

## RESEARCH ARTICLE

# Methyl Donor Status Influences DNMT Expression and Global DNA Methylation in Cervical Cancer Cells

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### Abstract

**Background:** Methyl donor status influences DNA stability and DNA methylation although little is known about effects on DNA methyltransferases. The aim of this study was to determine whether methyl-donor status influences DNA methyltransferase (Dnmt) gene expression in cervical cancer cells, and if so, whether there are associated effects on global DNA methylation. **Materials and Methods:** The human cervical cancer cell line, C4-II, was grown in complete medium and medium depleted of folate (F-M+) and folate and methionine (F-M-). Growth rate, intracellular folate, intracellular methionine and homocysteine in the extracellular medium were measured to validate the cancer cell model of methyl donor depletion. Dnmt expression was measured by qRT-PCR using relative quantification and global DNA methylation was measured using a flow cytometric method. **Results:** Intracellular folate and methionine concentrations were significantly reduced after growth in depleted media. Growth rate was also reduced in response to methyl donor depletion. Extracellular homocysteine was raised compared with controls, indicating disturbance to the methyl cycle. Combined folate and methionine depletion led to a significant down-regulation of Dnmt3a and Dnmt3b; this was associated with an 18% reduction in global DNA methylation compared with controls. Effects of folate and methionine depletion on Dnmt3a and 3b expression were reversed by transferring depleted cells to complete medium. **Conclusions:** Methyl donor status can evidently influence expression of Dnmts in cervical cancer cells, which is associated with DNA global hypomethylation. Effects on Dnmt expression are reversible, suggesting reversible modulating effects of dietary methyl donor intake on gene expression, which may be relevant for cancer progression.

**Keywords:** Cervical cancer - folate - methionine - DNA methylation - DNA methyltransferase

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### Introduction

Cervical cancer is the fourth major cancer in women worldwide (GLOBOCAN, 2012). The main cause of cervical cancer is infection with high-risk strains of the human papillomavirus (HR-HPV), but not every woman who acquires HR-HPV infection develops cervical cancer. Whilst the majority of infections are transient and resolve of their own accord, HR-HPV infection persists in about 40% of cases (Rositch et al., 2013). Moreover, a high percentage of women who are diagnosed with moderate grade pre-cancerous lesions regress to normal (Wang et al., 2013). Clearly, there are factors additional to HPV infection which influence the development of cervical cancer. Among the factors known to influence risk of cervical cancer are high parity, use of oral contraceptives, and diet (Guo et al., 2015; Roura et al., 2016). Dietary factors influencing the methyl donor cycle may be important in determining risk of cervical cancer (Ziegler, Weinstein and Fears, 2002).

Methyl-donor nutrients are important to DNA methylation and to the maintenance of a functional methyl donor cycle. S-adenosylmethionine (SAM) is the major methyl donor and is derived from two main methyl donor pathways; the folate pathway and the choline (betaine) pathway. Studies suggest an interdependency of methyl donors such that a disturbance to one methyl donor pathway brings about change in another (James, Cross and Miller, 1992; Niculescu and Zeisel, 2002). Some studies have shown that altering methyl donor status can induce changes in DNA methylation, although results are not entirely consistent (Rampersaud et al., 2000; Duthie et al., 2004; Farias et al., 2015). Changes in DNA methylation can have downstream effects on the expression of genes important to carcinogenesis and cancer progression (Brunaud et al., 2003; Jones and Baylin, 2007; Lande-Diner et al., 2007). For these reasons factors which influence DNA methylation are considered relevant to cancer risk and progression. We have previously reported low folate status and aberrant

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DNA methylation to be associated with HR-HPV infection and cervical carcinogenesis (Flatley et al., 2009) and there have been numerous reports of aberrant DNA methylation in cervical cancer (Narayan et al., 2003; Cheng, Gao and Lou, 2010; Woo et al., 2015).

The DNA methylation process is catalysed by DNA methyltransferases (Dnmts), therefore, together with the availability of methyl donors, factors influencing their expression and activity might be expected to influence DNA methylation. Early studies suggested that aberrant Dnmt expression influences the DNA methylation profile, but results were not consistent (Fatemi et al., 2002; Sawada et al., 2007). There are three distinct enzymatically active forms of Dnmts in mammals: Dnmt1, Dnmt3a, and Dnmt3b. Dnmt1 is classified as a DNA maintenance enzyme, preferring hemimethylated CpG sites as substrate. The essential role of Dnmt1 in human embryonic stem cells is evidenced by the rapid cell death seen upon targeted disruption of the DNMT1 gene (Liao et al., 2015). Dnmt3a and 3b catalyse the addition of methyl-groups to unmethylated CpG sites on both strands of the DNA (Fatemi et al., 2002). There are numerous observational studies reporting over-expression of Dnmts in tumour tissue, including cervical cancer (Sawada et al., 2007), liver cancer (Oh et al., 2007) and gastric cancer (Etoh et al., 2004). There is also some evidence that Dnmt expression may influence cancer progression, possibly by epigenetic silencing (Ning et al., 2015). Various post-translational modifications are thought to regulate Dnmts, including methylation itself (Denis, Ndlovu and Fuks, 2011; Kinney and Pradhan, 2011). This study explores causative links between methyl donor status, Dnmt expression and effects on DNA global methylation in a cervical cancer cell model of methyl donor depletion.

## Materials and Methods

Unless otherwise specified, laboratory chemicals were obtained from Sigma-Aldrich and cell culture plastic ware was obtained from Fisher Scientific or Corning Life Sciences.

