ^a 5'-CTC CTT CAT GAG CTT CAC TG-3'	208	699	492	2.5
<i>BHMT2</i>	AF257473	5'-CAA GTG TGT GCA TAT TGA GC-3' ^c 5'-GTA TCT AGC TTA GTC ACT TG-3'	1245	1492	248	2.5
<i>cSHMT</i>	NM_004169	5'-CTA CTC CCG AAA CCT GGA-3' 5'-ACC ATC TGT GCC TTT GGA-3'	612	1110	499	2.5
<i>mSHMT</i>	BC044211	5'-GCT ACA TGT CTG ACG TCA-3' 5'-CTT CAC CCC TTT CCG GTA-3'	524	891	368	1.5
<i>MTHFR</i>	BC053509	5'-CTC TTC TAC CTG AAG AGC AA-3' 5'-GTT GAT GTT GGG CTG TGA-3'	1213	1467	255	2.5
<i>ADC</i>	BC000171	5'-GGA CTA TTC ACA TCA CTC CA-3' 5'-CAT CTA GGG CTT TCT GCA A-3'	719	1133	415	2.5
<i>ODC</i>	NM_002539	5'-GCT GTA CCG ATC CTG AGA-3' 5'-CGT GGT CAT AGA GTA TGC A-3'	602	1000	399	1.5
<i>FRα</i>	BC002947	5'-TGT AGT AGG GGA GGC TCA GA-3' 5'-CCT CGG CTG TAG TTG CTG A-3'	51	614	564	1.5
<i>FRβ</i>	NM_000803	5'-GAC AGG ACT GAT CTC CTC A-3' 5'-CTG ACA GTC CTC TTT GCA-3'	67	402	336	1.5
<i>DNMT1</i>	NM_001379	5'-GTG GGG GAC TGT GTC TCT GT-3' 5'-TGA AAG CTG CAT GTC CTC AC-3'	2512	2715	204	1.5
<i>DNMT3A</i>	AF067972	5'-GGG GAC GTC CGC AGC GTC ACA C-3' 5'-CAG GGT TGG ACT CGA GAA ATC GC-3'	2053	2332	280	1.5
<i>DNMT3B</i>	AF76228	5'-ACC TGC TGA ATT ACT CAC GCC-3' 5'-ATG TCC CTC TTG TCG CCA AC-3'	2006	2105	100	1.5
<i>DNMT3L</i>	AF194032	5'-CCC CTT GAG ATG TTC GAA-3' 5'-GAA CTG GAA CAG GTA CCA-3'	640	906	267	3.5

^aAvila et al. (2000).^bLevonon et al. (2000).^cChadwick et al. (2000).

Results

Expression of methyl cycle enzymes

The gene expression profiles of methionine/folate cycle-related enzymes in BGN01 human embryonic stem cells are shown in **Figure 2**.

Formation of S-adenosyl methionine, methyl group transfer from SAM and homocysteine fate

Methionine adenosyl transferase (*MAT*), which converts methionine to *S*-adenosyl methionine (SAM), has two isoforms that differ in their tissue expression and catalytic properties (LeGros *et al.*, 2000). *MAT2A* and *MAT2B*, but not *MAT1* expression was detected in BGN01 with all three genes being detected in the human liver positive control. The expression of the DNA methyltransferases *DNMT1*, *DNMT3A*, *DNMT3B* and *DNMT3L* was detected in the hESC as well as glycine *N*-methyltransferase (*GNMT*), which transfers a methyl group from SAM to glycine for sarcosine production, indicating the potential for methyl group transfer and production of SAH. The possibility of SAM methyl group donation to the polyamine biosynthetic pathways is also indicated by the expression of SAM (AdoMet) decarboxylase (*ADC*) and ornithine

decarboxylase (*ODC*). *S*-adenosylhomocysteine hydrolase (*SAHH*) RNA, and protein (**Figure 2A, C**) expression suggests the ability of hESC to convert SAH to adenosine and homocysteine. Homocysteine can then form cystathionine from cytoplasmic serine in the transulphuration pathway catalysed by cystathione beta synthase (*CBS*) and then cystathionine gamma lyase (*CGL*) converts cystathionine to cysteine. At least one isoform of *CBS* was detected in the hESC. Alternatively, homocysteine can be remethylated to methionine by methionine synthase (*MTR*), also expressed in the hESC at the RNA and protein (**Figure 2A, C**) concentrations alongside methionine synthase reductase (*MTRR*), which regenerates cobalamin to the methylcobalamin co-factor required in this reaction using methyl groups from SAM. The final potential mechanism for homocysteine remethylation uses betaine as a methyl donor rather than SAM, and expression of betaine homocysteine methyltransferase (*BHMT*) was also detected. The human *BHMT2* gene was also detected, albeit at a relatively low concentration.

