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Altered folate metabolism modifies cell proliferation and progesterone secretion in human placental choriocarcinoma JEG-3 cells

Carolyne Moussa[1,2], Nikia Ross[1], Philippe Jolette[1,3] and Amanda J. MacFarlane[1,2,3]*

[1]*Nutrition Research Division, Health Canada, Ottawa, ON, Canada K1A 0K9*
[2]*Department of Biology, Carleton University, Ottawa, ON, Canada K1S 5B6*
[3]*Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, ON, Canada K1H 8M5*

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Abstract

Folate is an essential B vitamin required for *de novo* purine and thymidylate synthesis, and for the remethylation of homocysteine to form methionine. Folate deficiency has been associated with placenta-related pregnancy complications, as have SNP in genes of the folate-dependent enzymes, methionine synthase (*MTR*) and methylenetetrahydrofolate dehydrogenase 1 (*MTHFD1*). We aimed to determine the effect of altered folate metabolism on placental cell proliferation, viability and invasive capacity and on progesterone and human chorionic gonadotropin (hCG) secretion. Human placental choriocarcinoma (JEG-3) cells cultured in low folic acid (FA) (2 nm) demonstrated 13 % ($P < 0·001$) and 26 % ($P < 0·001$) lower proliferation, 5·5 % ($P = 0·025$) and 7·5 % ($P = 0·004$) lower invasion capacity, and 5 to 7·5 % ($P = 0·004–0·025$) lower viability compared with control (20 nm) or supplemented (100 nm) cells, respectively. FA concentration had no effect on progesterone or hCG secretion. Small interfering RNA (siRNA) knockdown of *MTR* gene and protein expression resulted in 17·7 % ($P < 0·0001$) lower proliferation and 61 % ($P = 0·014$) higher progesterone secretion, but had no effect on cell invasion and hCG secretion. siRNA knockdown of *MTHFD1* gene expression in the absence of detectable changes in protein expression resulted in 10·3 % ($P = 0·001$) lower cell proliferation, but had no effect on cell invasion and progesterone or hCG secretion. Our data indicate that impaired folate metabolism can result in lower trophoblast proliferation, and could alter viability, invasion capacity and progesterone secretion, which may explain in part the observed associations between folate and placenta-related complications.

Key words: Folate: Folic acid: Placenta: Human chorionic gonadotropin: Progesterone

Folate is an essential water-soluble B vitamin required for fetal and placental development. Folate deficiency is a risk factor for pre-eclampsia(1–3) and placental abruption(4). The use of folic acid (FA)-containing multivitamins in the peri- and postconceptional periods has been associated with a reduced risk for neural tube defects(5) and preterm delivery(6). Use of antifolate drugs during pregnancy has been associated with increased risk for pre-eclampsia, placental abruption, fetal growth restriction and fetal death(1). Further implicating folate in placental health is the association of SNP in genes of the folate-dependent enzymes methionine synthase (*MTR*) and methylenetetrahydrofolate dehydrogenase 1 (*MTHFD1*) with placenta-related pathologies.

The *MTR* 2756 A > G SNP was associated with maternal hyperhomocysteinemia, uteroplacental insufficiency(7) and recurrent pregnancy loss(8). MTR transfers a methyl group from 5-methyltetrahydroflate (5-methylTHF) to homocysteine (Hcy) to form methionine and tetrahydrofolate (THF)(9). Reduced *MTR* expression would result in a 'methyl trap' where 5-methylTHF and Hcy accumulate, and methionine and THF production is reduced(10). Reduced THF could lower *de novo* nucleotide biosynthesis and cell proliferation, and reduced methionine could lower cellular methylation capacity through reduced production of the universal methyl donor *S*-adenosylmethionine. As a consequence, reduced *MTR* expression could lead to altered placental and fetal development.

The *MTHFD1* 1958 G > A SNP, a genetic polymorphism in the 10-formylTHF synthetase (FTHFS) domain of MTHFD1, was associated with an increased risk for placental abruption(11,12), spontaneous second-trimester pregnancy loss(13), intra-uterine growth restriction(7) and neural tube defects(11,12,14). The *MTHFD1* gene encodes three different enzymatic activities, including the FTHFS, methenylTHF cyclohydrolase (MTHFC) and methyleneTHF dehydrogenase (MTHFD) activities(9). The product of the FTHFS activity, 10-formylTHF, can be used in *de novo* purine synthesis or be sequentially reduced by the MTHFC and MTHFD activities to form 5,10-methyleneTHF(9).

Abbreviations: BrdU, bromodeoxyuridine; dTMP, thymidylate; FA, folic acid; hCG, human chorionic gonadotropin; MTHFD1, methyleneTHF dehydrogenase 1; MTR, methionine synthase; siRNA, small interfering RNA; THF, tetrahydrofolate.

* **Corresponding author:** A. J. MacFarlane, email amanda.macfarlane@hc-sc.gc.ca

5,10-MethyleneTHF can either be used in thymidylate (dTMP) synthesis or be reduced further to 5-methylTHF and used for methionine synthesis(9). Elimination of FTHFS activity in mice results in impaired purine synthesis and negatively impacts embryo development and viability(15,16).

