RESEARCH ARTICLE

Maternal folate supply and sex influence gene-specific DNA methylation in the fetal gut

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Scope: Epidemiological evidence supports the developmental origins of health and disease hypothesis that developmental under/over-nutrition increases adulthood disease risk. Epigenetic markings are one potential mechanism mediating these effects. Altered folate supply may influence methyl group availability for DNA methylation. We reported low folate supply in utero was associated with reduced global DNA methylation in the murine small intestine of adult offspring. We hypothesised that aberrant methylation would be observed during early development.

Methods and results: Female C57BL/6J mice were fed diets containing 2 mg folic acid/kg or 0.4 mg folic acid/kg 4 wk before mating and during pregnancy. At 17.5 day gestation, gene methylation in fetal gut was analysed by Pyrosequencing®. Low folate reduced overall methylation of *Slc394a* by 3.4% ($p = 0.038$) but did not affect *Esr1* or *Igf2* differentially methylated region (DMR) 1. There were sex-specific differences in *Slc394a* and *Esr1* methylation (2.4% higher in females ($p = 0.002$); 4% higher in males ($p = 0.0014$), respectively).

Conclusion: This is the first study reporting causal effects of maternal folate depletion on gene-specific methylation in fetal gut. These observations support reports that altered methyl donor intake during development affects DNA methylation in the offspring. The consequences of epigenetic changes for health throughout the life course remain to be investigated.

Keywords:
DNA methylation / Folate / Gene-specific / Maternal diet / Sex

1 Introduction

The developmental origins of health and disease (DOHaD) hypothesis proposes that early life exposures modulate adulthood disease risk. Indeed, there is substantial evidence for an association between lower birth weight and increased risk of type 2 diabetes, coronary heart disease, and hypertension, which has been attributed to poor nutrition in utero [1]. These observations indicate a degree of plasticity during development, in which the fetal phenotype may be altered due to environment cues [2] in order to prepare it for the anticipated post-natal environment [3]. These environmentally orchestrated programming events must ''mark'' the animal at a molecular, cellular and/or tissue level and be sustained through the life course to impact on the processes leading to disease. Currently, the mechanistic bases of programming events are poorly understood. Epigenetic mechanisms are attractive potential candidates to mediate the long-term effects of early life exposures since they are modifiable by dietary and other environmental exposures [4, 5], have the potential to change gene expression [6] and are established during development in utero.

There is increasing evidence that DNA methylation (the most commonly investigated epigenetic mark) can be altered in offspring in response to maternal nutrition and

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Abbreviations: CGI, CpG island; **CRC,** colorectal cancer; **DMR,** differentially methylated region

that these changes are associated with changes in gene expression and the phenotype of the progeny [7–9]. Since folate is a key source of methyl groups for synthesis of *S*-adenosyl methionine (SAM) – the universal methyl donor – it is an attractive candidate nutrient for the modulation of DNA methylation. Indeed, altered intakes of folate (and other methyl donors) during pregnancy can have profound effects on DNA methylation patterns in the offspring as has been reported in both animal models and human studies [8–12].

Previously, we reported DNA hypomethylation in the small intestine of adult mice born to folate-depleted mothers [13]. Given that aberrant DNA methylation is an early event in the pathogenesis of colorectal cancer (CRC) [14], it is plausible that maternal diet may influence tumour development in adult offspring through manipulation of the epigenome in utero. Indeed, it was observed that early life exposure to famine was associated with the decreased risk of developing a CpG island methylator phenotype (CIMP) CRC [15]. This suggests that early life exposures may indeed result in persistent epigenetic alterations that may influence CRC development in later life.

To test the hypothesis that aberrant epigenetic marks can be established during early mammalian development via maternal nutrition, we investigated the effects of maternal folate depletion during pregnancy on DNA methylation in the fetal gut. The *Esr1, Igf2,* and *Slc39a4* genes were selected for investigation based upon the previous reports of DNA methylation changes due to altered folate supply to ascertain whether marking of these loci within the fetal genome is susceptible to maternal folate depletion, or if it is protected from such changes [10, 12, 16, 17]. Furthermore, these genes are known to have altered methylation in CRC [18–20]. Given the propensity for sex-specific differences in response to early life exposures [21, 22], we investigated these effects in both sexes.