Expression of folate cycle enzymes

The single carbon units for methionine synthesis are donated by methylenetetrahydrofolate, which is methylated by cytosolic serine hydroxymethyltransferase (cSHMT) during the

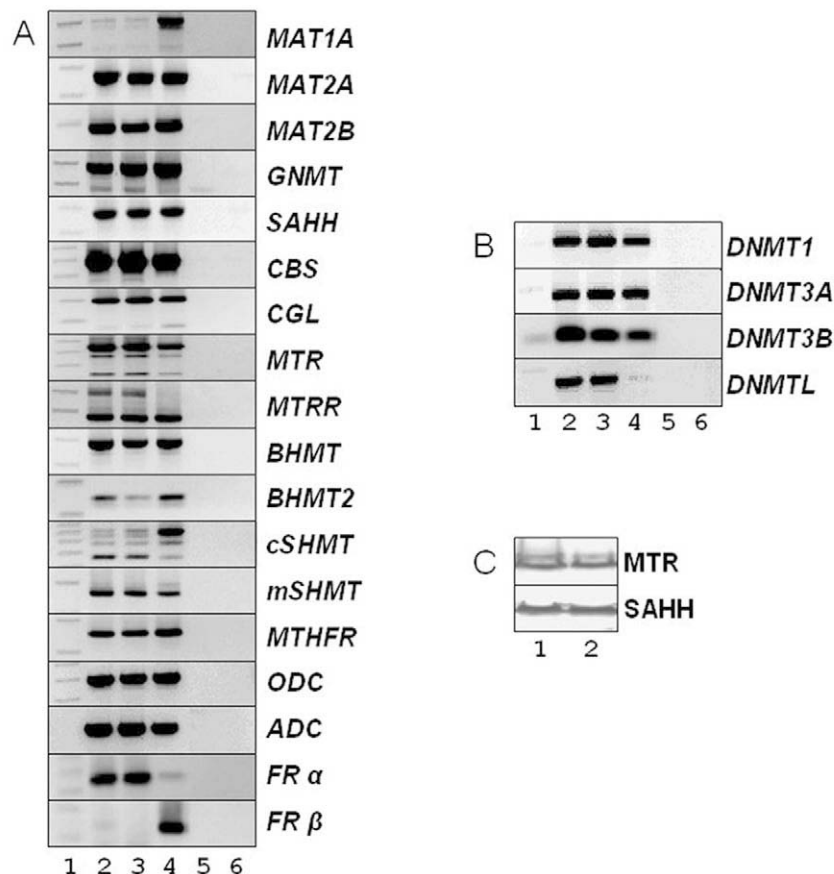


Figure 2. Methyl/folate cycle enzyme gene (A, B where 1 = 100-bp DNA ladder; 2 = BGN01; 3 = BGN01; 4 = adult human liver; 5 = reverse transcriptase negative control; 6 = cDNA negative control) and protein (C where 1 and 2 = duplicate samples of BGN01) expression in BGN01 human embryonic stem cells.

conversion of serine to glycine. The predominant 259-bp isoform detected in BGN01 lacked both exons 9 and 10 (Girgis *et al.*, 1998), with the 376- or 382-bp isoforms (lacking either exons 9 or 10), and a 499-bp transcript (containing both exons 9 and 10) also being detected, although expression of all isoforms was relatively low (**Figure 2A**). The independent, mitochondrial *SHMT* gene (*mSHMT*), on the other hand, was expressed at an equivalent level to that detected in the adult liver. The folate cycle is completed by reducing 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, with the catalytic methylenetetrahydrofolate reductase (MTHFR) enzyme also being expressed in hESC. Expression of the membrane bound receptors responsible for uptake of folate to mammalian cells was also examined. In contrast to adult liver, where the folate receptor β (FR β) is expressed, the main isoform expressed in BGN01 was the α receptor (FR α).