### Cell culture

The C-4 II and the SiHa human Caucasian cervical carcinoma cell lines were obtained from PHE Culture Collections UK. The C-4 II cell line was selected because it has been shown to express a gene profile that is very strongly correlated with that of cervical cancer cells from biopsies (Carlson and Marcotte, 2007) and has been reported to contain HPV-18 DNA sequences and to express HPV-18 RNA (Pater and Pater, 1985), while the SiHa cell line is a second cervical cancer line reported to harbour HPV-16 (Pater and Pater, 1985). For maintenance, cells were grown and passaged in Waymouth's MB 752/1 medium (F+M+ medium: GIBCO, Cat. No. 31220072). For experiments, a customized Waymouth's MB 752/1 medium lacking both folate and methionine was used (F-M- medium: GIBCO, Cat. No. 04196306). The folate and methionine concentrations in this medium were estimated to be <30 nM and 20 µM respectively, compared with 900 nM and 300 µM respectively in F+M+

medium. For experiments, C-4 II cells were sub-cultured in F-M- medium, in F-M- medium to which methionine (Sigma-Aldrich, Cat. No. M5308) was added back to a concentration of 300 µM (F-M+) or in F+M+ medium. All media were supplemented with 10% foetal bovine serum (PAA, FCS Gold Cat No. A15-151), 200 nM L-glutamine (Lonza, Cat. No. LZBE17-605E) and 1% penicillin-streptomycin (Lonza, Cat. No. LZDE17-603E). Cells were maintained at 37°C, 5% carbon dioxide and 95% humidity. Growth rates (seeding density 20,000 cells/cm<sup>2</sup>) were determined by counting cells every 4 days over 12 days, with a change of medium every 4 days. Cells were harvested for counting using trypsin-EDTA (Lonza, Cat. No. LZBE17-161F). For methyl-donor repletion experiments, C-4 II cells were grown in F-M+ or F-M-media for 8 days and then transferred to F+M+ medium for a further 4 days. Initially, the investigation also included the use of SiHa cells but it proved extremely time-consuming to deplete these cells of folate and methionine. Consistent effects on cell growth required depletion for at least 6 weeks, imposing practical constraints on the use of this cell line. Data for SiHa cells are restricted to the protein expression of Dnmts, shown later.

### Intracellular folate and methionine

More than 5 million cells were harvested and washed with PBS. The cells were suspended in 500 µl of 0.5% (w/v) ascorbic acid in water for the measurement of total folate, or suspended in 500 µl of serum-free, F-M- medium for the measurement of methionine. Cell lysates were prepared by cell disruption using a Diagenode UCD-200TM Bioruptor sonicator (high pulse, 10 min) followed by homogenization using a Precellys® 24 lysis and homogenization system (Bertin Technologies: 6000 rpm/pulse: 2 x 30 sec / pause: 20 sec). Total folate concentration was determined using an automated competitive folate protein binding assay (Beckman: Access Folate Kit, Cat. No. A98032) and expressed as pmol/10<sup>6</sup> cells. Methionine was measured using the Biochrom 30 amino acid analyzer under control of Biosys software v2.05, and analysed using EZChrom Elite software v3.4 (Biochrom).

### Extracellular homocysteine

Total homocysteine concentration in the incubation medium was determined by reverse-phase high-performance liquid chromatography (Gilson, UK) using a Chromsystems HPLC Kit (Cat. No. 45000). Sample preparation was according to the manufacturer's protocol. Absorbance of eluted compounds was monitored using  $\lambda_{\text{ex}} = 385 \text{ nm}$  and  $\lambda_{\text{em}} = 515 \text{ nm}$ . Chromatograms were recorded with UniPoint™ LC System Software Version 5.1 with quantification accomplished by automated peak area integration (Chromsystems).

### RNA extraction and cDNA synthesis

Total RNA was extracted using an RNeasy® Mini kit from Qiagen (Cat. No. 74104) following the manufacturer's protocol. First-strand cDNA synthesis was carried out using SuperScript™ III Reverse Transcriptase (Invitrogen, Cat. No. 18080051) according to the manufacturer's recommendations for random primers.

cDNA synthesis was performed on 1.5-2 µg of total RNA in a volume of 20 µl. In brief, 50 ng of random hexamer (Promega, Cat. No. C1181) and 0.5 mM of dNTP mix (Invitrogen, Cat. No. 18427-088) were added to each RNA sample. The mixture was heated at 65°C for 5 min and incubated on ice for at least 1 min. A master mix, comprised of 5x first-strand buffer (250 mM Tris-HCl pH 8.3 at room temperature, 375 mM KCl, 15 mM MgCl<sub>2</sub>), 5 mM DTT, 40U RnaseOUT™ (Invitrogen, Cat. No. 10777-019) and 200 U SuperScript™ III RT, was added to the mixture and incubated at 25°C for 10 min followed by 50°C for 50 min. The reaction was terminated by heating at 85°C for 5 min and chilled at 4°C. The cDNA was diluted to a concentration of 25 ng/µl and kept in aliquots at -80°C.

#### Expression of DNA methyltransferases

Dnmt gene expression was determined by qRT-PCR using relative quantification. Two µl of cDNA product (50 ng) were used for PCR amplification. The reaction was in a total volume of 25 µl, which included TaqMan® Fast Universal PCR Master Mix 2x, No AmpErase UNG (Applied Biosystems, Cat. No. 4367846), 125 nM of Taqman® probe (Applied Biosystems, custom synthesis), 300 nM of forward primer, and 300 nM of reverse primer (Sigma-Aldrich, custom synthesis). All reactions were run in triplicate in Fast optical 96-well barcoded reaction plates (Applied Biosystems, Cat. No. BC-1900), sealed with optical adhesive covers (Applied Biosystems, Cat. No. 430611) on a StepOnePlus™ Real-time PCR System (Applied Biosystems). Sequences for forward (F) and reverse (R) primers and Taqman® dual probes (P) were as follows:

β-actin:	F: 5'-TCACCCACACTGTGCCCATCTACGA-3' R: 5'-CAGCGGAACCGCTCATTGCCAATGG-3' P: 5'-VIC-ATGCCCTCCCCATGCCATCTCGCT-TAMRA-3'
DNMT1:	F: 5'-CGGTTCTCTCCTCGGAGAATGTCA-3' R: 5'-CACTGATAGCCCATGCCGACCA-3' P: 5'-6-FAM-AACTTGTCTCCTTCAAGCGCTCCATGGTC-TAMRA-3'
DNMT3A:	F: 5'-CAATGACCTCTCCATCGTCAAC-3' R: 5'-CATGCAGGAGGCGGTAGAA-3' P: 5'-6-FAM-AGCCGCCAGTGCCCTCGTAG-TAMRA-3'
DNMT3B:	F: 5'-CCATGAAGGTTGGCGACAA-3' R: 5'-TGGCATCAATCATCACTGGATT-3' P: 5'-6-FAM-CACTCCAGGAACCGTGAGATGTCCCT-TAMRA-3'

Thermocycling parameters were 20 sec at 95°C then 40 cycles consisting of 1 sec at 95°C for denaturation and 20 sec at 60°C for annealing/extension. Negative controls (in which water was added instead of cDNA) were run in each plate. Contamination of genomic DNA in RNA samples was monitored by including RNA samples processed in the absence of reverse transcriptase enzyme. Analysis of all genes of interest were carried out in independent reactions. Gene expression was normalized to β-actin for each sample and expression ratios between the Dnmts from F+M+ (control), F-M+, and F-M- were calculated.