The placenta has specialised fetal trophoblasts important for implantation and development of the maternal–fetal interface(17,18). After 2–3 weeks of conception, the maternal spiral arteries are invaded by fetal cytotrophoblasts, which take on the characteristics of endothelial cells and allow the flow of a larger blood supply to the placenta(18). Human chorionic gonadotropin (hCG), secreted early in pregnancy, stimulates trophoblast migration and invasion, promotes angiogenesis and trophoblast differentiation and regulates progesterone secretion(19–22). Progesterone is a steroid hormone initially secreted by the corpus luteum, but after approximately 9 weeks of gestation its expression shifts to placental cytotrophoblasts and syncytiotrophoblasts(19,23). Progesterone promotes decidualisation of endometrial cells at the maternal–fetal interface(24) and reduces trophoblast migration and invasive capacity(22). Improper cytotrophoblast invasion is associated with the inappropriate secretion of hCG and progesterone(25,26).

The data suggest that altered folate metabolism due to nutritional deficiency or genetic polymorphisms may play a role in placental development and function. Here we investigate the effect of altered folate metabolism on trophoblastic choriocarcinoma JEG-3 cell proliferation, viability, invasion and hormone secretion in two ways. First, cells were cultured in physiologically relevant FA concentrations: low (2 nM); control (20 nM); supplemented (100 nM). The WHO defines folate deficiency as having a serum folate <6·8 nM, the normal range as 13·5–45·3 nM and elevated as >45 nM. Data from the National Health and Nutrition Examination Survey in the USA indicate that the distribution of serum folate concentration in a population exposed to FA fortification and FA supplements ranges up to, and is even higher than, 100 nM(27). We also used RNAi knockdown to lower the expression of two key folate-dependent enzymes, *MTR* and *MTHFD1*, which have been associated with placenta-related pathologies.

Methods

Cell culture

Human placental choriocarcinoma JEG-3 cells (ATCC) were cultured at 37°C in 5 % CO_2 in growth medium consisting of MEM Alpha Modification medium (Hyclone) supplemented with 10 % (v/v) fetal bovine serum (FBS) (Hyclone), 100 µg/ml streptomycin (Hyclone), 100 U/ml penicillin (Hyclone) and 100 µg/ml non-essential amino acids (Hyclone). Media were changed every 48 to 72 h. To determine the effect of FA concentration, cells were cultured in Defined medium containing 2 (low), 20 (control) or 100 (supplemented) nM-FA (Sigma). Defined medium consisted of MEM Modified (Hyclone; lacks methionine, pyridoxyl-L-phosphate and FA; gift from Patrick Stover, Cornell University) supplemented with 26 mM $NaHCO_3$ (Sigma), 1 mg/l pyridoxyl-L-phosphate (Sigma), 200 µM-L-methionine (Sigma), 10 % (v/v) FBS (dialysed and charcoal treated), 100 µg/ml streptomycin and 100 U/ml penicillin.

Small interfering RNA gene knockdown

Genes of interest were knocked down using the RNAi Human/Mouse Starter Kit (Qiagen) following the 'Fast-Forward Transfection of Adherent Cells with siRNA' protocol described by the manufacturer. Knockdown was achieved by dual transfection of two gene-specific small interfering RNA (siRNA) constructs for each gene (online Supplementary Table S1; Qiagen). The final concentrations of siRNA were 12·5 and 20 nM for *MTR* and *MTHFD1*, respectively. AllStars Negative Control siRNA and AllStars Hs Cell Death Control siRNA (Qiagen) were used as negative and positive transfection controls, respectively, at a final concentration of 10 nM. A vehicle-only control included only HiPerFect Transfection Reagent (Qiagen) to control for cytotoxicity or other non-specific effects.

JEG-3 cells were seeded at 5×10^6 cells/well in 2 ml of growth medium in six-well plates and incubated for 1 h. The siRNA constructs and HiPerFect Transfection Reagent were added to MEM Alpha Modification medium without FBS (online Supplementary Table S2). DEPC-H_2O was added to a final volume of 300 µl, which was added drop-wise to the cells. Cells were transfected for 24 or 72 h at 37°C. Cells maintained for 72 h were re-transfected at 48 h, following the same protocol, with each well subcultured into two wells.

RT-quantitative PCR

Total RNA was extracted using the RNeasy Mini Kit and Qiagen QIAshredder (Qiagen) following the manufacturer's instructions. A 10 min incubation period was added before elution of the RNA with RNAse-free water. cDNA was synthesised using the High Capacity RNA-to-cDNA Kit (Applied Biosystems) in a DNA Thermal Cycler (BIORAD). RNA of 1 µg was used to synthesise cDNA with a mix of oligodT and random nonomer primers (Applied Biosystems) in a final volume of 20 µl.

RT-quantitative PCR analysis was conducted using the TaqMan Fast Universal PCR Master Mix (2×) protocol (Applied Biosystems) and the CFX96 Real Time PCR Machine (BIORAD). Fluorescein amidite-labelled probes (Applied Biosystems) specific for *MTR*, *MTHFD1* and the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) were used. Samples were analysed in duplicate. Knockdown results were calculated using the $2^{-\Delta\Delta C_t}$ method. The mean C_t value of two technical replicates for each biological replicate was calculated, and two biological replicates were performed for every knockdown assay. In brief, the mean C_t value for each sample was normalised to the mean C_t value of *HPRT* achieving the ΔC_t value for each gene of interest. The ΔC_t values of knockdown samples were normalised to the ΔC_t value of the AllStars Negative Control resulting in a $\Delta\Delta C_t$ value representing the relative gene expression of *MTR* or *MTHFD1* to endogenous gene expression. Fold change in gene expression was determined using the equation $2^{-\Delta\Delta C_t}$.