Table 1. Composition of experimental diets

Ingredient	Normal folate diet (g/kg)	Low folate diet (g/kg)
Corn starch	427.986	427.9856
AIN-93 L-amino acid mix	175	175
Sugar	150	150
Soybean oil	70	70
Lard	80	80
Alphacel	50	50
AIN-93 Mineral mix	35	35
Vitamin mix with folic acid	10	0
Vitamin mix without folic acid	0	10
Choline bitartrate	2	2
Tert-Butylhydroquinone	0.014	0.014
Folic acid	0	0.0004

Table 2. Composition of vitamin mixes

Ingredient	g/kg
Nicotinic acid	3
Calcium pantothenate	1.6
Pyridoxine HCl	0.7
Thiamine HCl	0.6
Riboflavin	0.6
Biotin	0.02
Vitamin E actetate (500 IU/g)	15
Vitamin B12 (0.1%)	2.5
Vitamin palmitate (500 000 IU/g)	0.8
Vitamin D3 (400 000 IU/g)	0.25
Vitamin K1/dextrose mix (10 mg/g)	7.5
Folic acid	0[a)] or 0.2[b)]
Sugar	967.4323[a)] or 967.23[b)]

a) For vitamin mix without folic acid.
b) For vitamin mix with folic acid.

2 Materials and methods

2.1 Animal housing, husbandry, and diet intervention

All animal procedures were approved by the Newcastle University Ethics Review Committee and the UK Home Office. Animals were housed in the Comparative Biology Centre, Newcastle University at 20–22°C and with 12 h light and dark cycles. Fresh water was available ad libitum. Female C57BL/6J mice were allocated at random to either a low folate (0.4 mg folic acid/kg diet) or normal folate diet (2 mg folic acid/kg diet) (6 g/day), and maintained on this diet for 4 wk prior to mating. Diet compositions were modified from AIN-93G [23] and are detailed in Tables 1 and 2. L-Amino acids were used as a protein source. All ingredients, other than folic acid, were included in both diets in the same quantities to avoid any potential confounding through other dietary factors. Mice were time mated i.e. a male was added to a cage containing two females overnight and removed the following morning. Pregnant females, identified by the presence of a vaginal plug, were re-caged and offered 10 g/day of allocated diet throughout pregnancy. At 17.5 days gestation dams were killed for collection of blood and tissues.

2.2 Sample collection

Animals were anesthetised using gaseous isoflurane, blood was removed by cardiac puncture and animals were killed by cervical dislocation. Blood was collected and stored in EDTA tubes. The uterus, containing all fetuses and placentas, was removed and immediately placed in ice cold PBS. The liver, whole gut and placenta of each fetus were removed, weighed and snap frozen in liquid nitrogen and stored at −80°C until

DNA was extracted. The maternal liver was also removed, weighed and snap frozen in liquid nitrogen and stored at −80°C.

2.3 Whole blood 5′methylTHF and 5′10′methylTHF concentrations

Liquid chromatography–tandem mass spectrometry (LC-MS/MS) was used to measure 5′methylTHF and 5′10′methylTHF in whole blood, as described previously [24].

2.4 DNA extraction

DNA was extracted from fetal tails to determine the sex of the fetus. Briefly, tail tissue was minced using a scalpel blade and added to 200 µl Cetus PCR buffer (5 M KCl, 1 M Tris/HCl, 0.25 M $MgCl_2$, 0.45% v/v Nonident P40, 0.45% v/v Tween 20, 0.5% w/v Gelatin). Five microlitres of Proteinase K (20 mg/mL) was added and the mixture was incubated at 55°C for a minimum of 3 h, before heating to 95°C for 15 min to deactivate the Proteinase K. Samples were then centrifuged at 13 000 rpm for 5 min and supernatant fluid containing the DNA was removed for use in PCR.

Fetal gut DNA, for measurement of DNA methylation, was extracted and purified (including RNase treatment) from whole gut using an EZNA tissue DNA isolation kit (Omega Bio-Tek, GA, USA), following the manufacturer's protocol.