#### Western Blotting

Cytosolic protein extracts were prepared at day 4 and day 8 from C-4 II and SiHa cells cultured in the presence or absence of methyl donors. Cells were grown as described above with the inclusion of an F+M-condition and using RPMI as the base medium after confirming that cell growth rates were no different than in Waymouth's medium. Cells were lysed in RIPA buffer pH 8.0 containing 1.0 Nonidet NP-40 (Sigma-Aldrich, Cat. No. N6507) and cOmplete® protease inhibitor cocktail (Roche, Cat. No. 11697498001). Protein extracts were normalised for total protein content (20 µg per lane), separated by SDS-PAGE (BioRad, 7.5% mini-Protean TGX), at 100 V continuous, and transferred to HyBond Immobilon-P membrane (Millipore, Cat. No. IPVH00010) over 1 h at 100V with ice.

Blots were blocked with tris-buffered saline/Tween 20 containing 5% skimmed milk (Marvel), probed with Anti Human Dnmt1 (Abcam, ab19905) at 1:1000 dilution, Anti Human Dnmt3b (Abcam, ab119282) at 1:1000 or Anti Human GAPDH (Abcam, ab9485) at 1:2500 dilution for 1 h at room temperature, and visualized with Goat Anti-Rabbit Horse radish peroxidase (HRP) conjugated secondary antibody (1:160K) (Sigma-Aldrich, Cat. No. A9169) and Immobilon™ Western Chemiluminescent HRP Substrate (Millipore, Cat. No. WBKLS0500).

#### Global DNA methylation

C-4 II cells were grown under control and methyl donor deplete conditions for 4, 8 and 12 days. The immunocytochemistry method modified from Habib et al. (1999) and McManus et al. (2006) with flow cytometric detection was used as the basis for the development and optimization of the measurement of 5-methylcytosine in our system. Briefly, more than 5x10<sup>6</sup> cells were collected using trypsin-EDTA into 6 ml of appropriate experimental medium in a 50 ml centrifuge tube and pelleted by centrifugation. The supernatant was decanted and cells were washed twice by resuspending in 10 ml of washing solution (phosphate buffered saline (PBS: Oxoid, Cat. No. BR0014) containing 0.1% v/v Tween 20 (Sigma-Aldrich, Cat. No. P7949) + 1% w/v bovine serum albumin: PBST-BSA) and pelleted by centrifugation. The cell pellet was resuspended in 5ml of 4% formaldehyde (Sigma-Aldrich, Cat. No. F8775) for 30 min at 37°C and cooled at 4°C for 10 min for fixation. Cells were incubated with 9 volumes of 88% methanol/12% PBS at -20°C for 30 min. Cell pellets were centrifuged and the supernatants discarded. The cells were washed twice by centrifugation in 10ml PBST-BSA. Antigen retrieval was performed by incubating each cell pellet in 5 ml of 2N HCl for 30 min at 37°C, pelleting by centrifugation, and resuspending in 5 ml of 0.1 M sodium borate pH8.5 for 5 min at room temperature. Cells were washed twice by centrifugation in washing solution. The cells were blocked in blocking solution (10% FCS or donkey serum in PBST-BSA) overnight at 4°C. Cells were counted and aliquots of 0.5x10<sup>6</sup> cells placed into FACS tubes. Cell aliquots were immunostained with 100µl of a 1:250 dilution of anti 5-methylcytosine primary antibody (Merck Millipore, Cat. No. NA81, clone 162 33 D3) in PBST-BSA or 10 µl murine IgG1 isotype control (AbD Serotec, Cat. No. MCA928) + 90µl PBST-BSA per tube

and incubated at 37°C for 45 min. Cells were washed 3 times by centrifugation in washing solution. One hundred µl of 1:1000 Alexa fluor 488 donkey anti-mouse IgG (H+L) (Invitrogen, Cat. No. A21202) diluted in PBS+5% BSA solution was added to cells and incubated at 37°C for 45 min in the dark. Cells were washed 3 times by resuspending the pellet in washing solution and pelleted by centrifugation. Cells were kept at 4°C before analysis by BD FACSCalibur™. Appropriate controls included unstained cells, secondary antibody alone and 5-aza, 2-deoxycytidine treated cells as a demethylated control. Nonspecific antibody binding was monitored with a mouse IgG1 isotype. Data was analysed using BD CELLQuest software.

*Statistical analysis*

All experiments were carried out at least three times and are expressed as mean ± SEM. Statistical analysis was carried out using SPSS version 20 for Windows. Two-way ANOVA was used to examine effects of time and methyl donor depletion treatment on outcome variables. Where a significant effect of time or treatment was indicated, one-way ANOVA was carried out, followed by Bonferroni’s post-hoc test. Results were considered significant if the P-value was ≤ 0.05.

**Results**

*Cell growth*

C-4 II cell growth was influenced by time and methyl donor depletion treatment (P<0.001), and a significant interaction occurred between these factors (P<0.001). Cells grew at a similar rate under each of the three conditions of complete (F+M+), folate-deplete (F-M+) and folate and methionine-deplete (F-M-) medium up to day 4, from which time cells grown in the F+M+ medium grew faster than cells grown in either of the deplete media (Figure 1). By day 8 the number of cells was greater in F+M+ medium compared with cells grown in either of the deplete media (P<0.05) and the difference was also evident at day 12 (P<0.05). Cell growth was faster when grown in F-M+ medium than when grown in F-M- medium (P<0.05), with doubling times of 2.77, 3.62 and 5.08 days for cells grown in F+M+, F-M+ and F-M- media, respectively.