Effect of methotrexate treatment on methyl cycle activity and cell proliferation

BGN01 cells cultured with 1 $\mu\text{mol/l}$ MTX (with added thymidine and hypoxanthine) for 7 days demonstrated elevated cytosolic homocysteine (control 1.1 nmol/mg protein; MTX 1.8 nmol/mg protein), glutathione (control 270.0 nmol/mg protein; MTX 360.2 nmol/mg protein) and cysteine (control 65.9 nmol/mg protein; MTX 88.3 nmol/mg protein) concentrations. Medium concentrations of methionine, serine and sarcosine increased when cells were cultured with MTX (**Table 2**), while glycine concentration decreased. Cytosolic methionine and glycine concentrations were reduced by MTX, while cytosolic serine and sarcosine concentrations increased following MTX treatment.

Table 2. Amino acid concentrations in hESC culture media and cell lysates following 7 days of culture either in the presence or absence of methotrexate (MTX) with added thymidine and hypoxanthine.

Amino acid	Medium day 7		Cell lysate day 7	
	Control (μmol)	MTX	Control (nmol/mg protein)	MTX
Methionine	66.3	85.1	2.6	2.2
Serine	420.3	726.7	25.6	37.3
Glycine	524.5	383.0	29.2	22.4
Sarcosine	30.4	42.4	76.5	87.1

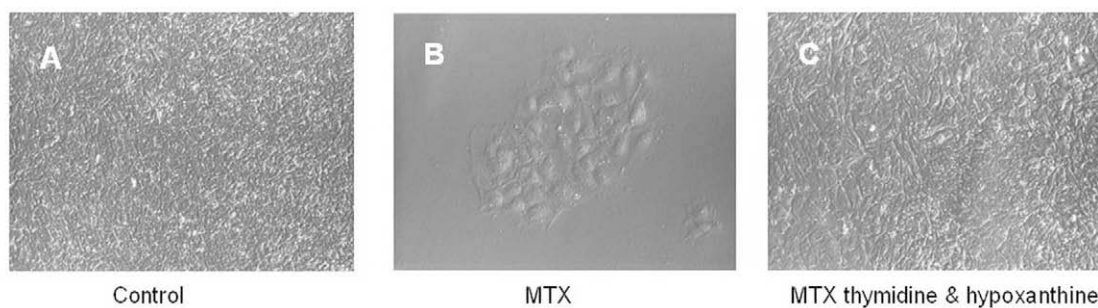


Figure 3. Effect of methotrexate treatment on BGN01 cells after 7 days of culture with (A) no methotrexate, (B) methotrexate, (C) methotrexate and thymidine and hypoxanthine.

While methotrexate-treated cells in the absence of hypoxanthine and thymidine demonstrated considerably reduced proliferation (control $1.2 \times 10^5/\text{cm}^2$; methotrexate $1.1 \times 10^4/\text{cm}^2$), supplemented cells showed no difference in cell counts from controls ($1.1 \times 10^5/\text{cm}^2$; **Figure 3**).

Discussion

With the exception of MAT1A and low expression of BHMT2, transcripts for all potential enzymes involved in the interrelated folate and methionine cycles, and transulphuration pathway, were detected in the hESC line, BGN01. The only tissues previously identified to exhibit the complete transulphuration pathway were liver, kidney, intestine and pancreas (James *et al.*, 2002). The breadth of enzyme gene expression detected in hESC suggests the potential for a number of mechanisms whereby cellular DNA methylation reactions could be altered by in-vitro culture conditions or maternal diet. These include alterations to SAM availability for methyl group donation, alterations to the concentration of decarboxylated SAM or SAH that can lead to inhibited DNA methyltransferase activity (Wu and Santi, 1987), and the indirect effects of folate-induced DNA damage and repair (Ross, 2004).

The presence of transcripts for all key components is likely to be a relevant indicator of functionality, or at least the potential for mRNA translation and protein function in response to changing environmental conditions. Since the hESC used were cultured on Matrigel for over 10 passages, it is extremely unlikely that contaminating mouse fibroblast feeder transcripts would remain detectable in the cell line. However, the possibility that enzymes not expressed in the human blastocyst are transcribed during hESC derivation or culture, although unlikely, cannot be excluded. hESC share the pluripotentiality of the inner cell mass, retain the ability to form trophoblast (Xu *et al.*, 2002), express the same pluripotent cell surface markers as the human (but not mouse) embryo (Henderson *et al.*, 2002) and can retain normal karyotype (Mitalipova *et al.*, 2005) at least at lower passage. In this study, these characteristics apply to cells cultured on Matrigel as well as on mouse feeder layers (L Young, unpublished data). Given the overall similarities between human embryo and hESC culture media, it is proposed that that human embryonic stem cells will provide an appropriate model for the human embryo for a range of epigenetic and other studies, at least for establishing mechanistic principles.