2.5 Determination of fetal sex by PCR-based assay

DNA extracted from fetal tails was used to identify the sex of the fetuses using the protocol described previously [25].

2.6 Bisulfite-pyrosequencing for quantification of gene-specific methylation

Bisulfite conversion of DNA was performed using the EZ DNA Methylation GoldTM kit (Zymo Research), following the manufacturer's protocol. Briefly, 1 µg of genomic DNA was incubated with CT conversion reagent and incubated at the following temperatures; 98°C for 10 min, 64°C for 2.5 h, held at 4°C. DNA was then transferred to a spin column, washed, desulphonated, purified, and eluted in a final volume of 10 µL.

Quantitative bisulfite pyrosequencing was used to determine the percentage methylation at individual CpG sites within the *Esr1, Igf2* differentially methylated region (DMR) 1, *Slc39a4* CpG island 1 (CGI1) and *Slc39a4* CGI2 loci, as described previously [17].

Briefly, 1 µL of bisulfite-treated DNA was added as a template in a PCR reaction using 6.25 µL Hot Star Taq mastermix (Qiagen), total volume 12.5 µL. Amplification was carried out in a G-storm thermocycler (GRI) using the following protocol; 95°C 15 min, then cycles of 95°C 15 s, annealing temperature 30 s, 72°C 15 s, followed by 72°C for 5 min. Biotin-labelled PCR products were captured with Streptavidin Sepharose beads (GE Healthcare), and made single stranded using a Pyrosequencing Vacuum Prep Tool (Qiagen). Sequencing primers were annealed to the single-stranded PCR product by heating to 80°C, followed by slow cooling. Pyrosequencing was carried out on a Pyromark MD system and quantification of cytosine methylation using the Pyro Q CpG 1.0.6 Software.

2.7 Statistical analysis

Data distributions were examined by the Kolmogorov–Smirnov test and all data sets were normally distributed. Analysis of variance was used to examine the effects of diet on maternal body and liver weight, whole blood 5′methylTHF and 5′10′methylTHF concentrations and numbers of fetuses. A separate analysis of variance was used to examine the effects of diet and sex, and the interaction between these fixed factors, on fetal body, liver and gut weights, and on methylation of selected genes in the fetal gut. A p-value of <0.05 was considered statistically significant.

3 Results

3.1 Effect of low dietary folate on maternal body and liver weight, maternal folate status, and numbers of fetuses per dam

Feeding a low folate diet prior to, and during, pregnancy did not alter maternal body or liver weights compared with dams fed a normal folate diet (Table 3). However, as expected, dietary folate depletion during pregnancy resulted in substantially lower concentrations of both biomarkers of maternal folate status viz. 5′methylTHF and 5′10′methylTHF in maternal whole blood ($p = 0.001$ and 0.007 respectively; Table 3). Despite the reduction in maternal folate status, the number of fetuses per dam was not affected by the reduced maternal folate supply (Table 3).

3.2 Effect of low maternal dietary folate on fetal body, liver, and gut weight

Reduced maternal folate supply prior to, and during pregnancy, was associated with increased fetal body weight compared with controls ($p = 0.006$) but there were no detectable effects of maternal diet on fetal liver or gut

Table 3. Dam and fetal body and organ weights and maternal whole blood folate metabolite concentrations. Data are estimated marginal means (SEM)

Variable	Normal folate	Low folate	*p*-Value
Dams			
N	9	6	
Body weight (g)	34.6 (0.75)	33.7 (0.91)	0.463
Liver weight (g)	1.44 (0.81)	1.49 (0.99)	0.692
Number of fetuses/dam	6.22 (0.89)	6.17 (1.09)	0.969
5′ methylTHF (nmol/L)	666 (40.9)	378 (50.0)	0.001
5′10′methylTHF (nmol/L)	20.7 (1.29)	14.2 (1.58)	0.007
Fetuses			
n[a)]	52	33	–
Total fetal weight (g)	0.79 (0.02)	0.86 (0.02)	0.006
Liver weight (g)	0.058 (0.002)	0.065 (0.003)	0.111
Gut weight (g)	0.043 (0.003)	0.045 (0.004)	0.600

a) Sex was undeterminable for four fetuses from the normal folate group and three fetuses from the low folate group. These were therefore excluded from this statistical analysis, hence the fetal '*n*' does not equal number of fetuses/dam multiplied by the number of dams. Theses fetal samples were not used for DNA methylation analysis.