*Intracellular folate concentration*

Intracellular folate fell by 90% within 4 days of culture in F-M+ or F-M- medium. Cells grown in either F-M+ or F-M- medium for only 4 days had an intracellular folate concentration of 0.1 – 0.3 pmol/10<sup>6</sup> cells compared with 2 pmol/10<sup>6</sup> cells in cells grown in F+M+ medium (Figure 2a). There was a significant effect of time and methyl donor status on intracellular folate concentration (P<0.001) and a significant interaction between these factors (P<0.001). On days 4, 8 and 12 cells grown in F-M+ or F-M- medium had significantly lower intracellular folate concentration than cells grown in F+M+ medium (P<0.001), but were not different from one another.

Intracellular folate concentration was lower at day 12

than day 4 for cells grown in F-M- medium (P=0.036), and lower at day 12 than that at day 8 for cells grown in F-M+ medium (P=0.05). There was an unexpectedly higher folate concentration at day 8 compared with day 4 and day 12 in cells grown in F+M+ medium (P=0.001).

*Intracellular methionine concentration*

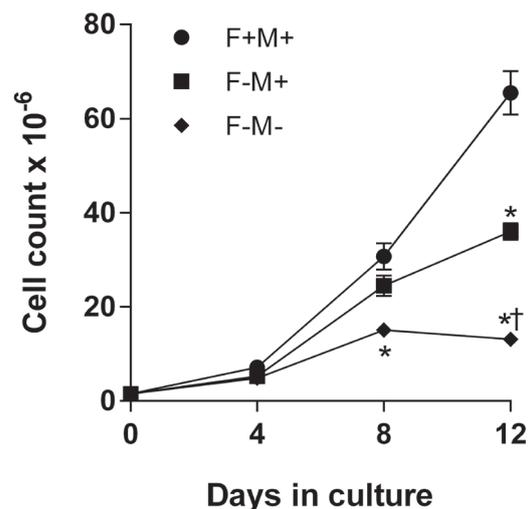
Time (P=0.05) and methyl donor depletion treatment (P=0.001) influenced the intracellular concentration of methionine. Cells grown in F-M- medium showed a reduction in intracellular methionine from about 300 pmol/10<sup>6</sup> cells in control cells to about 100 pmol/10<sup>6</sup> cells at day 4 (Figure 2b) and a further decrease to about 30 pmol/10<sup>6</sup> cells at day 8. By day 4, intracellular methionine concentration was lower for cells in F-M- medium than for cells grown in either F-M+ or F+M+ medium (P<0.01). A difference was also evident at day 8 (P<0.05), but not at day 12.

*Extracellular homocysteine concentration*

There was a significant effect of time and methyl donor depletion treatment (P<0.001) on extracellular homocysteine concentration and a significant interaction between these factors (P<0.001) (Figure 2c). At days 8 and 12 the concentration of homocysteine was greater in the medium of cells grown in F-M+ medium than for cells grown in F+M+ medium or F-M- medium (P<0.01). At day 12 the concentration of homocysteine was also greater in the medium of cells grown in F-M- medium than F+M+ medium (P=0.011).

*Expression of DNA methyltransferases*

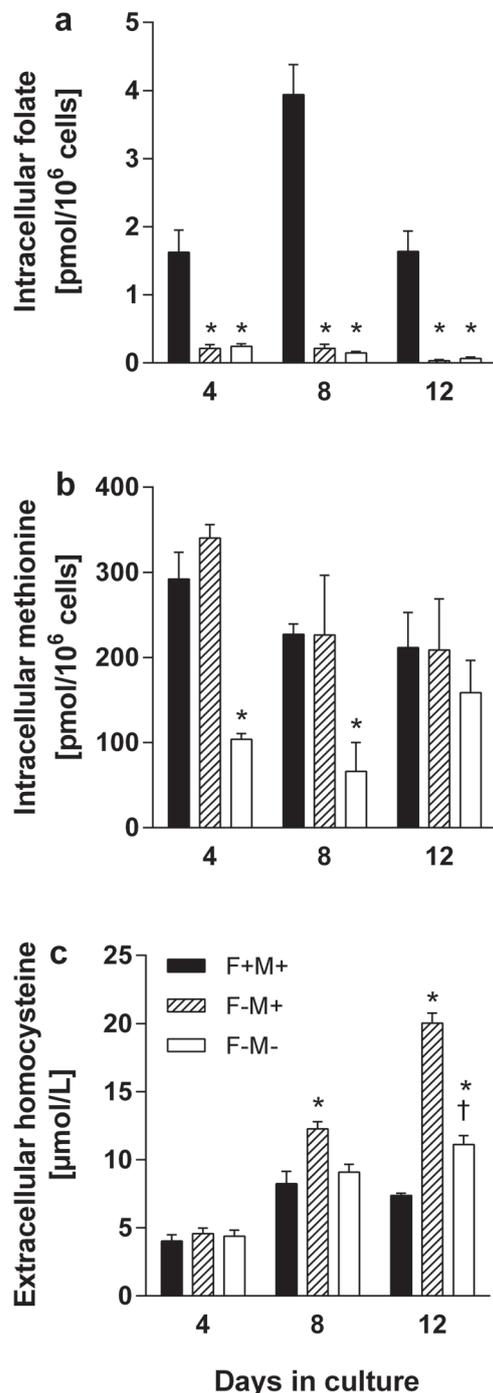
There was a down-regulation in expression of Dnmt3a and Dnmt3b mRNA relative to β-actin in response to methyl donor depletion, in contrast with Dnmt1 which