Comparisons with the expression profiles of human liver provide interesting clues as to methyl cycle activity in hESC. While methionine adenosyl transferase (MAT), which converts methionine to SAM, is expressed in most, if not all, tissues, there is a liver-specific form (MAT1; most methionine metabolism occurs in the liver) with a relatively high K_m for methionine, and an extrahepatic form (MAT2) with a low K_m . MAT1 is activated by its product, SAM, such that the liver is the only known tissue capable of responding to excessive methionine (Van der Put *et al.*, 2001). The lack of MAT1 expression in hESC is consistent with embryos encountering comparatively low concentrations of methionine in oviductal and uterine fluids (14–50 mmol/l; Casslen, 1987; Tay *et al.*, 1997) and its lack of induction in hESC culture media containing supraphysiological methionine may suggest an

intrinsic mechanism to attenuate potential cycle disruption during the crucial period of embryo epigenetic reprogramming, when the ability to significantly increase SAM production may be undesirable. Nevertheless, readily detectable expression of the MAT2A and MAT2B subunits is consistent with active production of SAM in hESC (De La Rosa *et al.*, 1995).

Conversion of SAM to S-adenosyl homocysteine (SAH) occurs when a variety of methyltransferase enzymes transfer the SAM methyl group to their targets, including DNA, RNA, proteins, polysaccharides, lipids and a range of small molecules (Cheng and Roberts, 2001). Expression of *DNMTs 1, 3A* and *3B* in BGN01 is consistent with SAM utilization for DNA methylation. Future studies on histone methyltransferase expression will determine SAM involvement in other epigenetic processes. The mouse embryo undergoes genome wide demethylation by the 8-cell stage, de-novo methylating the inner cell mass at the blastocyst stage (Santos *et al.*, 2002). In contrast, the sheep embryo only loses around 40% of genome wide DNA methylation in the cleavage stages, with no global increase in blastocyst methylation (Beaujean *et al.*, 2004a,b,c), but an apparent requirement for maintenance methylation. Thus the requirements for SAM as DNA methylation substrate are likely to vary between species and developmental stages. Preliminary studies on the human embryo suggest some demethylation around the 4-cell stage with only limited de-novo methylation in the inner cell mass and more pronounced de-novo methylation in the trophoblast (Fulka *et al.*, 2004). These results are consistent with both maintenance and de-novo methylation requirements of the human inner cell mass and with the methyl cycle expression profiles in hESC. Huntriss *et al.* (2004) have also reported expression of *DNMTs 1, 3A, 3B* and *3L* in human embryos and another hESC line isolated by Lanzendorf *et al.* (2001).

Other genes that act as methyl group acceptors from SAM were also detected in BGN01. Although glycine N-methyltransferase is generally considered a liver-specific enzyme in the fetus and adult (accounting for 1% of liver cytosolic protein), it has also been reported in the adult pancreas and prostate (Chen *et al.*, 2000). The primary function of GNMT is to remove excess SAM synthesized during a methionine load and thus regulate SAM:SAH ratio. Glycine is methylated to sarcosine, with the production of SAH. Abundant expression of *GNMT* in hESC indicates significant SAM synthesis, requiring effective removal when SAM utilization is low (Mato *et al.*, 2002). Since the human inner cell mass DNA exists in a relatively hypomethylated state (Fulka *et al.*, 2004), high GNMT expression may provide a means to prevent aberrant hypermethylation of the pluripotent genome. Methotrexate reduced glycine and increased sarcosine concentrations in hESC lysates and spent media (**Table 3**), effects consistent with the anti-folate actions of MTX in reducing 5-methyl THF, a known inhibitor of GNMT (Stipanuk, 2004).