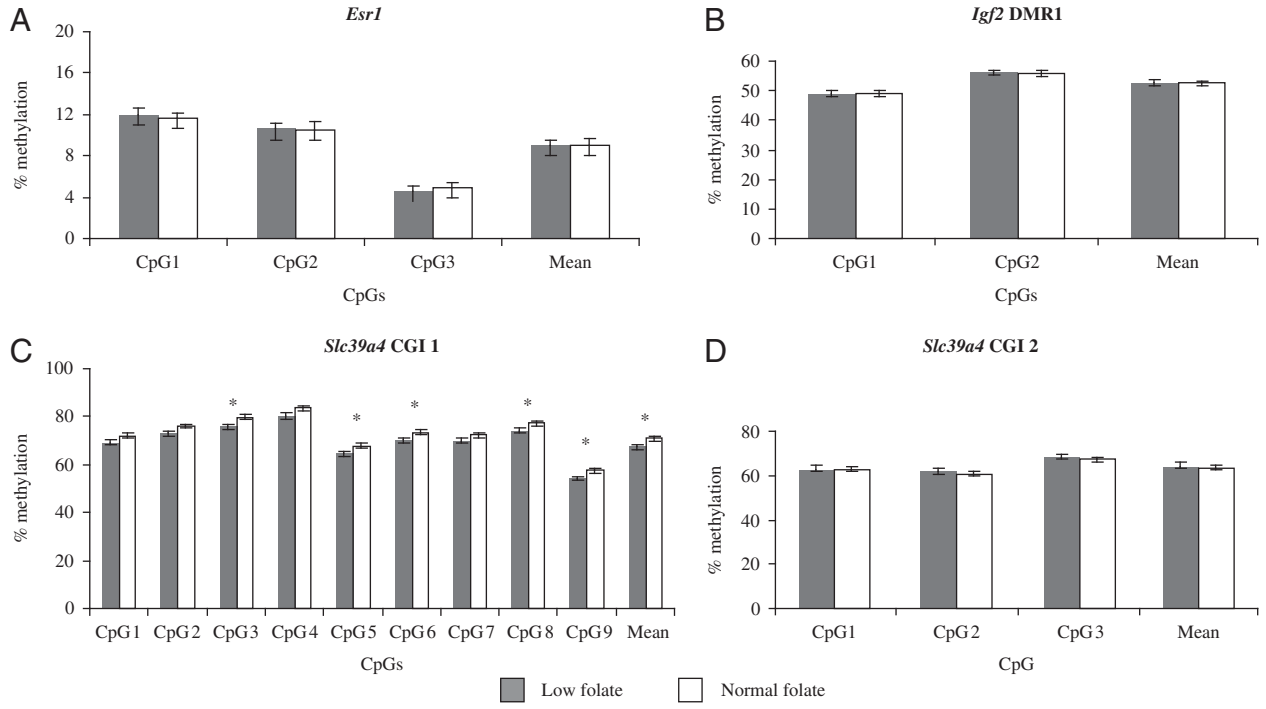

Figure 1. Effect of maternal folate supply during pregnancy on methylation of selected genes in the mouse fetal gut. Error bars represent standard error of the mean. Low folate $n = 16$, normal folate $n = 18$. $^{*}p < 0.05$.

weights (Table 3). The weights of fetal whole body, liver and gut were similar in males and females (data not shown).

3.3 Effect of low maternal dietary folate on methylation of selected genes in the fetal gut

Reducing maternal folate status during pregnancy had no effect on methylation at the *Esr1, Igf2* DMR 1, and *Slc39a4* CGI2 loci in fetal gut (Fig. 1A, B and D). However, mean methylation across the 9 CpG sites analysed within the *Slc39a4* CGI1 was lower in the gut of fetuses from dams fed the low folate diet (Fig. 1C) ($p = 0.038$). When investigated individually, there was significantly reduced methylation of 5 of these *Slc39a4* CGI1 CpG sites (sites 3, 5, 6, 8, and 9) in response to low maternal folate.