Western blotting

Transfected cells were treated with 200 µl protein lysis buffer (0·15 M-NaCl, 5 mM-EDTA (pH 8), 1 % Triton X100, 10 mM-Tris-Cl, 5 mM-DTT and 1 µM-PMSF). Samples were sonicated using a Misonix Sonicator (Misonix). Cells were centrifuged at 18 800 ***g*** for 15 min at 4°C. Protein concentration was determined using a modified Lowry assay(28) and was measured using a Multiskan Spectrum spectrophotometer (Thermo) at an absorbance of 740 nm. Cell lysates containing 40 µg of protein were electrophoresed on Criterion's pre-cast TGX Stain-free 8–16 % polyacrylamide gels (BIORAD).

The gel was equilibrated in transfer buffer containing 20 % (v/v) methanol for approximately 10 min. Proteins were transferred to a polyvinylidine fluoride membrane at 4°C for 1 h. The membrane was removed and placed in blocking buffer (5 % skim milk powder in TBST containing 0·1 % Tween 20) for 1 h. The membrane was incubated overnight at 4°C with primary antibody (1:5000 mouse polyclonal anti-MTR (Santa Cruz Biotechnology) or 1:1000 mouse monoclonal anti-MTHFD1 (Abnova)). An anti-mouse IgG secondary antibody conjugated to horseradish peroxidase was used. Membranes were washed and developed in Enhanced Chemiluminescence solution (BIORAD), and the image was acquired using an Alpha Innotech Fluor Chem HD 2 Imaging System.

Cell proliferation

Cell proliferation was measured by staining JEG-3 cells with bromodeoxyuridine (BrdU) (Sigma). Cells (5×10^5) were plated on sterile coverslips in Defined medium containing 2, 20 or 100 nM-FA in a six-well plate for 24 h. For analysis under knockdown conditions, BrdU staining was performed on cells transfected for 24 h. BrdU was added to a final concentration of 10 µM, and cells were incubated for 2 h at 37°C. Cells were washed twice with PBS, fixed in 70 % ethanol for 3 min, and washed twice more with PBS.

BrdU-labelled cells were quantified by the labelled streptavidin–biotin method using a BrdU IHC kit following the manufacturer's protocol with some modifications (Chemicon International). Fixed cells were incubated in 0·3 % hydrogen peroxide in methanol for 10 min and washed with PBS. Cells were incubated in denaturing solution for 30 min and washed twice with PBS. Cells were incubated in blocking solution for 10 min and incubated in primary antibody for 1 h at RT. Following two PBS washes, cells were incubated with streptavidin–HRP conjugate for 10 min and incubated with DAB (Dako Liquid DAB Substrate Chromagen System) for 10 min. Cells were counterstained with haematoxylin, dehydrated and mounted on slides with permanent mounting medium. BrdU-stained JEG-3 cells were photographed (eight images per slide) using a Zeiss Axiophot microscope and Axiovision software (Carl Zeiss Microscopy GmbH). Total and BrdU-positive cells were counted for each image and the ratio of BrdU-positive cells to the total number of cells was determined for each image. The mean ratio for each treatment was calculated. Experiments were performed in duplicate (transfections) or triplicate (FA concentration) with two replicates per group.

Cell viability

JEG-3 cells (1×10^6) were plated in a ninety-six-well plate and incubated in Defined medium with 2, 20 or 100 nM-FA for 24 h. Cell viability was measured using the MTT Cell Proliferation Assay (ATCC) following the manufacturer's protocol, and absorbance was measured at 570 nm. Cell viability was also measured using the Multitox-Fluor Multiplex Assay (Promega) following the manufacturer's protocol. Fluorescence was measured at 400 nm (excitation) and 505 nm (emission) using a ninety-six-well Multiscan Spectrum (Thermo Electron Corporation). Experiments were performed in duplicate with three replicates per group.

Invasion assay

The twenty-four-well BD Biocoat Matrigel Invasion Chambers (BD Biosciences) with 8-µm polyethylene terephthalate membrane inserts were prepared 24 h in advance. The inserts were coated with 100 µl of Basement Membrane Matrix, Growth Factor Reduced (BD Biosciences) according to the manufacturer's instructions, and incubated at 37°C to allow the matrix to solidify. Cells (5×10^4) were plated in 750 µl medium in each insert. The inserts were added to wells containing 750 µl of medium and incubated under standard growth conditions for 24 h. For transfected cells, the cells were transfected for 48 h, as described above, at which point they were seeded on the inserts and re-transfected in 1 ml of serum-free Defined medium containing 20 nM-FA for an additional 24 h.

Calcein blue staining was used to measure the number of cells that had invaded through the matrigel and transwell to the receiver well. Cells in inserts and the receiver wells were washed twice each with PBS. Calcein AM Dye (Invitrogen) was diluted 4 µg/ml in 1× StemPro Accutase Cell Dissociation Reagent (CDS) (GIBCO). A volume of 400 µl of 1× CDS–Calcein was added to each receiver well and the inserts were put back in the wells. The plate was incubated at 37°C for 30 min; the plate was tapped and incubated for an additional 30 min. The transwell was incubated in the dark for 1 h at room temperature. After thoroughly mixing the solution, 100 µl was added to a ninety-six-well plate, along with previously prepared standards. A five-point standard curve ranged from 0 to 5×10^4 cells suspended in 100 µl CDS. Fluorescence was measured at 485/520 nm excitation/emission. Experiments were performed in duplicate with three replicates per group (FA concentration), or, in the case of transfections, with two (negative siRNA control) or four (gene-specific siRNA) replicates per group.