Expression of key enzymes of the polyamine biosynthetic pathway, SAM (AdoMet) decarboxylase and ornithine decarboxylase, indicate a further role for SAM in hESC. Changes in polyamine concentrations are known to affect the degree of cellular DNA methylation, proliferation and

Table 3. Folate, methionine, vitamin B₁₂ and vitamin B₆ composition of common embryo culture media.

<i>Medium</i>	<i>Typical use (days)</i>	<i>Folate^a</i>	<i>Methionine^a</i>	<i>Vitamin B₁₂^a</i>	<i>Vitamin B₆^a</i>	<i>Information source and examples of use</i>
M16	0–blast (mouse)	0	0	0	0	Composition: Sigma M7292, e.g. Devgan and Seshagiri, 2003
KSOMaa	0–blast (mouse) (human)	0	50 µmol/l	0	0	Composition: 0.5× MEM Essential Amino Acids Invitrogen (Gibco) as in Ho <i>et al.</i> , 1995, e.g. mouse: Rinaudo and Schultz, 2004, e.g. human: Wiemer <i>et al.</i> , 2002
SOFaa (ruminant)	0–blast (ruminant)	0	112.5 µmol/l	0	0	Composition: with 2.25× BME Essential Amino Acids Sigma B6766, e.g. Holm <i>et al.</i> , 1999
TCM199	0–blast (ruminant)	23 nmol/l	0.2 mmol/l	0	120 nmol/l	Composition: Sigma M5017, e.g. Algriany <i>et al.</i> , 2004; Chohan and Hunter, 2004
P-1 medium	0–3 (human)	0	0	0	0	Composition: Irvine Scientific 99242, e.g. Barak <i>et al.</i> , 1998; Behr <i>et al.</i> , 1999; Milki <i>et al.</i> , 2000
HTF medium	0–3 (human)	0	0	0	0	Composition: Irvine Scientific 90125, e.g. Barak <i>et al.</i> , 1998; Artini <i>et al.</i> , 2004
Blastocyst medium	3–blast (human)	3 µmol/l	30 µmol/l	1 µmol/l	1 µmol/l	Composition: Irvine Scientific 99292, e.g. Behr <i>et al.</i> , 1999; Milki <i>et al.</i> , 2000
Ham's F12	2–blast (human)	2.95 µmol/l	30.2 µmol/l	1.03 µmol/l	0.291 µmol/l	Composition: Invitrogen (Gibco) 21765029, e.g. Conaghan <i>et al.</i> , 1998
G1 medium (Vitrolife)	0–3 (human)	0	0	0	0	Composition: Vitrolife personal communication e.g. Coskun <i>et al.</i> , 2000
G2 medium (Vitrolife)	3–blast (human)	0	50 µmol/l	0	4.9 µmol/l	Composition: Vitrolife personal communication, e.g. Coskun <i>et al.</i> , 2000
M3 medium	2–blast (human)	2.49 µmol/l	24.8 µmol/l	0.07 µmol/l	0.3 µmol/l	Composition: MediCult personal communication, e.g. Barak <i>et al.</i> , 1998
BlastAssist® medium 1	0–2 (human)	0	0	0	0	Composition: MediCult personal communication, e.g. Kovavic <i>et al.</i> , 2002; Zollner <i>et al.</i> , 2004
BlastAssist® medium 2	2–blast (human)	0	50.9 µmol/l	0	0	Composition: MediCult personal communication e.g.: Kovavic <i>et al.</i> , 2002; Zollner <i>et al.</i> , 2004
ISM1™	0–3 (human)	0	98 µmol/l	0	0	Composition: MediCult personal communication
ISM2™	3–blast (human)	4.5 µmol/l	103 µmol/l	0.7 µmol/l	5.9 µmol/l	Composition: MediCult personal communication
DMEM-F12	hESC (BGN01)	6 µmol/l	12 µmol/l	0.5 µmol/l	12 µmol/l	Composition: Invitrogen (Gibco) 11320
Human serum	N/A	14 nmol/l	21 µmol/l	0.299 nmol/l	12 nmol/l	Composition: Jauniaux <i>et al.</i> , 1999, Campbell <i>et al.</i> , 1993
Human uterine fluid	N/A	ND	0.05 mmol/l	ND	ND	Composition: Casslen, 1987
Human follicular fluid (serum in parentheses)	N/A	15.7 nmol/l (17.7)	15.9 µmol/l (19.9)	0.196 nmol/l (0.251)	36 nmol/l (53)	Composition: Steegers-Theunissen <i>et al.</i> , 1993

^aData in these columns are nutrient concentrations.

differentiation (Heby, 1995; Frostesjo *et al.*, 1997). This effect, however, is due to the increased generation of decarboxylated SAM by SAM decarboxylase, which acts as a competitive inhibitor of DNMT1 (Heby *et al.*, 1988). In scenarios where actively dividing cells manufacture spermine from ornithine via ornithine decarboxylase, increased SAM utilization will result in a concomitant clearance of decarboxylated SAM, thus removing the inhibition of DNA methylation.