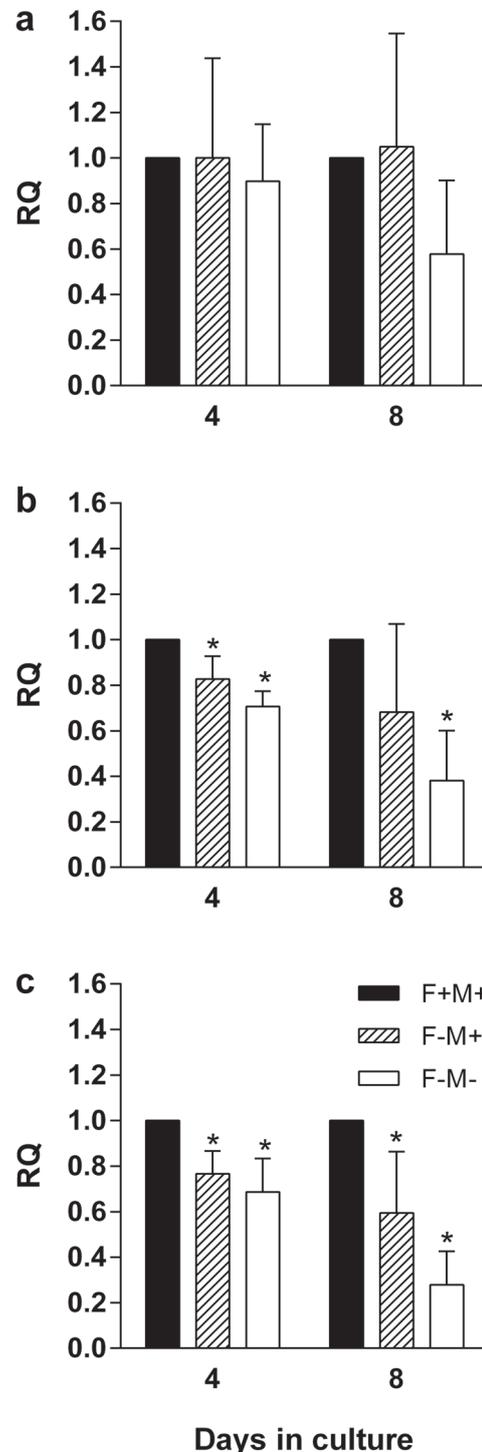
**Figure 1. Effect of Methyl Donor Depletion on the growth of C-4 II Cells C-4 II Cells were Grown in Folate-Deplete (F-M+), Folate and Methionine-Deplete (F-M-) or Complete Medium (F+M+) for 12 days.** Values are the mean of three independent experiments and error bars represent SEM. \* Significantly different from control (F+M+), P<0.05; † significantly different from F-M+, P<0.05 (one-way ANOVA, Bonferroni’s post hoc test)

showed no significant alteration relative to  $\beta$ -actin (Figure 3). Dnmt3a expression was significantly influenced by methyl donor depletion treatment ( $P=0.001$ ). At day 4 the expression was significantly lower in F-M+ ( $P=0.02$ ) and F-M- ( $P=0.001$ ) than in F+M+ medium. Dnmt3a

expression decreased from day 4 to day 8 in cells depleted of both folate and methionine ( $P=0.03$ ) and at day 8, the difference was only significant between cells in F-M- and F+M+ medium ( $P=0.024$ ), when expression of Dnmt3a was 2.62-fold lower in depleted cells than in controls. Expression of Dnmt3a in cells grown in F-M+ was not



**Figure 2. Intracellular Folate, Intracellular Methionine and Extracellular Homocysteine Concentrations from C-4 II Cells Grown In Methyl Donor-deplete and Complete Medium** (a) Intracellular Folate; (b) Intracellular Methionine; (c) Extracellular Homocysteine. C-4 II cells were grown in folate-deplete (F-M+), folate and methionine-deplete (F-M-) or complete medium (F+M+) over 12 days. Each value is the mean of three independent experiments  $\pm$  SEM. \* Significantly different from control (F+M+),  $P<0.05$ ; † significantly different from F-M+,  $P<0.05$  (one-way ANOVA, Bonferroni's post hoc test)



**Figure 3. Down-regulation of Dnmt gene expression in C-4 II cells in response to methyl donor depletion** (a) Dnmt1; (b) Dnmt3a; (c) Dnmt3b. C-4 II cells were grown in folate-deplete (F-M+), folate and methionine deplete (F-M-) and complete (F+M+) medium. Each value is the mean of four-independent experiments  $\pm$  SEM. \* Significantly different from control (F+M+),  $P<0.05$  (one-way ANOVA, Bonferroni's post hoc test)

different from that in F-M- on either day.

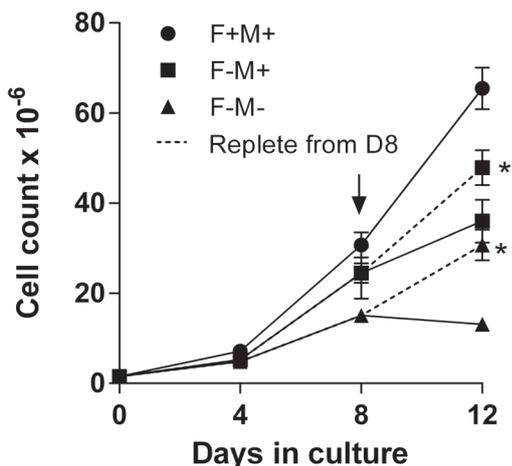
Dnmt3b expression was also significantly influenced by methyl donor depletion treatment ( $P<0.001$ ) as well as by time ( $P=0.004$ ), and a significant interaction was observed between these factors ( $P=0.038$ ). On day 4, there was a lower expression of Dnmt3b in F-M+ ( $P=0.032$ ) and F-M- cells ( $P=0.006$ ) than in F+M+, and this difference was also evident on day 8 ( $P<0.05$ ). On this day, Dnmt3b expression was 3.6-fold lower in F-M- cells compared with F+M+. Expression of Dnmt3b in F-M+ was not different from that in F-M- on either day. There was a significant fall in Dnmt3b expression in F-M- from day 4 to day 8 ( $P=0.008$ ).