Progesterone/human chorionic gonadotropin ELISA

ELISA analyses were performed on supernatants collected from a six-well plate of JEG-3 cells incubated in Defined medium for 3, 6 and 24 h. *MTR* and *MTHFD1* siRNA-transfected cells were incubated in Defined medium containing 20 nM-FA. At 48 h post-transfection, cells were re-transfected and supernatants were collected 3, 6 and 24 h post-transfection. Progesterone and hCG were measured using progesterone (Medicorp) and hCG (Phoenix Peptides) ELISA kits according to the manufacturers' protocol. Experiments were performed in duplicate with three replicates per group (FA concentration), or, in the case of the transfections, with two (negative siRNA control) or four (gene-specific siRNA) replicates per group.

Statistical analyses

For each experimental endpoint, a replicate effect was assessed by two-way ANOVA with the independent variables being replicate number and FA concentration or siRNA transfection. In the absence of a replicate effect, the mean values and their standard errors were calculated using all data points from all experimental replicates. In the case of a significant replicate effect, which was evident for hormone secretion, data from each experimental replicate were normalised to one group at 3 h. For FA concentrations, values within a replicate were normalised to the low FA (2 nM) group at 3 h. For the siRNA transfection experiments, values within a replicate were normalised to the negative siRNA transfection group at 3 h. This eliminated the replicate effect, and the mean values and their standard errors for all normalised values from all experimental replicates was then calculated.

Differences between experimental groups were determined by Student's *t* test or one-way ANOVA and the Holm–Sidak *post hoc* test. Folate × time effects on hormone concentration were analysed by two-way ANOVA and the Holm–Sidak *post hoc* test. Groups were considered significantly different when the *P* value was $\leq 0{\cdot}05$. All statistical analyses were performed using SigmaPlot software, version 11.0.

Results

Effect of folate concentration on JEG-3 cells

JEG-3 cell proliferation was higher with increasing FA concentration. The percentage of BrdU-positive cells was 13 % lower in cells cultured in low FA (2 nM) ($P < 0{\cdot}001$) and 13 % higher in FA-supplemented cells (100 nM) ($P < 0{\cdot}001$) compared with control (20 nM) cells (Fig. 1(a)).

JEG-3 cell invasion capacity in low FA was 5·5 ($P = 0{\cdot}025$) and 7·5 % ($P = 0{\cdot}004$) lower than that of control and supplemented cells, respectively (Fig. 1(b)). There was no difference in invasion capacity between the control and supplemented cells.

Cells cultured in low FA had a modest but significant approximate 5–7·5 % ($P = 0{\cdot}004–0{\cdot}025$) lower cell viability compared with cells cultured in control and supplemented FA conditions, respectively, using the MTT and Multitox assays (Fig. 1(c) and (d)). Cell viability was not different between control and supplemented cells.

Progesterone and hCG concentrations were higher over time, but there were no differences among the FA treatment groups (Fig. 1(e) and (f)).

Effect of MTR knockdown

At 24 and 72 h post-transfection with siRNA, *MTR* gene expression was 68 and 72 % lower, respectively, than that of negative siRNA-transfected cells (Fig. 2(a)). MTR protein was not detectable by Western blot in *MTR* siRNA-transfected cells (Fig. 2(b)).

JEG-3 cells demonstrated lower proliferation after 24 h of lower *MTR* gene expression. The *MTR* siRNA-transfected cells had 17·7 % ($P < 0{\cdot}001$) fewer BrdU-positive cells than did cells transfected with the negative control siRNA (Fig. 3(a)).

There was no difference in JEG-3 cell invasion capacity in *MTR* siRNA-transfected cells compared with negative control siRNA-transfected cells (Fig. 3(b)).

Progesterone and hCG concentrations were higher over time. At 24 h, *MTR* siRNA-transfected cells had 61 % higher ($P = 0{\cdot}014$) progesterone compared with negative control siRNA-transfected cells (Fig. 3(c)). *MTR* knockdown had no effect on hCG secretion (Fig. 3(d)).

Effect of MTHFD1 knockdown

MTHFD1 siRNA-transfected cells had 32 and 69 % lower gene expression compared with the negative control siRNA-transfected cells at 24 and 72 h post-transfection, respectively (Fig. 2(c)). However, *MTHFD1* siRNA knockdown did not result in detectable changes in MTHFD1 protein expression (Fig. 2(d)).

JEG-3 cell proliferation was lower 24 h after transfection with the *MTHFD1 siRNA*. JEG-3 cells transfected with *MTHFD1* siRNA had 10·3 % ($P = 0{\cdot}001$) fewer BrdU-positive cells compared with negative control siRNA-transfected cells (Fig. 3(a)).

There was no difference in JEG-3 cell invasion capacity in *MTHFD1* siRNA-transfected cells compared with negative control siRNA-transfected cells (Fig. 3(b)).

Progesterone and hCG concentrations were higher over time. However, no significant differences in progesterone or hCG were observed between *MTHFD1 siRNA*-transfected cells and negative control siRNA-transfected cells (Fig. 3(e) and (f)).

Discussion

Using JEG-3 cells, which are morphologically and functionally similar to extravillous trophoblasts(29), we demonstrate that folate deficiency resulted in lower cell proliferation, viability and invasive capacity, albeit with modest differences. We also show that functional folate deficiency due to lower gene expression of folate-dependent enzymes may impact JEG-3 cell proliferation. Lower *MTR* gene and protein expression resulted in lower cell proliferation and higher progesterone secretion. Lower gene expression of *MTHFD1* resulted in lower cell proliferation, but this was in the absence of detectable changes in protein expression, leaving this finding open to interpretation. Together, the data indicate that placental cell growth and function is at least in part dependent on folate metabolism, which may underlie the associations between folate intake and/or status, and SNP in folate-dependent enzymes, and placenta-related pathologies.