DNA methylation reactions in the cell are thought to be regulated by SAM:SAH ratio, since SAH is also a potent inhibitor of DNMT1 (Kredich and Hershenfield, 1980; Cantoni *et al.*, 1986; Chiang *et al.*, 1996). Although SAM conversion to SAH occurs via 1:1 stoichiometry, differential utilization of SAM in the cell's many methylation reactions and differential clearance of homocysteine via the alternative MTR, BHMT and CBS-catalysed reactions can result in altered SAM:SAH. Furthermore, the intracellular accumulation of homocysteine drives SAHH to catalyse the energetically favourable reverse reaction and synthesize SAH (Yi *et al.*, 2000). Methionine synthase has a low K_m for homocysteine (<0.1 mmol/l) while the K_m for cystathionine β -synthase is high (>1 mmol/l; Medina *et al.*, 2001). Thus, at low homocysteine concentrations methionine conservation is favoured, whilst at high homocysteine concentrations, immediate and long-term clearance of homocysteine via the trans-sulphuration pathway is ensured. Three species of MTR have been detected in humans by northern blot, although the relative distribution varies little between tissues (Medina *et al.*, 2001). Highest transcript levels in the adult are seen in the pancreas, skeletal muscle and heart, but transcript expression is relatively low in the liver. In the current study, BGN01 cells showed similar relative expression of the three detected MTR isoforms to that of adult human liver, with the highest molecular weight band (554 bp) being the most abundant in both cases. Tissue-specific isoforms of CBS have also been demonstrated with differences between the adult and fetus (Bao *et al.*, 1998; Quere *et al.*, 1999), but their significance in terms of enzyme activity is not clear.

The low and almost undetectable expression of BHMT2 in BGN01 is consistent with expression largely confined to the liver and kidney in humans (Park *et al.*, 1999), where betaine provides an alternative methyl donor for homocysteine remethylation, although a similar BHMT expression level to adult human liver does suggest the potential for betaine metabolism in hESC. Betaine also increases the availability of 1-carbon units in the folate pool, allowing increased methylation by MTR and a secondary route via which potentially toxic homocysteine concentrations can be lowered in highly active tissues (Heil *et al.*, 2000). Since SAHH conversion of SAH to homocysteine helps to remove the potential inhibition of DNMTs by SAH, the abundance of this enzyme is likely to reflect relative DNA methylation activity. With equivalent apparent abundance in hESC to adult liver, DNA methylation activity is also likely to be high. Liver activity of SAHH is also 10-fold higher in liver than in lung, for example (Merta *et al.*, 1995).

Expression of folate cycle enzymes in BGN01 provides the potential for environmental folate concentrations to alter methyl metabolism in early human embryonic cells, a scenario demonstrated by increased concentrations of homocysteine

and transulphuration pathway metabolites (cysteine and glutathione) with methotrexate. Both the cellular and mitochondrial forms of SHMT (*cSHMT* and *mSHMT*) were detected in hESC. These enzymes catalyse the reversible interconversion of serine/THF to glycine/methylene THF and produce 1-C units for methionine synthesis. Since mitochondria provide the majority of 1-C units required in the cytoplasm, cytosolic SHMT function is unknown. Human embryonic stem cells (Sathananthan *et al.*, 2002) contain abundant mitochondria thus would be expected to provide sufficient 1-C units from mSHMT activity. mSHMT concentrations are relatively constant in all cell types, with slight enrichment in liver and kidney (Stover *et al.*, 1997). *cSHMT*, however, is predominantly in kidney and liver, with at least six isoforms generated from alternative splicing (Girgis *et al.*, 1998). The present study detected the same four *cSHMT* isoforms in BGN01 as in adult liver. However, the isoform containing both exons 9 and 10 was most abundant in liver, but the isoform lacking exons 9 and 10 was most abundant in the hESC. *mSHMT* expression was also similar to liver.