*Reversibility of effects on growth and Dnmt expression*

Cells grown in F-M- medium for 8 days were subsequently grown in F+M+ medium for a further 4 days and there was a significant increase in cell number ( $P=0.006$ ) compared with cells maintained in F-M- medium over the same period (Figure 4). The increase in cell number following repletion did not reach statistical significance for cells grown in F-M+ medium. By day 12, cell number was greater in folate-replete cells and folate and methionine-replete cells compared with the relevant depleted cell condition ( $P<0.05$ ).

In this depletion/repletion experiment, F-M- depletion led to a statistically significant down-regulation in expression of Dnmt1 (Figure 5), such that by day 8 Dnmt1 expression was lower in depleted cells than controls ( $P<0.05$ ). Following transfer to F+M+ medium, there was an increase in expression of Dnmt1 from day 8 to day 12 ( $P<0.05$ ) and there was no longer any significant difference in Dnmt1 expression between treatments (Figure 5).

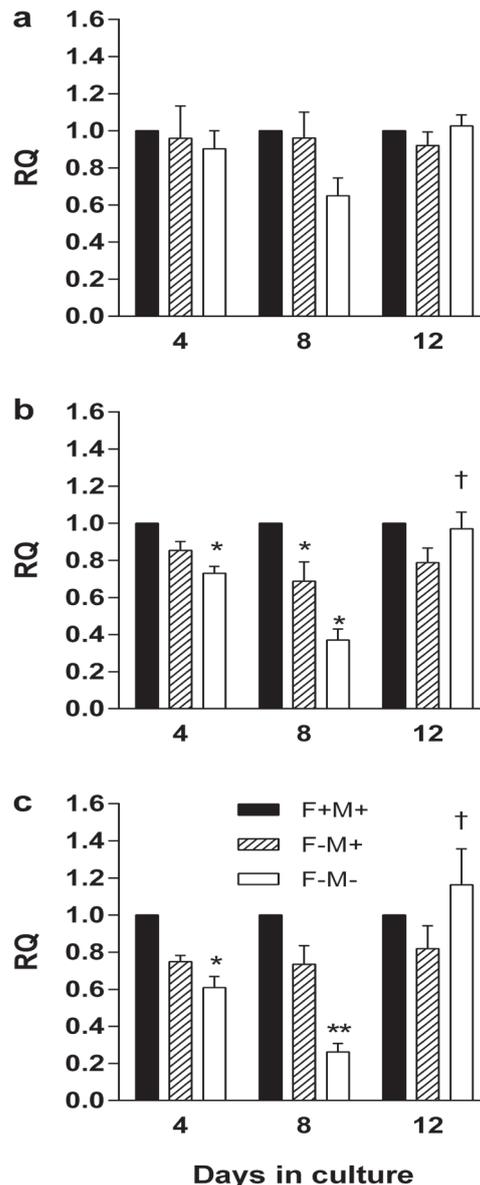
There was a significant fall in Dnmt3a gene expression (Figure 5) from days 4 to 8 in folate and methionine-depleted cells ( $P=0.002$ ) such that after 8 days of depletion



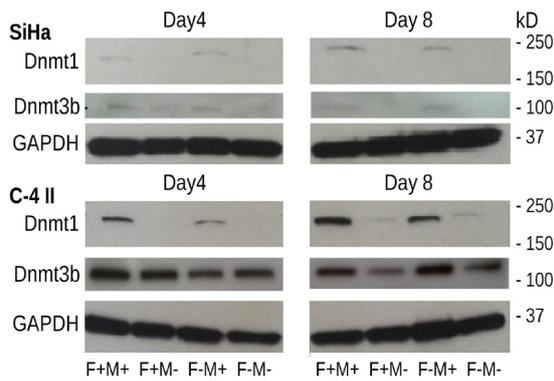
**Figure 4. Growth of C-4 II cells in response to methyl donor repletion** C-4 II cells were grown in folate-deplete (F-M+), folate and methionine deplete (F-M-) or complete (F+M+) medium for 8 days, then transferred to complete medium (F+M+) or maintained in deplete medium for a further 4 days. Dashed lines indicate deplete conditions and solid lines indicate repletion conditions between days 8 - 12. \* Statistically significant between replete and deplete conditions on day 12,  $P<0.05$  (one-way ANOVA, Bonferroni's post-hoc test)

Dnmt3a expression was significantly lower in F-M+ ( $P=0.016$ ) and F-M- treated cells ( $P<0.001$ ) than in F+M+ and lower in F-M- than in F-M+ treated cells ( $P=0.014$ ). Repletion led to a significant increase in Dnmt3a gene expression ( $P<0.001$ ) so that by day 12 there was no significant difference in Dnmt3a expression between treatments (Figure 5).

Similarly, Dnmt3b expression fell (Figure 5) in response to 8 days of growth in F-M- medium ( $P=0.008$ ); following 4 days of repletion Dnmt3b gene expression increased ( $P=0.038$ ). After 8 days of depletion, expression



**Figure 5. Dnmt Gene Expression in C-4 II Cells in Response to Methyl Donor Repletion** C-4 II cells were grown in folate-deplete (F-M+), folate and methionine deplete (F-M-) or complete (F+M+) medium for 8 days, then transferred to complete medium (F+M+) or maintained in deplete medium for a further 4 days. Gene expression was determined relative to  $\beta$ -actin and is expressed standardised to the mean of the control (F+M+) conditions. (A) Dnmt1. (B) Dnmt3a. (C) Dnmt3b. Values are the mean of seven independent experiments and error bars indicate SEM. \* Significantly different from control (F+M+)  $P<0.05$ ; § significantly different from F-M+,  $P<0.05$ ; † significantly different by time in culture,  $P<0.05$  (one-way ANOVA, Bonferroni's post hoc test)



**Figure 6. Dnmt1 and Dnmt3b Protein Expression in C-4 II and SiHa Cells in Response to Methyl Donor Depletion** C-4 II and SiHa cells were grown in folate-deplete (F-M+), methionine-deplete (F+M-), folate and methionine deplete (F-M-) or complete (F+M+) medium for 4 or 8 days. Protein expression was determined by Western blotting relative to GAPDH

**Table 1. Reduction in global DNA methylation in C-4 II cells grown in methyl donor deplete medium over 4 - 12 days**

Days of growth	Percent decrease in global DNA methylation	
	F-M+	F-M-
4	4.39 ± 4.93	2.03 ± 11.85
8	8.99 ± 4.35	13.6 ± 1.46 *
12	6.94 ± 9.66	17.5 ± 2.78

Mean values ± SEM from three independent experiments, expressed as a % decrease relative to cells grown in F+M+ medium). \* Significantly different from F+M+, P<0.05 (one-way ANOVA, post hoc test)

of Dnmt3b was lower in F-M- treated cells than F-M+ and F+M+ treated cells (P<0.01). This difference was reversed by 4 days of repletion (Figure 5).