Folate metabolism is important in maintaining nucleotide synthesis, specifically the *de novo* synthesis of dTMP and purines, and as such supports cell proliferation. *De novo* synthesis of dTMP occurs when thymidylate synthase mediates the

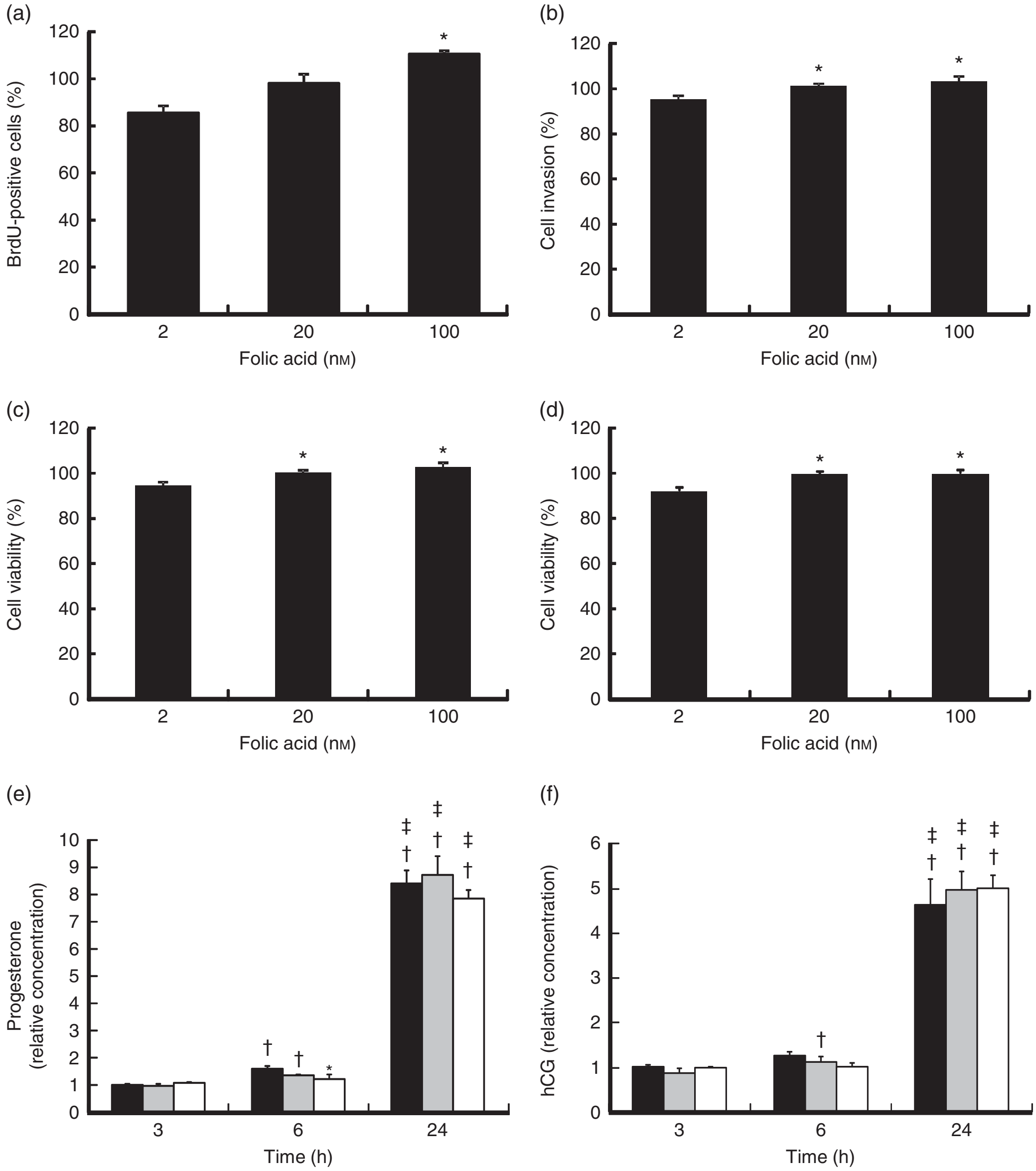

Fig. 1. Effect of low (2 nM), control (20 nM) and supplemented (100 nM) folic acid (FA) on cell proliferation, invasive capacity and viability and on progesterone and human chorionic gonadotropin (hCG) production in JEG-3 cells. (a) Cell proliferation in response to FA after 24 h, as measured by the percentage of bromodeoxyuridine (BrdU)-positive cells. Data are from three independent experiments, two replicates/group per experiment (*n* 6 per group). (b) JEG-3 invasive capacity after 24 h in response to FA. Data are from three independent experiments, two replicates/group per experiment, *n* 6 per group. (c) JEG-3 cell viability in response to FA at 24 h as measured by the Multitox and (d) MTT assays. Data are presented as relative to cell viability of the 20 nM-FA group at 24 h. Data are from two independent experiments, three replicates/group per experiment, *n* 6 per group. Secretion of (e) progesterone and (f) hCG in response to FA after 3, 6 and 24 h. Data from each independent experiment were normalised to the 2 nM-FA group at 3 h. Data are from two independent experiments, three replicates/group per experiment, *n* 6 per group. All data are presented as mean values and their standard errors. * Statistically significant difference with the low FA (2 nM) group, as determined by one-way ANOVA, Holm–Sidak *post hoc* test, $P \leq 0·05$. † Statistically significant difference compared with 3 h, ‡ statistically significant difference compared with 6 h, as assessed by two-way ANOVA, Holm–Sidak *post hoc* test ($P \leq 0·05$). ■, 2 nm; ▨, 20 nm; □, 100 nm.