There were no differences between male and female fetuses in methylation at the *Igf2* DMR 1 and *Slc39a4* CGI2 loci (Fig 2B and D). However, methylation was lower in females at the *Esr1* locus at all CpGs analysed (mean across

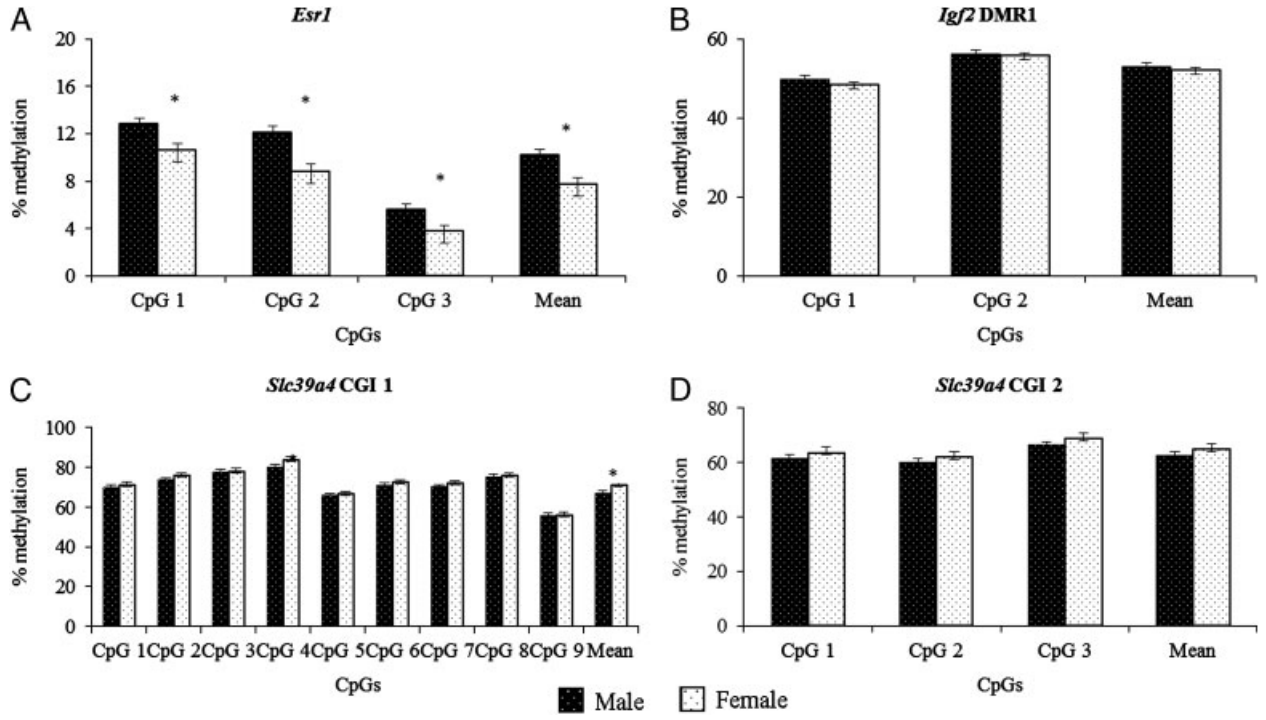

Figure 2. Methylation of selected genes in DNA from male and female mouse fetal gut. Error bars represent standard error of the mean. Females $n = 16$, Males $n = 18$. $^{*}p < 0.05$.

all CpG sites $p = 0.002$), whereas mean methylation across the 9 CpG sites analysed within the *Slc39a4* CGI1 locus was significantly ($p = 0.014$) higher in females (Fig. 2A and C respectively). When analysed individually, only methylation at CpG site 4 within the *Slc39a4* CGI1 locus was significantly ($p = 0.047$) more methylated in females compared with males.

There was no evidence of interactions between maternal folate intake, DNA methylation and sex (data not shown).