#### Western blotting

Dnmt1 protein expression was slightly reduced when C-4 II and SiHa cells were grown for 4 or 8 days in folate deplete (F-M+) medium (Figure 6). In contrast, Dnmt1 protein expression was almost completely abolished when cells were grown in methionine-depleted medium (F+M- and F-M-) (Figure 6). A similar effect was observed in both C-4 II and SiHa cells on Dnmt3b (Figure 6).

#### Global DNA methylation

There was a significant effect of time on global DNA methylation status from day 4 to day 8 (P=0.016) (Table 1). The mean global DNA methylation showed a progressive decrease from day 4 to day 12 in F-M- conditions. By day 8, global DNA methylation was 13.6% lower than in F+M+ conditions (P<0.05) and by day 12 it was 17.5% lower than in F+M+ conditions. Effects of folate depletion alone were more modest, resulting in a mean fall of 4-9% over 12 days compared with F+M+ conditions.

## Discussion

To our knowledge this is the first model of a methyl-depleted cervical cancer cell to be developed. Growth of

C-4 II cells in folate- or folate and methionine-deplete medium led to a rapid depletion of intracellular folate and methionine, respectively. There was an associated increase in the concentration of homocysteine in the extracellular medium, consistent with disturbance to the methyl cycle, impaired re-methylation of homocysteine, and increased export. The increase in homocysteine export occurred after the decrease in intracellular methyl donor concentration, supporting a cause-effect relationship and compatible with a functional methyl donor deficiency. The decrease in intracellular folate and intracellular methionine concentration occurred earlier than the decline in growth rate, reflecting a cellular requirement for these nutrients to support DNA synthesis and cell division. Cells deficient in both folate and methionine grew more slowly than cells deficient in folate alone, indicative of the need for 5-methyl-tetrahydrofolate for the synthesis of methionine, which is an essential amino acid.

We carried out similar studies of cell growth using the SiHa cervical cancer derived cell line and observed a significant reduction in growth in F-M- medium compared with F+M+, but only after 20 days of culture (ca. 30% of control, data not shown). Consistent effects of methyl donor depletion on cell growth and mRNA expression in this cell line required culture periods of six weeks and longer, and this and the lower yields of cellular material from this cell line imposed practical constraints on exploring a replication of the model further.

Although others have shown the essentiality of folate or methionine for cell growth in vitro (Stempak et al., 2005; Crott et al., 2008; Hu and Cheung, 2009), no research has previously been conducted in combined folate and methionine deficiency. An increase in extracellular homocysteine is a reflection of intracellular homocysteine concentration, resulting from disturbance to the methyl cycle as we have previously shown for human umbilical vein endothelial cells (HUVECs) (Nakano et al., 2005). Folate-depleted HUVECs showed an increase in extracellular homocysteine, and addition of folic acid back to the growth medium mitigated the effect. Furthermore, the presence of methionine in the medium enhanced the effect of folate depletion on extracellular homocysteine, reflecting the use of methionine in the synthesis of SAM and thereby homocysteine (Nakano et al., 2005). Conversely, Buemi et al (2001) showed that adding folic acid to human vascular monocytes in culture decreased extracellular homocysteine, presumably through re-methylation of intracellular homocysteine.

C-4 II cells grown in folate and methionine-deficient medium demonstrated a loss of Dnmt3a and 3b mRNA expression, and subsequent repletion with methyl donors reversed this effect, providing strong evidence for a modulating effect of methyl donor availability on Dnmt gene expression. A dose response was observed, such that cellular depletion of both folate and methionine elicited a greater effect than folate depletion alone. The effect of folate and methionine depletion on Dnmt1 expression was equivocal in the initial depletion experiment shown in Figure 4 as it did not reach statistical significance. A greater number of independent experiments were conducted for the depletion/repletion study in which

statistical significance was reached for Dnmt1 down-regulation following folate and methionine depletion by day 8, and the effect was reversible upon repletion. We observed an identical pattern of effects on Dnmt mRNA expression using GAPDH as a reference gene, however there was slightly greater variance in GAPDH expression across experimental conditions than for  $\beta$ -actin.

In Western blots, we observed a down-regulation of Dnmt1 protein when C-4 II and SiHa cells were grown in folate and methionine deplete medium and methionine deplete medium, but there was a much smaller effect when folate alone was depleted. The rapid down-regulation in Dnmt protein expression in SiHa cells is in contrast with effects on cell growth in this cell line. We observed a similar pattern of modulation for both Dnmt1 and Dnmt3b in both cell lines, with strong down-regulation in conditions that included methionine depletion, but little effect with folate depletion alone. We have also observed a similar pattern using an antibody to Dnmt3a, however interpretation of the Western blots with that particular antibody is complicated by the appearance of additional higher molecular weight bands under our experimental conditions (data not shown). We conclude that methionine depletion leads to a significant down-regulation of Dnmt1 and Dnmt3b protein expression, while folate depletion alone has little or no effect on Dnmt protein expression.

The mechanism leading to Dnmt protein loss is uncertain, but it is not an indiscriminate effect on protein expression more generally, since GAPDH protein expression was not affected under any of the depletion conditions (Figure 6). We cannot rule out an indirect effect of reduced cell growth rate, although effects on Dnmt 3a and 3b mRNA expression occurred after only 4 days of methyl donor depletion in C-4 II cells and preceded the effects on cell proliferation rate.