transfer of a methyl group from 5,10-methyleneTHF to deoxyuridine monophosphate(9). Impairment of *de novo* dTMP synthesis increases cellular deoxyuridine triphosphate (dUTP) and can result in misincorporation of uracil into DNA(30,31). DNA repair mechanisms excise misincorporated uracils via an endonuclease; however, in the context of low available dTMP and high dUTP, single- and double-strand DNA breaks can occur(30,31). As DNA damage accumulates, cells will arrest in the S phase of the cell cycle, ultimately reducing cell proliferation(32). In addition, when purines are not available through the salvage pathway, purine bases are synthesised *de novo* in a 10-formylTHF-dependent fashion(33). *De novo* purine synthesis is required in highly proliferative cells and has been shown to regulate cell division(34). Aberrant purine synthesis results in cell cycle arrest, lower cell proliferation and cell death.

Previous studies suggested that folate deficiency lowers cell viability in human cytotrophoblasts isolated from placentas, as

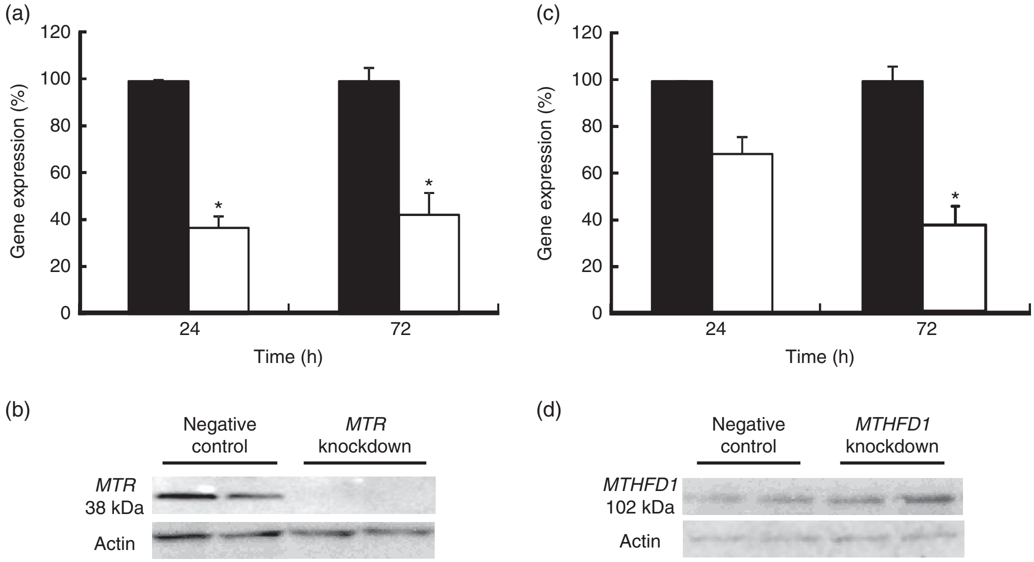

Fig. 2. *MTR* and *MTHFD1* gene and protein expression in JEG-3 cells transfected with *MTR* or *MTHFD1*-specific small interfering RNA (siRNA). *MTR* (a) mRNA expression at 24 and 72 h, and (b) protein expression. *MTHFD1* (c) mRNA expression at 24 and 72 h, and (d) protein expression. mRNA expression was measured by RT-quantitative PCR. Protein knockdown was confirmed by Western blot. Data are presented as mean values and their standard errors, *n* 3–6 replicates per group. * Statistically significant difference from the negative (Neg) siRNA transfection, as assessed by Student's *t* test ($P \leq 0{\cdot}0004$). ■, Neg; (a) □, *MTR* knockdown; (c) □, *MTHFD1* knockdown.

shown by significantly higher *in vitro* cell apoptosis under folate-free medium conditions(35). We found that, under low FA conditions, JEG-3 cell proliferation and viability was lower; the difference was modest but significant. In light of previous findings associating folate deficiency with pregnancy and fetal complications, our data suggest that folate deficiency could restrict placental growth and viability likely due to impaired nucleotide synthesis. This could lead to an underdeveloped placenta and poor placental function. Reduced placental function increases the risk for poor nutrient exchange, which could lead to a small-for-gestational-age fetus(36) or an increased risk for congenital anomalies(5).

Trophoblast invasion is another key step in placental development that may be influenced by folate metabolism. In a study by Williams *et al.*(37), culture of placental explants of gestational age 7 weeks in increasing FA resulted in higher extravillous trophoblast invasion. We also observed lower JEG-3 invasive capacity, albeit modest, when cells were cultured in FA-deficient media compared with those cultured in control or supplemented media. Of note, supplemental concentrations of FA did not improve JEG-3 cell invasion relative to the control. These differences occurred in the absence of changes in progesterone and hCG. The data indicate that folate adequacy supports trophoblast invasion in a possibly hormone-independent manner. Folate deficiency could therefore result in reduced vascularisation and nutrient exchange at the maternal–fetal interface(38,39), leading to placental hypoxia and insufficiency. A hypoxic placental environment and placental insufficiency may hinder nutrient exchange at the maternal–fetal interface, increasing the risk for fetal complications.