4 Discussion

In an earlier study, we reported a significant reduction in global DNA methylation in the small intestine of adult murine offspring born to folate-depleted mothers [13], an observation which suggested that maternal folate status may have enduring effects upon the gut epigenome. Given the evidence that DNA hypomethylation is an early event in CRC development [26, 27], our previous findings established the possibility that susceptibility to intestinal cancer might be enhanced by lower maternal folate status. Here, we report epigenetic changes in the fetal gut in response to reduced maternal folate status during pregnancy. These epigenetic effects were gene and locus specific with reduced methylation at the *Slc39a4* CGI1 locus in the fetal gut from offspring of low folate-fed dams but no significant differences in methylation at the *Esr1*, *Igf2* DMR1 or *Slc39a4* CGI2 loci.

Others have reported that low folate intake during pregnancy in rats reduced placental DNA methylation [11] and restricting vitamin B_{12}, folate, and methionine supply periconceptionally in sheep resulted in differential methylation of 4% of 1400 CpG islands (CGI) in fetal liver [28]. In addition, supplementation with methyl donors during pregnancy can alter methylation of specific loci in the offspring of rodents and humans [8–10, 12]. The observations presented here support the hypothesis that maternal methyl donor supply can affect patterns of DNA methylation in the offspring. In addition, because the effects were observed in fetuses collected at 17.5 day of gestation, they demonstrate unequivocally that the changes are attributable to maternal nutrition via exposure in utero. Finally, they demonstrate that a degree of maternal depletion of a single micronutrient, folate, which has no adverse effects on maternal body or liver weights or on fetal numbers or fetal organ weights (Table 3), is sufficient to alter gene-specific DNA methylation patterns.

Although we have not measured gene expression changes in this study, it is plausible that the reduced DNA methylation observed in *Slc39a4* due to folate depletion may increase gene expression in some cells within the fetal gut. Methylation of the *SLC39A4* gene has been observed to be less frequent in CRC tumour tissue when compared with either corresponding macroscopically normal colon mucosa or with normal epithelium from non-cancer patients (although differences were not statistically significant) which suggests a relationship between methylation at this

locus and CRC [20]. Aberrations in *Slc39a4* gene expression have been observed in several cancer types [29–32]. These observations suggest that increased *Slc39a4* expression may be detrimental with respect to the development and/or progression of cancer. In addition, they highlight the possibility that epigenetic changes at the gene locus are responsible for altered gene expression. The reduction in *Slc39a4* methylation, which we observed in mouse fetal gut in response to folate depletion, may therefore have consequences for the development of cancer if sustained into adulthood.

We observed sex-specific differences in DNA methylation patterns in the *Esr1* and *Slc39a4* CGI1 loci in the fetal gut. Although the differences were relatively small, they were consistent and *Esr1* was more methylated in males whereas *Slc39a4* CGI1 was more methylated in females. Differences in DNA methylation between males and females has been observed in several human studies [33–35], but, to date, such information is fragmentary and most examples have not been replicated in independent studies. This emerging evidence for sex-related differences in DNA methylation patterns may be of importance in understanding phenotypic differences between the sexes. For example, DNA hypermethylation of *KCNH5*, *KCNH8*, and *RARB* was more frequent in lung tumour samples from female non small cell lung cancer patients compared with males, suggesting possible differences in weighting of risk factors or disease pathways between the sexes [36].

In summary, we have demonstrated that maternal diet, and in particular maternal dietary folate supply, can affect DNA methylation in the fetal gut in a locus-specific manner. Our observations support the hypothesis that DNA methylation is a mechanism through which exposure of the fetus to environmental challenges (e.g. maternal diet) can be recorded [37]. Such effects could contribute to lifelong health if they are sustained throughout the life course, and may play a role in modulating cancer risk in adulthood if gene expression is altered as a result of altered methylation at this locus. In this respect, it will be pertinent to determine whether effects of maternal dietary folate on *Slc39a4* methylation observed in the fetal gut are manifested in older (adult) animals. A deeper understanding of the mechanisms linking early life exposure with subsequent health, and more specifically, cancer, risk will serve to help in the development of effective preventative strategies.

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The authors have declared no conflict of interest.

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