Others have studied effects of methyl donor depletion on Dnmt expression in various model systems. Stempak et al (2005) reported a decrease in the expression of Dnmt1 and Dnmt3a protein in folate-depleted NIH/3T3 mouse fibroblasts and human colon cancer cells HCT116 and Caco-2 cells. Similarly, Hayashi et al (2007) observed a down regulation of Dnmt3a and 3b in folate-depleted human colon adenocarcinoma cells but Dnmt1 was up-regulated in this system. They did not propose a mechanism for the effects. Importantly, in our studies the additional depletion of methionine generated more pronounced effects than folate depletion alone, and the effects were reversible. This suggests a mechanism that is not specific to either folate or methionine but is rather underpinned by methyl group deficiency.

It is possible that methyl-donor depletion could affect Dnmt expression through effects on DNA methylation, and we observed a reduction in global DNA methylation under our depletion conditions (Table 1). Several other factors are thought to influence the expression of Dnmts, such as the expression level of microRNAs (miRNAs) and non-coding RNAs (ncRNAs), and chromatin conformation, known to be affected by methyl donor deficiency [35-38]. Changes in DNA methylation might also be accompanied by altered histone methylation and acetylation, with consequent effects on the accessibility of transcription

factors to binding sites of chromatin (Jones et al., 1998; James et al., 2003; Pogribny et al., 2007; Delage and Dashwood, 2008).

Dnmts have been found to be over-expressed in some human cancers (De Marzo et al., 1999; Mizuno et al., 2001; Etoh et al., 2004; Su et al., 2013). Choi et al., (2003) demonstrated a loss of cytoplasmic expression of Dnmt3a in hepatic carcinogenesis and suggested that dysregulation of Dnmt3a expression might be involved in cancer progression. More recently, Sun et al., (2015) have demonstrated that miR-182 is significantly down-regulated in cervical tumours and that it inhibits proliferation of C4-II cells by enhancing the rate of cell apoptosis. They also found that miR-182-induced apoptosis is mediated through down-regulation of Dnmt3a, and that siRNA inhibition of Dnmt3a mimicked the pro-apoptotic effect of miR-182. The functional significance to cancer progression of altered expression of Dnmts in response to methyl donor depletion is unclear, but the evidence from Sun et al (2015) supports the notion that down-regulation of Dnmt3a may be an important anti-tumour strategy in cervical cancer. There is also limited evidence that silencing of Dnmt1 may be associated with demethylation and reactivation of some tumour suppressor genes, in various cancer cell lines, although effects were not wholly consistent (Zhang et al., 2011). Two potentially important studies suggest an inverse relationship between Dnmt3a or 3b expression and carcinogenesis. Hlady et al (2012) conducted a study in which Dnmt3b was inactivated in a mouse model of lymphomagenesis and observed an accelerated lymphomagenesis. A gradual demethylation of a tumour suppressor gene *Ment* (“methylated in normal thymocytes”, approved symbol *C1orf56*) was observed in the Dnmt3b knock-out model. These findings suggested that Dnmt3b has a role in maintaining tumour suppressor gene methylation in cancer and Dnmt3b was proposed as a tumour suppressor (Hlady et al., 2012). Similarly, Gao et al (Gao et al., 2011) showed that deletion of Dnmt3a in a mouse model of lung cancer led to an enhanced tumour progression associated with increased expression of genes important to the cancer phenotype. Dnmt3a was proposed to be a tumour-suppressor-like gene (Gao et al., 2011). Taken together these findings suggest that a down-regulation of Dnmt3a and 3b in response to methyl donor depletion may have cancer- promoting effects.

In our model of methyl donor depletion in cervical cancer cells, global DNA methylation was only modestly affected, despite the marked decrease in intracellular folate and methionine and the pronounced fall in Dnmt3a and 3b expression. DNA is only one of several substrates for methylation and it is possible that the DNA methylation profile is preserved to some extent at the expense of other methyl acceptors and the effects of methyl-donor depletion may be evident in changes in other methylated substrates prior to effects on DNA methylation (Brosnan et al., 2004). The methyl groups spent on the methylation of guanidinoacetate (GAA) to form creatine are estimated to be greater than all other methylation substrates combined (Stead et al., 2001). Furthermore, others have shown interactions between the activities of dietary methyl donors (Niculescu and Zeisel, 2002), notably choline,

may mitigate effects of folate depletion. For example, Craciunescu et al (Craciunescu, Johnson and Zeisel, 2010) showed that choline supplementation mitigated effects of folate depletion on the rate of mitosis of neural progenitor cells in mice. As the medium used to grow the C-4 II cells was particularly rich in choline, this may have compensated for the depletion of folate and methionine and maintained methionine synthesis and therefore SAM synthesis, through the betaine pathway.

The effect of folate depletion on DNA global methylation has been investigated in several folate-deplete systems, from cultured cells to dietary depletion in human studies. Findings are not consistent, perhaps reflecting differences in the severity of the depletion, and the capacity of the choline/betaine pathway to compensate for inadequate folate. Furthermore, the methods available to quantify DNA methylation differ in sensitivity and precision and this also contributes to the lack of consistency of findings. DNA hypomethylation in response to folate depletion has been reported in human colon cells (Wasson et al., 2006), immortalized human colon cells and non-transformed cells (NIH/3T3 and CHO-KI) (Duthie et al., 2000), but not for normal human colonic epithelial cells (Stempak et al., 2005), which seemed resistant to DNA global hypomethylation, despite effects on gene expression. Moderately folate-depleted rats demonstrated no significant alteration of DNA methylation level in liver or colonic mucosa, measured using the [<sup>3</sup>H]-SAM donor assay (Kim et al., 1995), and mice fed a folate-deficient diet for 8 months exhibited a modest 7.2% reduction of DNA methylation in colon epithelial cells (P<0.04), a 9% reduction of global DNA methylation in splenocytes, and a 4% fall in small intestinal epithelial cells but this was not statistically significant (Linhart et al., 2009). However, moderately folate-depleted women did show global DNA hypomethylation (Rampersaud et al., 2000). The exchangeability of dietary sources of methyl groups for DNA methylation probably contributes to the heterogeneity in responses to depletion of a single methyl donor in different model systems.

Despite a modest effect of methyl donor depletion on global DNA methylation our studies show that methyl donor status can reversibly influence expression of Dnmts in cervical cancer cells.

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