We also observed changes in JEG-3 characteristics when the expression of specific folate-dependent genes was knocked down. Similar to when folate was limited, JEG-3 cells in which *MTR* gene and protein expression were knocked down demonstrated lower cell proliferation. MTR transfers the methyl group from 5-methylTHF to Hcy to form methionine and THF. Therefore, it might be expected that lower MTR protein expression would limit the availability of THF for *de novo* purine and dTMP synthesis, and, as such, lower cell proliferation. Interestingly, lower *MTR* expression also resulted in higher progesterone secretion, but had no effect on hCG secretion. Progesterone negatively regulates trophoblast invasion(22), and poor trophoblast invasion is a key characteristic of uteroplacental insufficiency(7,8). Therefore, the effect of reduced *MTR* functionality on progesterone secretion may explain, at least in part, the association between the 2756 A > G SNP in *MTR* and uteroplacental insufficiency(7). It must be stated that in these short-term experiments we did not observe an effect of *MTR* expression on cell invasion; however, trophoblast invasion could possibly be affected after longer term exposure to higher progesterone due to lower *MTR* expression and/or function.

Two possible mechanisms may explain the higher progesterone secretion in cells with lower *MTR* expression. First, higher progesterone secretion may be a result of methylation-dependent gene expression changes. Methionine derived from the MTR enzyme activity can be used to generate S-adenosylmethionine, the major methyl donor for cellular methylation reactions, including that of DNA and histones(33). The gene expression of type I 3β-hydroxysteroid dehydrogenase (*HSD3B1*), a steroidogenic enzyme required for progesterone synthesis from pregnenolone, has been shown to be methylation dependent(40,41). Hypomethylation of a non-CpG region in exon 2 of the *HSD3B1* gene was associated with higher gene expression in placenta, and with risk for early- and late-onset pre-eclampsia(40). In addition, treatment of BeWo and JEG-3 cells with the hypomethylation-inducing chemical 5-azacytidine resulted in reduced methylation of exon 2 of *HSDB1* and higher gene expression(41). Thus, it is plausible that