The expression of folate receptors, used to internalize dietary folates for cytosolic reactions, was markedly different in BGN01 compared with adult human liver. BGN01 expressed the folate receptor α , but not the β form detected in liver. A similar scenario also exists in mouse embryonic stem cells (Bolton *et al.*, 1999) and liver (Gates *et al.*, 1996). The β form is thought to bind methyltetrahydrofolate with 50-fold less affinity than folic acid, whereas the α form binds both equally. Interestingly, FR α expression is up-regulated in a range of cultured tumour cells, but this may not reflect the expression pattern *in vivo* (Elnakat and Ratnam, 2004). Whether this is a response to culture in supraphysiological concentrations of folate or to recombination events during the transformation process is not known (Elnakat and Ratnam, 2004). However, nasopharyngeal carcinoma cells, grown in physiological concentrations of folate (2–10 nmol/l), showed significantly higher levels of FR α expression than cells maintained in Dulbecco's modified eagle medium containing >2000 nmol/l folic acid. Intracellular SAH was also increased 100% by folate deficiency (Jhaveri *et al.*, 2001). In the present study, methotrexate-induced folate depletion of the hESC medium (originally containing 6000 nmol/l folate), resulted in a 63% increase in homocysteine concentrations, indicating a similar effect on the methyl cycle. The range of animal and human embryo culture media investigated contained between 0 and 4.5 μ mol/l, i.e. around 300-fold the concentration reported in maternal serum or human follicular fluid (Table 3). While these are still lower than the approximately 400-fold physiological concentrations in the hESC culture medium, the principle that either a complete lack of folate in the embryo culture environment or supraphysiological concentrations could alter methyl cycle metabolism is evident. Since SAH concentrations are considered an important indicator of inhibiting DNA methylation reactions (Capdevila *et al.*, 1997), measurement in commercially available media where concentrations are not disclosed, may be a useful initial measure of any potential methyl cycle shift.

Several other components of embryo culture media that may be present in non-physiological concentrations, could also impact on the methyl cycle. For example, vitamin B₁₂ is either absent or is present in up to 3000–5000 fold and vitamin B₆ is

also present in up to 160–500-fold the concentration reported in maternal serum or human follicular fluid (**Table 3**). The addition of serum to cultures also adds an unknown dimension.

Overall, these expression studies indicate that the methionine/folate cycles are active in hESC, providing a putative mechanism for epigenetic perturbation in assisted reproduction. Serum supplementation of bovine embryo culture medium can result in a significant alteration of SAM:SAH in blastocysts (Rooke *et al.*, 2003), indicating folate/methyl cycle enzyme activities, at least in this species. Interestingly, the serum supplementation conditions were those that resulted in high incidence of large offspring syndrome (LOS) after sheep embryo culture (Sinclair *et al.*, 1999; Young *et al.*, 2001). The parallels in phenotype and epigenetic disruption in imprinted genes between LOS in ruminants with Beckwith–Wiedemann syndrome found to be more prevalent amongst human assisted reproduction births (reviewed by Maher *et al.*, 2003) suggest some commonality in mechanism (see Young, 2003). Microarray studies showing the effects of folate depletion in tumour cells have demonstrated a gene-specific response in only a subset of the genome (Jhaveri *et al.*, 2001; Crott *et al.*, 2004); perhaps a similar effect in embryos might result in non-lethal, altered developmental programming. The stochastic nature of early epigenetic programming (reviewed by Blewitt *et al.*, 2004; Moore and Ball, 2004) may result in variations in genes affected in different embryos. Furthermore, common polymorphisms in MTR, MTHFR and CBS in the human population (reviewed by Ueland *et al.*, 2001; Friso and Choi, 2002) may result in different hESC lines or individual embryos in culture, eliciting differential responses even within the same culture environment. This possibility could underlie the heterogeneity in response to identical culture conditions evident for example in imprinting phenotypes (see Young *et al.*, 2004).

It is believed that refining key dose-response experiments in the hESC model should ultimately allow the design of more limited studies that can be carried out on animal embryos and on research-donated human embryos to further test the proposed hypothesis. Revealing the composition of commercially available culture media would also be a prudent and important step in the ultimate safety evaluation of clinical assisted reproduction.

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