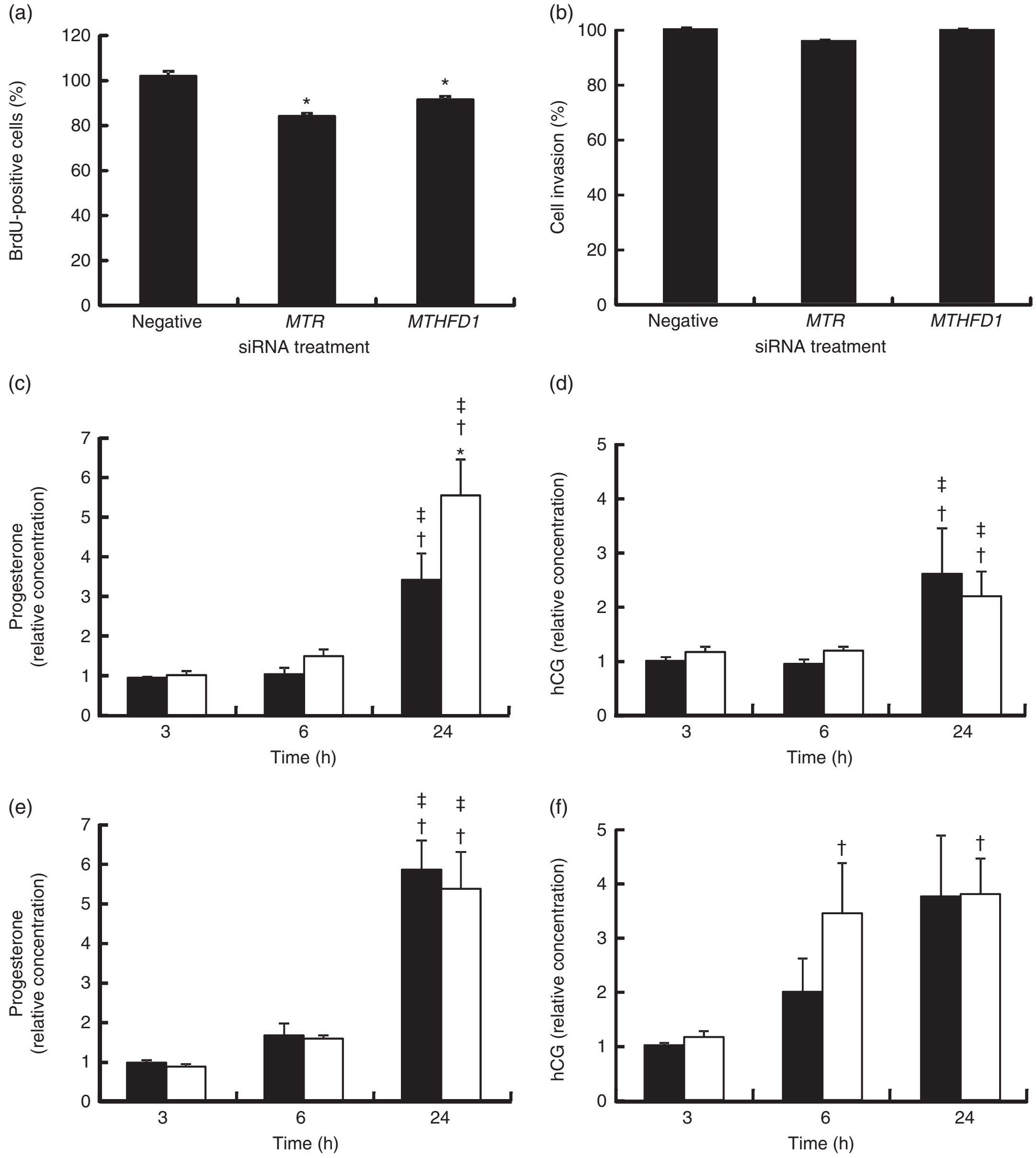

Fig. 3. Effect of *MTHFD1* and *MTR* small interfering RNA (siRNA) knockdown (KD) on JEG-3 cell proliferation, invasive capacity and progesterone and hCG production. (a) Cell proliferation was measured as the percentage of BrdU-positive cells. Data are from two independent experiments, two replicates/group per experiment, *n* 4 per group. (b) JEG-3 cell invasion after 24 h. Secretion of (c) progesterone and (d) human chorionic gonadotropin (hCG) in response to *MTR* KD. Secretion of (e) progesterone and (f) hCG in response to *MTHFD1* KD. For cell invasion and hormone secretion, data for each endpoint are from two independent experiments. For the negative (Neg) siRNA control, there were two replicates/group per experiment, *n* 4 per group, and for the *MTR* and *MTHFD1* siRNA transfections, there were four replicates/group/experiment, *n* 8 per group. All data are presented as mean values and their standard errors. * Statistically significant difference compared with the negative siRNA transfection, as determined by one-way ANOVA, Holm–Sidak *post hoc* test, $P \leq 0{\cdot}05$. †,‡ Statistically significant effect of time, as assessed by two-way ANOVA, Holm–Sidak *post hoc* test ($P \leq 0{\cdot}05$), † difference compared with 3 h, ‡ difference compared with 6 h. ■, Neg; (c, d) □, *MTR* KD; (e, f) □, *MTHFD1* KD.

lower *MTR* expression altered cellular methylation capacity, which in turn resulted in methylation-dependent gene expression differences in enzymes required for progesterone synthesis or secretion. A second possible explanation for the higher progesterone secretion in cells with lower *MTR* expression may be that, because of the inhibition of cell proliferation, a portion of the JEG-3 cells prematurely differentiated into hormone-secreting cytotrophoblasts.

Knockdown of *MTHFD1* mRNA expression also resulted in lower cell proliferation, but to a lesser degree than either folate deficiency or *MTR* knockdown. It had no effect on cell invasion or hormone secretion. These observations must be interpreted carefully as they were observed in the absence of detectable changes in MTHFD1 protein. The null effect on cell invasion and hormone secretion may simply be a result of no change in MTHFD1 protein expression. As for cell proliferation, it could be that the siRNA transfection procedure alone resulted in lower cell proliferation. However, as a negative siRNA transfection control was included for all experiments, it seems less likely that changes in proliferation were due to the transfection. Perhaps the *MTHFD1* siRNA specifically affected proliferation independent of protein expression. Another possibility is that cells are exquisitely sensitive to changes in the level of this essential enzyme, such that even small changes in protein expression below the level of detection by Western blotting may impact cell proliferation. Either way, the lack of MTHFD1 protein knockdown leaves the relationship between MTHFD1 and placental health undetermined.

This study has a number of strengths and weaknesses. We used a physiologically relevant range of folate concentrations that is observed in populations exposed to FA fortification and supplements. Placental cells, and specifically JEG-3 cells, a model of extravillous trophoblasts(29), express multiple folate transporters, including the folate receptor alpha, which can transport FA with high affinity(42). We initially performed parallel experiments with 5-formylTHF and FA and found similar effects on cell proliferation and viability (data not shown). We therefore continued all subsequent experiments with FA because of its relative stability. Although 5-methylTHF is the major circulating form of folate, unmetabolised FA is observed in >95 % of individuals who consume FA-containing foods and/or supplements, and therefore direct exposure of the placenta to unmetabolised FA is common(43). Our use of RNAi technology allowed us to assess the effect of lower expression of key folate-dependent enzymes that have been associated with placenta-related pathologies. It should be noted that these experiments cannot be directly related to the effect of a particular SNP as RNAi reduces the expression of the mRNA/protein, whereas an SNP could result in the loss of a particular function of a protein. In addition, the response to RNAi varied by the gene target; *MTR* mRNA and protein expression were both lower, whereas only *MTHFD1* mRNA expression was lower.

Overall, our findings suggest that folate deficiency impairs placental cell proliferation and function, which may underlie the placenta-related risks observed in pregnant women with low folate status or genetic polymorphisms in folate-dependent enzymes. Placental pathologies are multifactorial, requiring the interaction of genetic and environmental factors. Although the observed effects of JEG-3 cells in response to altered folate metabolism were modest, the short duration of the studies makes them noteworthy. Longer exposure conditions or conditions in which FA concentration and gene expression are simultaneously modified may result in additional or exacerbated phenotypes. Further studies will allow us to elucidate the mechanisms by which altered folate metabolism affects JEG-3 cells, which by proxy will inform its role in placental development and pregnancy.

Supplementary material

For supplementary material/s referred to in this article, please visit http://dx.doi.org/doi:10.1017/S0007114515002688

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A. J. M. formulated the research question and designed the study. C. M., N. R. and P. J. carried out the experiments and analysed the data. C. M. and A. J. M. wrote the manuscript. All authors have read and approved the manuscript.

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